

Interferon- α suppresses hepatitis B virus enhancer II activity via the protein kinase C pathway

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ABSTRACT

HBV has two enhancer (En) regions each of which promotes its own transcription. En II regulates production of pregenomic RNA, a key product of HBV replication, more strongly than En I. Although IFN- α has been found to suppress En I activity, its effect on En II activity has not been examined. Here we used luciferase assay to demonstrate that IFN- α suppresses En II activity. Analysis with several deletion/mutation constructs identified two major segments, nt 1703–1727 and nt 1746–1770, within the En II sequence as being responsible for the suppressive effects of IFN- α . Pre-treatment with protein kinase C (PKC) inhibitors blocked this effect regardless of the expression levels of phospho-STAT1 and Mx upon IFN- α stimulation. These results indicate that IFN- α suppresses En II activity via the PKC pathway, which may be an alternative suppressive pathway for HBV replication. (136 words).

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Introduction

Hepatitis B virus (HBV) causes acute and chronic hepatitis in humans, and chronic infection is closely associated with the development of liver cirrhosis and hepatocellular carcinoma (Lok and McMahon, 2009). HBV has a partially double-stranded 3.2-kb DNA genome (relaxed circular (RC) DNA) in its nucleocapsid. When HBV invades host cells, RC-DNA is converted into a plasmid-like covalently closed circular DNA (cccDNA) inside the nucleus. From the cccDNA, the 3.5-, 2.4-, 2.1-, and 0.8-kb mRNAs are transcribed by cellular RNA polymerase II (Beck and Nassal, 2007). Among these RNAs, 3.5-kb pregenomic RNA (pgRNA) serves as the template of reverse transcription for synthesis of negative-strand DNA. Thus, transcription of pgRNA from cccDNA is one of the key steps in HBV replication.

In the HBV genome, there are four promoters (CP, SPI, SPII, and XP) and two transcriptional enhancer regions. Both enhancers stimulate transcription from the promoters (Antonucci and Rutter, 1989; Moolla et al., 2002; Su and Yee, 1992; Vannice and Levinson, 1988; Yee, 1989). Enhancer I (En I), which is located upstream of the X gene, activates transcription in a relatively cell-independent manner (Vannice and Levinson, 1988). In contrast, enhancer II (En II) (Fig. 1), located just upstream of CP, specifically activates

transcription in hepatocytes (Wang et al., 1990; Yee, 1989; Yuh and Ting, 1990). Hepatocytes selectively express transcription factors which activate En II activity, such as HNF1 (Wang et al., 1998), HNF3 (Johnson et al., 1995; Li et al., 1995), HNF4 (Guo et al., 1993; Raney et al., 1997), CCAAT/enhancer binding protein (C/EBP) (López-Cabrera et al., 1990, 1991; Yuh and Ting, 1991) and FTF (Ishida et al., 2000; Li et al., 1998). This characterizes En II as a hepatocyte-specific *cis*-acting element. A previous report showed that, upon transfection with HBV genome, human hepatic cells, but not non-hepatic cells, were able to express pgRNA (Sureau et al., 1986). For this reason, En II is considered to regulate the production of pgRNA more strongly than En I (Yee, 1989).

Interferon- α (IFN- α) has been used as an anti-viral agent against HBV. It suppresses HBV viral load and ameliorates hepatic inflammation (Jonas et al., 2010; Liaw, 2009). Type I IFN activates the Janus kinase (JAK) bound to the cytoplasmic domain of its receptor. JAK phosphorylates transcription factors such as signal transducers and activators of transcription (STAT) 1 and STAT2. Phosphorylated STAT1 and STAT2 bind to IFN regulatory factor 9 (IRF9). These transcription factors form a complex, IFN-stimulated gene factor 3 (ISGF3). This complex binds to IFN stimulation response element (ISRE) in the promoter region of various genes, and activates interferon-stimulated genes (ISGs) (Der et al., 1998). Some of the ISGs including RNA-activated protein kinase (PKR), 2',5'-oligoadenylate synthetases (OAS), and Mx have been shown to possess antiviral activity. ISG induction by type I IFN is considered to be the main pathway to suppressing viral replication.

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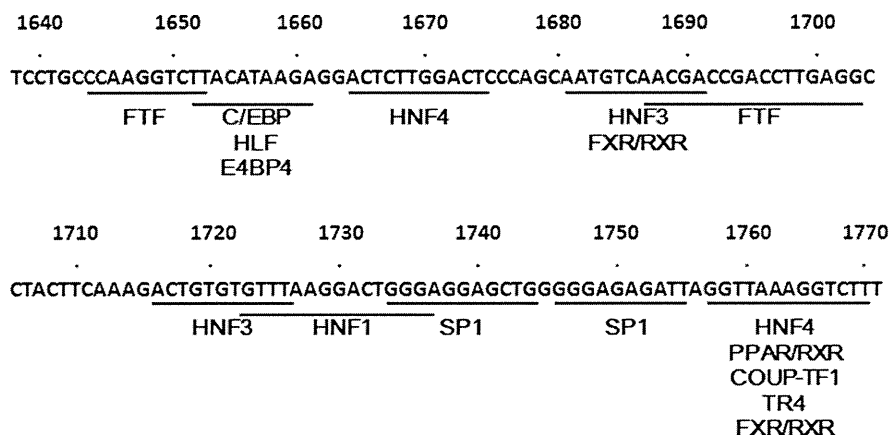


Fig. 1. Nucleotide sequences of the HBV En II region. The HBV sequence used in this study was of the *adw2* subtype (GenBank accession no. X02763). Numbering of the HBV sequence started at the unique *EcoRI* site. The underlined sequences represent the transcription factor binding sites mentioned in previous reports.

Type I IFN has been reported to inhibit HBV En I and core promoter activities (Nakao et al., 1999; Romero and Lavine, 1996; Schulte-Frohlinde et al., 2002; Tur-Kaspa et al., 1990). Nakao et al. demonstrated that IFN- α suppressed En I transcriptional activity by the binding of ISGF3 to the ISRE-like sequence in En I region (Nakao et al., 1999). However, there has been no study on the effect of IFN- α on HBV En II activity. In this study, we demonstrated that IFN- α suppressed En II activity via activation of PKC. Notably, STAT1 activation and ISG induction may be dispensable for IFN- α -mediated suppression of En II activity. This might shed light on understanding the inhibition of HBV replication by IFN- α .

Results

En II activity is down-regulated by IFN- α

We constructed a luciferase gene expression vector by inserting the En II sequence (nt 1640 to 1771) into pGL4LUC (pGL4LUC-En II). Huh-7 cells were transfected with pGL4LUC or pGL4LUC-En II, treated with or without IFN- α , and luciferase activities were evaluated. Insertion of En II increased the luciferase activity (about 228-fold) (Fig. 2A). IFN- α down-regulated the luciferase activity of pGL4LUC-En II, but did not affect that of pGL4LUC (Fig. 2B). This result suggested that IFN- α inhibited the activity of En II, and we examined the time course of IFN- α -induced suppression of En II activity. The suppressive effect of IFN- α on En II activity appeared at 3 h after administration of IFN- α , peaked at 6–12 h, and was gradually attenuated (Fig. 2C). Next, dose-response analysis showed that the En II activity was down-regulated by IFN- α in a dose-dependent manner, with the maximal suppressive effect at 300–1000 IU/m (Fig. 2D). We also examined the IFN- α -mediated suppression of En II activity in other hepatoma cell lines, PLC/PRF/5 and Hep3B. IFN- α significantly suppressed En II activities in both these cell lines (Fig. 2E). We next assessed whether or not IFN- α regulated HBV transcription in the HBV genome transfected cells by RT-PCR. HBV-RNA levels were significantly reduced by IFN- α (Fig. 2F). These results indicate that IFN- α suppresses HBV En II activity as well as its expression at a transcriptional level.

Both nt 1703–1727 and nt 1746–1770 within the En II region are required for suppression of En II activity by IFN- α

To determine the region responsible for the inhibitory effect of IFN- α on En II activity, we divided the En II sequence into six segments (Fig. 3A), and constructed plasmids containing En II

sequences with deletion of each segment (pGL4LUC-En II-D1~6). Huh-7 cells were transfected with these deleted constructs, treated with IFN- α , and then assayed for luciferase activity. None of the deletions could restore the suppressive activity by IFN- α (Fig. 3B), suggesting that there are several responsible regions for the IFN- α -induced suppression of En II activity. Next, we constructed plasmids containing four iterations of each segment within the En II sequence in tandem (pGL4LUC-En II-T1~6) to examine the contribution of individual short fragments. IFN- α significantly suppressed the activities of pGL4LUC-En II-T2, T3, T4, T5 and -T6 in luciferase assay. Among them, the activities of pGL4LUC-En II-T4 and -T6 showed the largest suppression by IFN- α (Fig. 3C). On the basis of this result, we constructed a luciferase reporter vector with deletions of both segment 4 (nt 1703–1727) and segment 6 (nt 1746–1770) (pGL4LUC-En II-D4+6). The activity of this dual-deleted construct did not show a significant change due to IFN- α (Fig. 3D). These results suggest that both nt 1703–1727 and nt 1746–1770 within the En II region are required for the suppression of En II activity by IFN- α .

IFN- α -mediated suppression of En II activity is dependent on JAK activation

IFN-induced signal transduction occurs through the sequential activation of JAKs and STATs (Darnell et al., 1994). We examined the role of JAK in the inhibition of En II activity. JAK inhibitor alone did not affect En II activity. But the pre-treatment of the cells with JAK inhibitor completely blocked the suppressive effect of IFN- α on En II activity (Fig. 4A). The effect of JAK inhibitor was confirmed by the reduction of Mx induction in Western blot analysis (Fig. 4B). This result demonstrates that JAK activation is necessary for the IFN- α -induced suppression of En II activity.

The PKC pathway is involved in IFN- α -mediated suppression of En II activity

Previous reports demonstrated that type I IFN activated various kinases such as MAPK family members (MEK/ERK and p38 MAPK) (David et al., 1995; Goh et al., 1999), PI3K/Akt (Uddin et al., 1995), JNK (Caraglia et al., 1999) and protein kinase C (PKC) (Uddin et al., 2002). Here we examined the involvement of alternative pathways by pre-treatment with inhibitors for various kinases, including MEK, p38 MAPK, PI3K/Akt, JNK and PKC. The name of each inhibitors and its target kinase is commented in Table 1. As shown in Fig. 5A, only staurosporine, a PKC inhibitor, blocked the inhibitory effect of IFN- α , and other inhibitors did

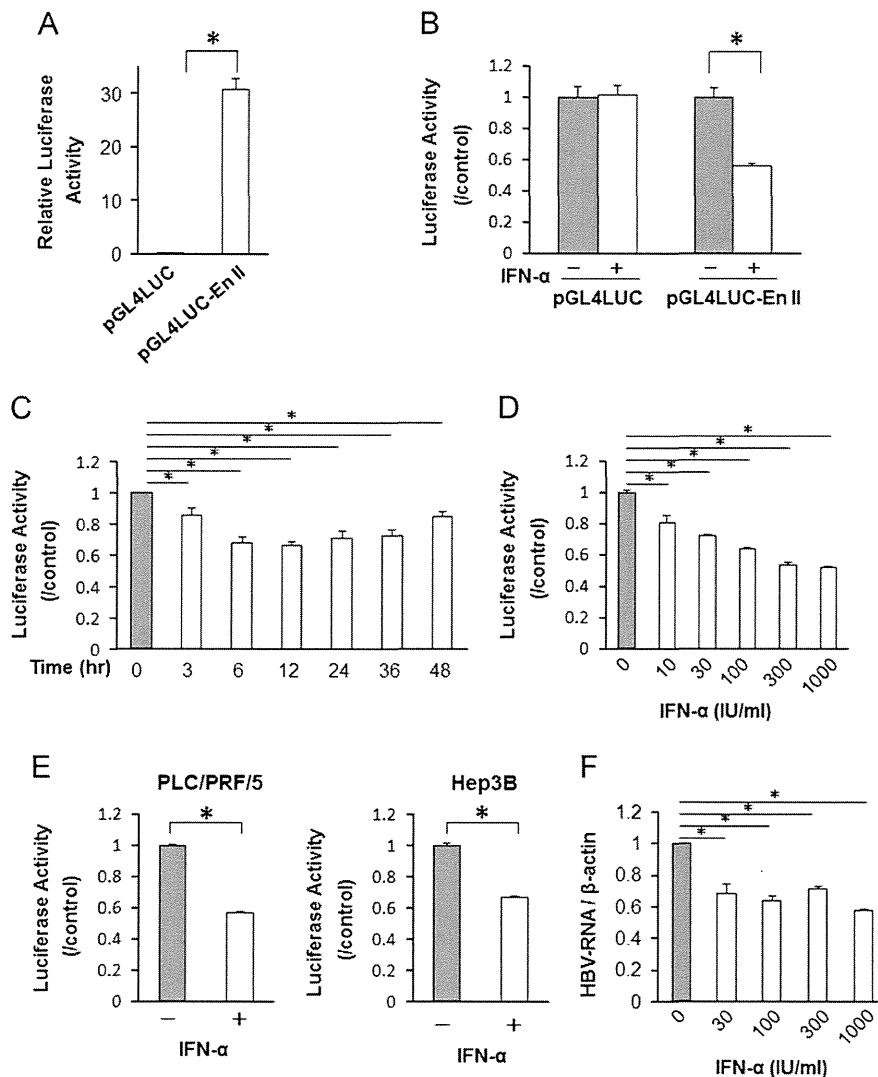


Fig. 2. Suppression of HBV En II transcriptional activity and reduction of HBV-RNA by IFN- α . A, B. Huh-7 cells were transfected with pGL4LUC or pGL4LUC-En II or incubated with or without IFN- α (100 IU/ml). After 24 h, the activity of firefly luciferase was evaluated. C. Huh-7 cells were transfected with pGL4LUC-En II, and incubated with IFN- α (100 IU/ml). Luciferase activities were evaluated at the indicated times. D. Huh-7 cells were transfected with various concentrations (0–1000 IU/ml) of IFN- α for 12 h and luciferase activities were evaluated. E. PLC/PRF/5 cells (left panel) and Hep3B (right panel) cells were transfected with pGL4LUC-En II, and incubated with or without IFN- α (300 IU/ml). Luciferase activities were evaluated. F. Huh-7 cells were transfected with pHBV1.5, and treated with IFN- α at various concentrations (0–1000 IU/ml). At 72 h after IFN- α treatment, cells were harvested, and the abundances of HBV-RNA were evaluated by quantitative RT-PCR. The HBV-RNA level of the IFN- α treated cells was normalized with that of non-treated cells. * $p < 0.05$. “/control” on the vertical axis means the ratio of luciferase activity of IFN- α treated cells normalized with that of non-treated cells.

not. Since staurosporine is a PKC inhibitor showing broad-spectrum activity (Marte et al., 1994), we also examined other inhibitors specific for PKC isoforms. Previous reports demonstrated that IFN- α activated PKC- α/β and PKC- δ (Pfeffer et al., 1990; Uddin et al., 2002). Indeed, activation of PKC- α/β and PKC- δ by IFN- α was confirmed by immunoblot analysis (Fig. 5B). Thus, we examined the PKC inhibitors rottlerin and Gö6976 (Gschwendt et al., 1994; Martiny-Baron et al., 1993). All PKC inhibitors blocked the suppression of En II activity by IFN- α (Fig. 5C). These results suggest that several isoforms of PKC are involved in the IFN- α -mediated suppression of En II activity. We also examined STAT1 activation and ISGs induction by IFN- α in cells pre-treated with these PKC inhibitors using immunoblot analysis (Fig. 5D). Expression levels of phospho-STAT1 and Mx differed among these PKC inhibitors. Staurosporine and Gö6976 slightly diminished the activation of STAT1, but rottlerin did not. This result suggests that PKC isoforms might not strongly regulate

activation of STAT1. Rottlerin, a specific inhibitor for PKC- δ , inhibited the induction of Mx, which agreed with previous findings (Kaur et al., 2005). Staurosporine and Gö6976 did not suppress Mx expression. Taken together, all these PKC inhibitors blocked the suppression of En II activity by IFN- α regardless of the expression levels of phospho-STAT1 and Mx. These results suggest that STAT1 activation and ISG induction may be dispensable for the IFN- α -mediated suppression of En II activity. Next, we examined the effect of phorbol 12-myristate 13-acetate (PMA), a PKC activator (Castagna et al., 1982; Griner and Kazanietz, 2007). PMA suppressed En II activity (Fig. 5E), and PMA stimulation did not result in STAT1 phosphorylation and Mx induction (Fig. 5F), suggesting that suppression of En II by PMA is independent of STAT1 activation and ISG induction. On the basis of these findings, we conclude that IFN- α suppresses En II activity via the PKC pathway, which may not involve STAT1 activation and ISG induction.

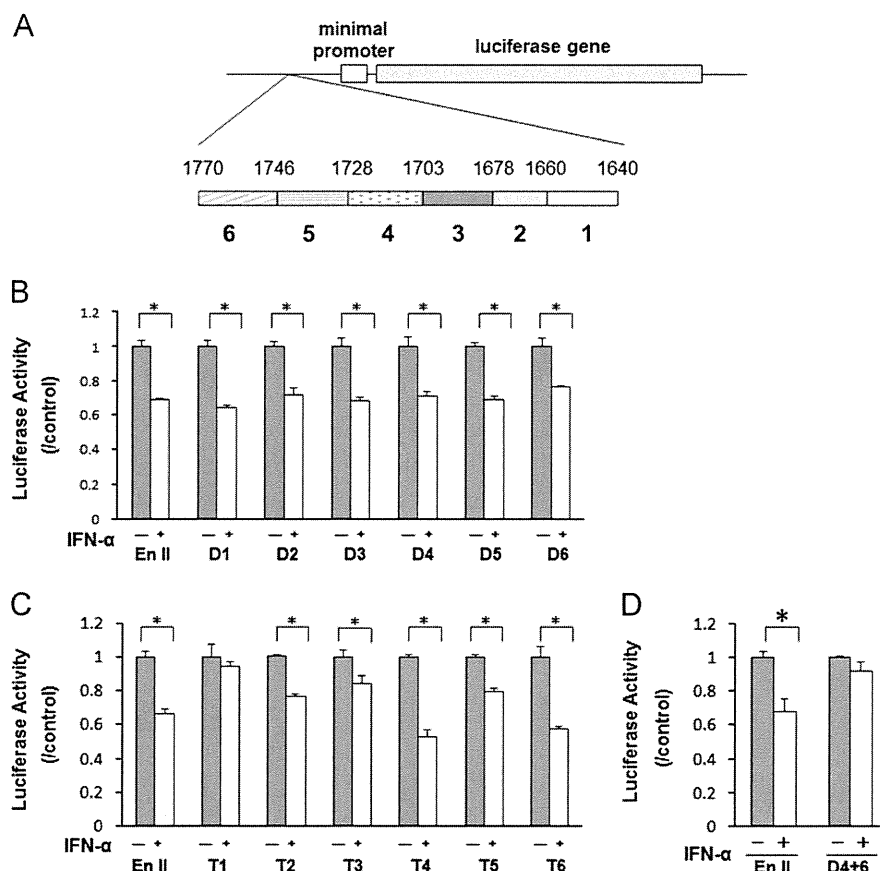


Fig. 3. Deletion/mutational analysis to identify the responsive sequence for the suppressive effect of IFN- α on En II. A. Scheme of pGL4LUC-En II and six segments defined within the En II sequence. The En II sequence was integrated just upstream of the minimal promoter of pGL4LUC. B. Huh-7 cells were transfected with the reporter vectors with deletion of each segment (pGL4LUC-En II-D1~6), incubated with 300 IU/ml IFN- α for 12 h, and luciferase activities were evaluated. C. Plasmids containing four iterations of each segment within En II sequence in tandem (pGL4LUC-En II-T1~6) were generated and luciferase activities were evaluated similarly. D. Plasmid with deletion of both nt 1703–1727 and nt 1746–1770 (pGL4LUC-En II-D4+6) was constructed and luciferase activities were evaluated similarly. * $p < 0.05$. "/control" on the vertical axis means the ratio of luciferase activity of IFN- α treated cells normalized with that of non-treated cells.

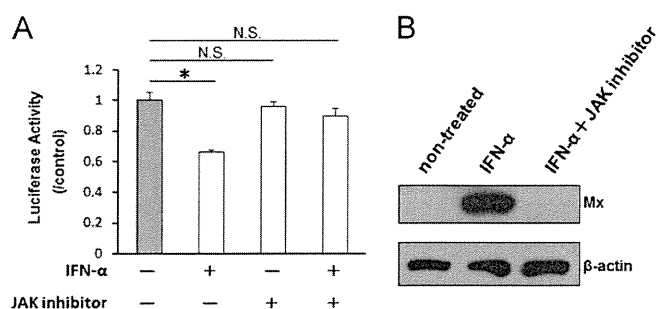


Fig. 4. Involvement of JAK activation in the IFN- α -induced suppression of En II activity. A. Huh-7 cells were transfected with pGL4LUC-En II and treated with JAK inhibitor (1 μ M) for 1 h. The cells were then incubated with IFN- α (150 IU/ml) for 12 h, followed by luciferase assay. B. Huh-7 cells were pre-treated with JAK inhibitor for 1 h, and then incubated with IFN- α (150 IU/ml) for 12 h, followed by immunoblot analyses to detect Mx protein. * $p < 0.05$. "/control" on the vertical axis means the ratio of luciferase activity of IFN- α treated cells normalized by that of non-treated cells.

Knockdown of a single transcription factor does not influence IFN- α -induced suppression of En II activity

We anticipated that IFN- α suppressed En II activity by functional down-regulation of some transcription factor(s) phosphorylated in a PKC-dependent manner. Among transcription factors which bind the En II region, previous reports showed that Specificity Protein 1 (Sp1) (Mahoney et al., 1992; Pal et al., 1998; Rafty and Khachigian,

Table 1

A comment of the inhibitors and its target kinase.

PD98059	MEK inhibitor
SB203580	P38MAPK inhibitor
LY294002	PI3K inhibitor
Akt-1-1/2	Akt inhibitor
SP600125	JNK inhibitor
Staurosporine	PKC inhibitor with broad spectrum
Rottlerin	Inhibitor specific for PKC- δ
Gö6976	Inhibitor specific for Ca ²⁺ -dependent PKC isoforms

2001), Retinoid X Receptor α (RXRA) (Delmotte et al., 1999) and C/EBP (Mahoney et al., 1992) were inactivated by PKC. Thus, we examined the En II response to IFN- α after knockdown of these transcription factors. C/EBP, RXR and Sp1 expression was efficiently reduced by siRNA (Fig. 6A). We observed no significant change in the suppression of En II activity compared with control siRNA (Fig. 6B). This result suggests that several transcription factors (including unknown proteins) might be involved in the IFN- α -mediated suppression of En II activity.

Discussion

In the present study, we demonstrated that IFN- α suppressed HBV En II activity. The inhibition by IFN- α of En II activity could be blocked by pre-treatment with PKC inhibitors, and this

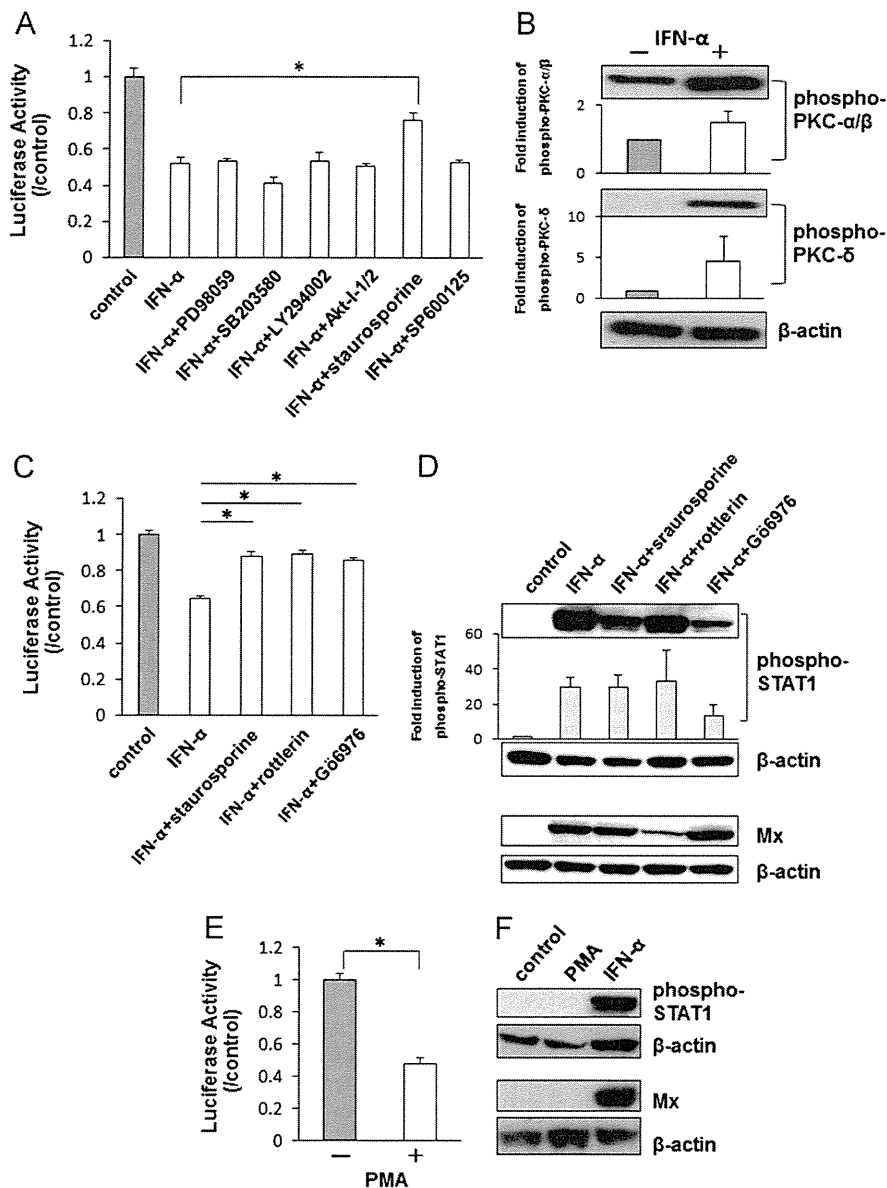


Fig. 5. PKC-dependent suppression of En II activity by IFN- α . **A** and **C**. Huh-7 cells were transfected with pGL4LUC-En II, treated separately with each kinase inhibitor for 1 h. The cells were then treated with IFN- α (1000 IU/ml) for 12 h, and luciferase activities were evaluated. **B**. Huh-7 cells were treated with IFN- α (1000 IU/ml) for 12 h. Immunoblot analyses were performed to detect phosphorylated PKC- α/β and phosphorylated PKC- δ . Quantitative analysis of the expression level of phospho-PKC- α/β and - δ was performed by using ImageJ. Each level was normalized with that of IFN- α -non-treated cells. **D**. Huh-7 cells were harvested at 30 min to detect phosphorylated STAT1 and at 12 h to detect the expression of Mx after administration of IFN- α (1000 IU/ml), and immunoblot analyses were performed. Quantitative analysis of the expression level of phospho-STAT1 was performed by using ImageJ. Each level was normalized with that of IFN- α -non-treated cells. **E**. Huh-7 cells were transfected with pGL4LUC-En II, treated with PMA (100 nM) for 12 h, and luciferase activities were evaluated. **F**. Huh-7 cells were treated with PMA (100 nM) or IFN- α (1000 IU/ml). The cells were harvested at 30 min to detect phosphorylated STAT1 and at 12 h to detect the expression of Mx, and immunoblot analyses were performed. * $p < 0.05$. "/control" on the vertical axis means the ratio of luciferase activity of IFN- α /PMA treated cells normalized with that of non-treated cells.

blocking effect may not involve STAT1 activation and ISG induction. The latter, ISG induction via the JAK-STAT pathway, has been considered to be the main mechanism suppressing viral replication. Our findings suggest a pathway for IFN- α repression of HBV transcription other than ISG induction.

PKCs are involved in a wide variety of cell functions and signal transduction pathways regulating cell migration and polarity, proliferation, differentiation and cell death (Nishizuka, 1988). In the PKC family, there are at least ten isoforms which can be divided into three sub-groups based on their structural characteristics and cofactor requirements. These include the classical PKC (cPKC: α , β I, β II, and γ), the novel PKC (nPKC: δ , ϵ , η and θ), and the atypical PKC (aPKC: ζ and ι/λ) (Azzi et al., 1992;

Breitkreutz et al., 2007; Kikkawa et al., 1989). IFN- α can activate multiple PKC isoforms: not only PKC- δ , but also PKC- α/β (Pfeffer et al., 1990), PKC- ϵ (Pfeffer et al., 1991), and PKC- θ (Srivastava et al., 2004). Despite the variety of PKC isoforms, most phosphorylate similar sequences (Breitkreutz et al., 2007). Both the PKC- α/β inhibitor (Gö6976) and PKC- δ inhibitor (rottlerin) blocked the inhibitory effect of IFN- α on En II activity. Thus, it was speculated that each PKC isoform might be similarly involved in suppressing of En II activity.

Other studies have examined the role of the PKC pathway in HBV replication. Kang et al. (2008) reported that PKC-mediated phosphorylation increased capsid assembly and stability (von Hahn et al., 2011), and von Hahn et al. (2011) reported that the

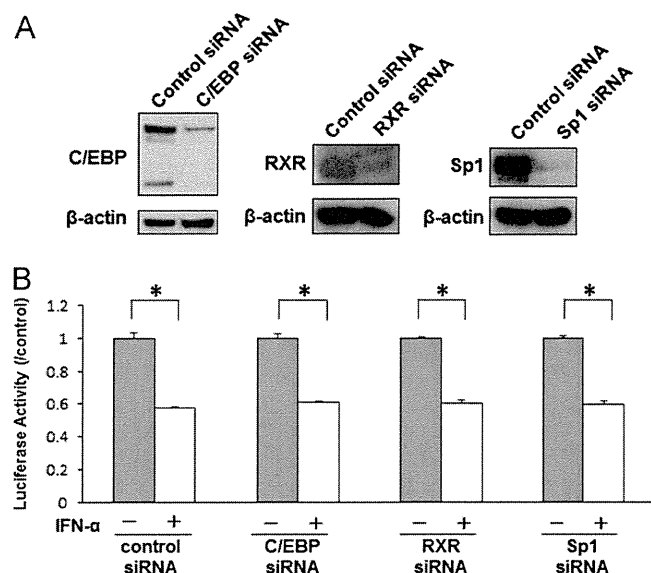


Fig. 6. IFN- α -mediated suppression on En II activity with knockdown of C/EBP, RXR and Sp1. A. Huh-7 cells were transfected with 10 nM siRNA (negative control or specific for C/EBP, RXR and Sp1). Immunoblot analyses for expressions of C/EBP, RXR, Sp1 and β -actin were performed at 48 h post siRNA transfection. B. Huh-7 cells were transfected with 10 nM siRNA (negative control or specific for C/EBP, RXR and Sp1). On the next day, si-RNA treated cells were transfected again with pGL4LUC-En II. On the following day, these transfected cells were incubated with IFN- α (1000 IU/ml) for 12 h, and luciferase activities were evaluated. “/control” on the vertical axis means the ratio of luciferase activity of IFN- α treated cells normalized with that of non-treated cells.

pan-PKC inhibitor sotrastaurin did not affect HBV replication. While the role of PKC in the HBV life cycle is still controversial, our findings suggest that PKC isoforms activated by IFN- α play inhibitory roles in HBV transcription by down-regulation of En II activity. As von Hahn et al. reported, sotrastaurin alone did not affect HBV replication. But, based on our present data about another pan-PKC inhibitor, staurosporine, we speculate that sotrastaurin may also block the inhibitory effect of IFN- α on En II activity.

We showed that knockdown of a single transcription factor did not influence the IFN- α -mediated suppression of En II activity, suggesting that several transcription factors might be involved in this suppression. We also showed that both segment 4 (nt 1703–1727) and segment 6 (nt 1746–1770) within the En II region are required for the IFN- α -induced suppression of En II activity. Although these two regions seem to be more important than the others, all the deleted version of reporter constructs showed almost completely similar suppression activities (Fig. 3B). We speculate that there may be some transcription factors which affect both the segment 4 and 6. Even if one of these regions is deleted, some factors may affect the other region, and result in the suppression of En II activity. Further study will be needed to clarify the mechanism.

Indeed, there are no identified transcription factors which could bind both segment 4 and 6. Only two transcription factors (HNF1 and 3) were reported to bind segment 4 (Johnson et al., 1995; Wang et al., 1998), and there have been no reports indicating that IFN- α or PKC inactivates HNF1 or 3. We also examined the expression levels of HNF1 and 3 of the IFN- α treated and the non-treated cells by RT-PCR. There was no significant difference in the expression of these transcription factors between the IFN- α treated and the non-treated cells (Nawa et al., unpublished data). Thus, we speculate that HNF1 or 3 might not be involved in the IFN- α mediated suppression of En II activity. There may be unknown transcription factors in the PKC pathway.

Previous reports showed that IFN- α suppressed En I activity (Nakao et al., 1999; Tur-Kaspa et al., 1990). Nakao et al. (1999) indicated that this occurred due to the binding of ISGF3 to an ISRE-like motif within the En I region. However, Rang et al. (2001) demonstrated that IFN- α reduced HBV-RNA levels derived from both HBV genome wild type and mutated ISRE-like motifs. This result contradicted the Nakao's result that the activity of the En I mutated ISRE-like motif was not suppressed by IFN- α . Schulte-Frohlinde et al. (2002) reported that IFN- α suppressed HBV core promoter regulated transcriptional activity, even when the ISRE-like motif of En I was deleted. The results of Rang et al. and Schulte-Frohlinde et al. suggest that IFN- α might suppress the activity of regions other than En I. In the present study, we demonstrated that IFN- α suppressed En II activity via the PKC pathway. En II might be one of the candidate regions down-regulated by IFN- α within the HBV genome.

Since En II activates viral transcription only in hepatocytes, it is responsible for the hepatocyte-specific gene expression of HBV. There had been no study on the effect of IFN- α on En II activity. Our study clarified that the PKC pathway is involved in the IFN- α -mediated suppression of En II activity, but may not involve ISG induction. Our result should aid in establishing better treatment with IFN- α against HBV infection. As we could not determine the molecule which inhibits En II activity by IFN- α , further study is needed to clarify this molecule and to control hepatitis B by IFN- α treatment.

Materials and methods

Plasmids

The HBV sequence used in this study was of the *adw2* subtype (GenBank accession no. X02763). Numbering of the HBV sequence started at the unique *EcoRI* site. The En II region in this study was defined as nt 1640–1771 of HBV sequence (Fig. 1) (Ishida et al., 2000). To construct pGL4LUC-En II, a plasmid containing the HBV En II region, the DNA fragment was amplified with PCR and inserted between *Hind* III and *Nhe* I site of pGL4 Luciferase Reporter Vector (pGL4LUC) (Promega, Madison, WI). The PCR primers were as follows: 5'-CCAAGCTTCTGCCAAGGTC-3' and 5'-CCCCTAGCAAAGACCTTTAACCTAATCTCTCC-3'. The constructs of the En II sequence with various deletions were generated by modifying pGL4LUC-En II using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The constructs containing four tandem repeats of short fragment in En II sequence were generated by inserting duplexes of synthesized oligonucleotides into the multicloning site of pGL4LUC. All of the En II sequences were inserted in the antisense orientation to evaluate their enhancer activity.

Plasmid pHBV1.5 containing a 1.5-fold-overlength genome of HBV-DNA (GenBank accession no. AF305422) has been described previously (Bruss and Ganem, 1991).

Cell lines and reagents

The human hepatocellular carcinoma cell lines Huh-7, PLC/PRF/5, and Hep3B were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO) in a humidified incubator at 5% CO₂ and 37 °C. Human natural IFN- α was kindly provided by Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan).

The inhibitors/activators and the final concentrations used were: JAK inhibitor I (1 μ M), PD98059 (10 μ M), SB203580 (10 μ M), LY294002 (10 μ M), Akt-I-1/2 (5 μ M), staurosporine (10 or 20 nM), rottlerin (5 μ M), G66976 (1 μ M), SP600125 (10 μ M)

(Calbiochem, San Diego, CA), phorbol 12-myristate 13-acetate (PMA) (100 nM) (Sigma-Aldrich, St. Louis, MO).

Plasmid transfection and luciferase assay

Huh-7 cells were co-transfected with the firefly luciferase plasmid and pGL4-RL-tk, an expression vector of renilla luciferase, which was used as an internal control, using FuGENE HD reagent (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol. Activities of firefly luciferase and renilla luciferase were measured using the Dual-Glo Luciferase Assay System (Promega, Madison, WI), and then relative luciferase activity was calculated by normalizing firefly luciferase activity to renilla luciferase activity.

RNA extraction

Total RNA was isolated from cells using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. The isolated RNA was treated with DNase I (Promega, Madison, WI) to avoid contamination with transfected plasmid, and then purified with a mixture of phenol, chloroform, and isoamylalcohol (pH 7.9), followed by ethanol precipitation.

Western blot analysis

Cultured cells were lysed with a lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and protein inhibitor cocktail (Nacalai Tesque), in PBS, pH 7.4). Equal amounts of protein were electrophoretically separated by polyacrylamide gel and transferred onto PVDF membrane. For immunodetection, the following antibodies were used: anti-STAT1 antibody, anti-phospho-STAT1 antibody, anti-phospho-PKC- α/β II (Thr 638/641) antibody, anti-phospho-PKC- δ (Thr 505) antibody, anti-C/EBP antibody, anti-RXR antibody, anti-Sp1 antibody, anti- β -actin antibody from Cell Signaling Technology (Beverly, MA), and anti-Mx antibody from Abcam (Cambridge, UK). The signals of phosphorylated proteins such as phospho-PKC- α/β , - δ and phospho-STAT1 were analyzed quantitatively using image analyzing software (ImageJ; version 1.45).

Small RNA interference

Stealth Select RNAi specific for STAT1 (HSS 10273) was purchased from Invitrogen (Carlsbad, CA). Silencer Select siRNA specific for C/EBP (ID: S2890), RXR (ID: S12386) and Sp1 (ID: S13319) were purchased from Ambion (Austin, TX). Stealth RNAi Negative Control Low GC Duplex (Invitrogen, Carlsbad, CA) was used as a control for the off-target effect following Stealth Select RNAi delivery. The transfections were carried out using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to the reverse transfection protocol.

Real-time reverse-transcription PCR

For cDNA synthesis, 1 μ g of total RNA was reverse-transcribed using High Capacity RNA-to-DNA Master Mix (Applied Biosystems, Foster City, CA). cDNA, equivalent to 20 ng RNA, was used as a template for real-time reverse-transcription PCR (RT-PCR) using Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). mRNA expressions of C/EBP, FTF, HNF1, HNF3, and HNF4 were measured using TaqMan Gene Expression Assays and were corrected with the quantified expressions level of β -actin mRNA. Assay IDs for the genes were as follows: C/EBP (Hs00269972_s1), FTF (Hs00187067_m1), HNF1 (Hs00167041_m1), HNF3 (Hs00232754_m1), and HNF4 (Hs01023298_m1).

For the detection of pgRNA and pre-C mRNA, the primers and the probes were designed as follows according to a previous study (Laras et al., 2002): the sense primer was 5'-TCTTGACATGTCCTCACTGTTCAA-3' (nt 1843–1866); the anti-sense primer was 5'-AATGCCATGCCCCAAAGC-3' (nt 1890–1909); the probe was 5'-FAM-CTCCAAGCTGTGCCTT-3' (nt 1869–1884). Since they were within precore/core coding sequence, only the total abundance of pgRNA and pre-C RNA could be detected.

Statistical analysis

Data were presented as mean \pm SD. Differences between two groups were determined using Student's t-test for unpaired observations. $p < 0.05$ was considered statistically significant.

Disclosures

All authors have nothing to disclose.

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

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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.

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 dis-

ease-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 evolutionarily 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 sequesters 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

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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% CO₂.

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 × 10⁶ 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 = $\pi/6 \times (\text{major axis}) \times (\text{minor axis})^2$. 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 ± 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

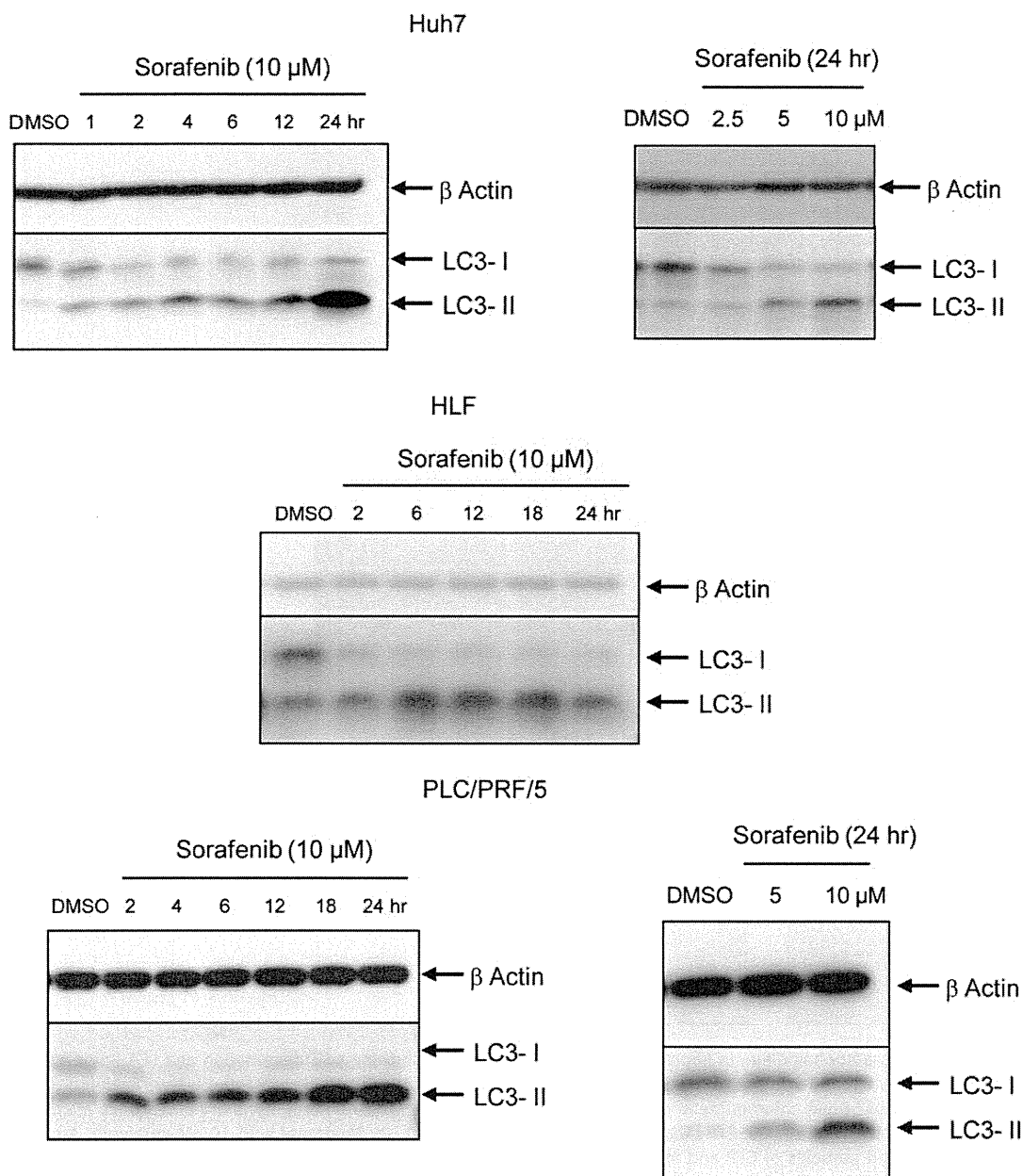


Figure 1. Sorafenib induces accumulation of autophagosomes in hepatoma cells. Western blot showing an increase in LC3-II in Huh7, HLF and PLC/PRF/5 hepatoma cells after treatment with sorafenib. Hepatoma cells were treated with 2.5, 5 or 10 μM sorafenib for the indicated times and analyzed for LC3 expression 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.

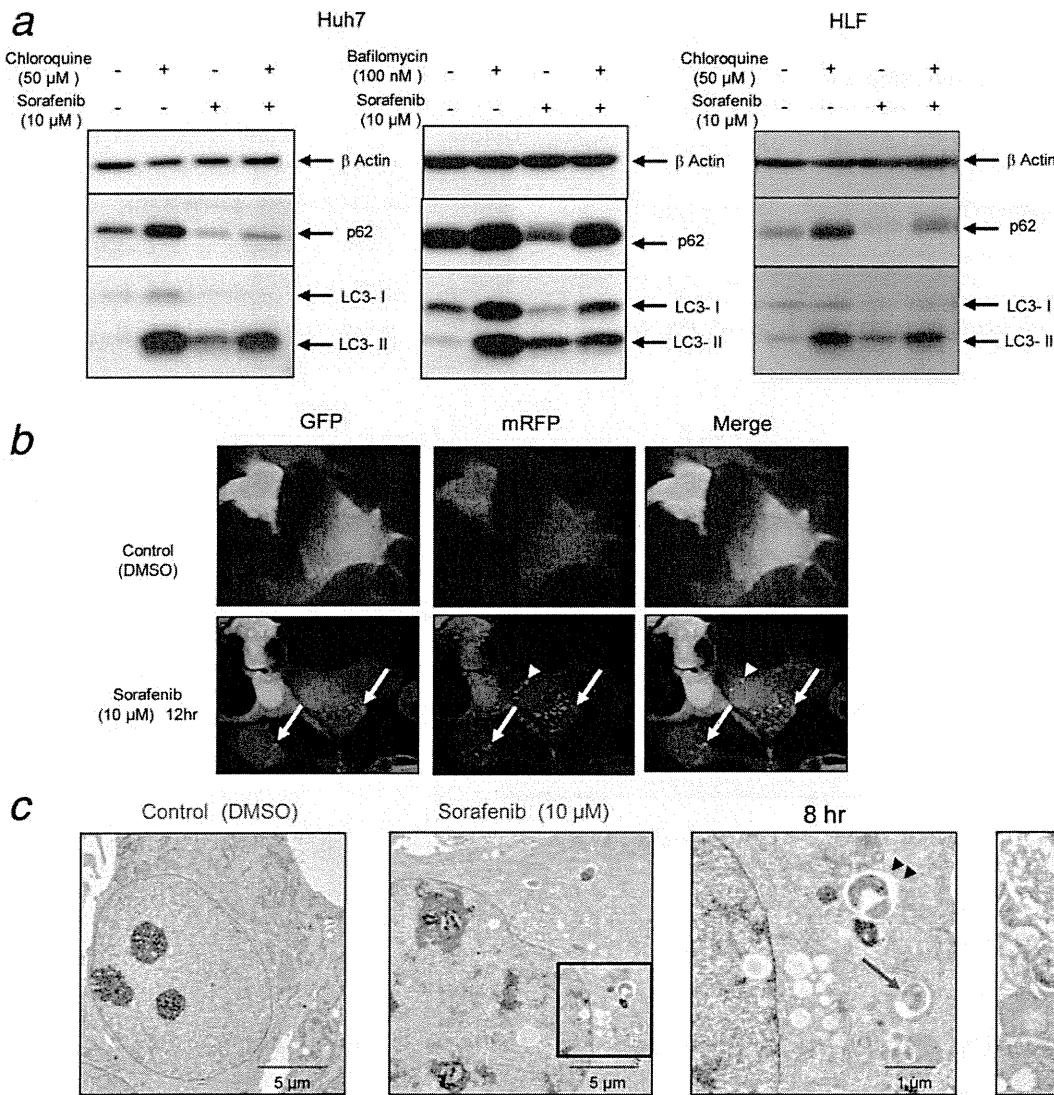


Figure 2. Sorafenib activates autophagic flux in hepatoma cells. (a). Western blot showing p62 degradation and LC3 lipidation in Huh7 cells and HLF cells treated with sorafenib and/or lysosomal inhibitors. Huh7 cells or HLF cells were treated with or without 10 μ M sorafenib in the presence or absence of 50 μ M 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 μ M 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 μ M 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. 2a). 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. 2b). 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. 2c).

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 VEGFR-2, Flt-3 and c-Kit.¹⁷ The inhibitory effect of sorafenib on the Raf/MEK/ERK pathway¹⁸ or the STAT3 pathway¹⁹ 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. 3a). 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 Torin1²³ 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. 3b). 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 anti-tumor 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. 4a). 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. 4b). 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 anti-tumor effects. We performed an ATG7 knockdown experiment using HLF cells as well and obtained a similar result (Fig. 4c).

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 2a. 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. 6a). 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. Co-administration of chloroquine and sorafenib for 7 days led to significant suppression of tumor growth compared with

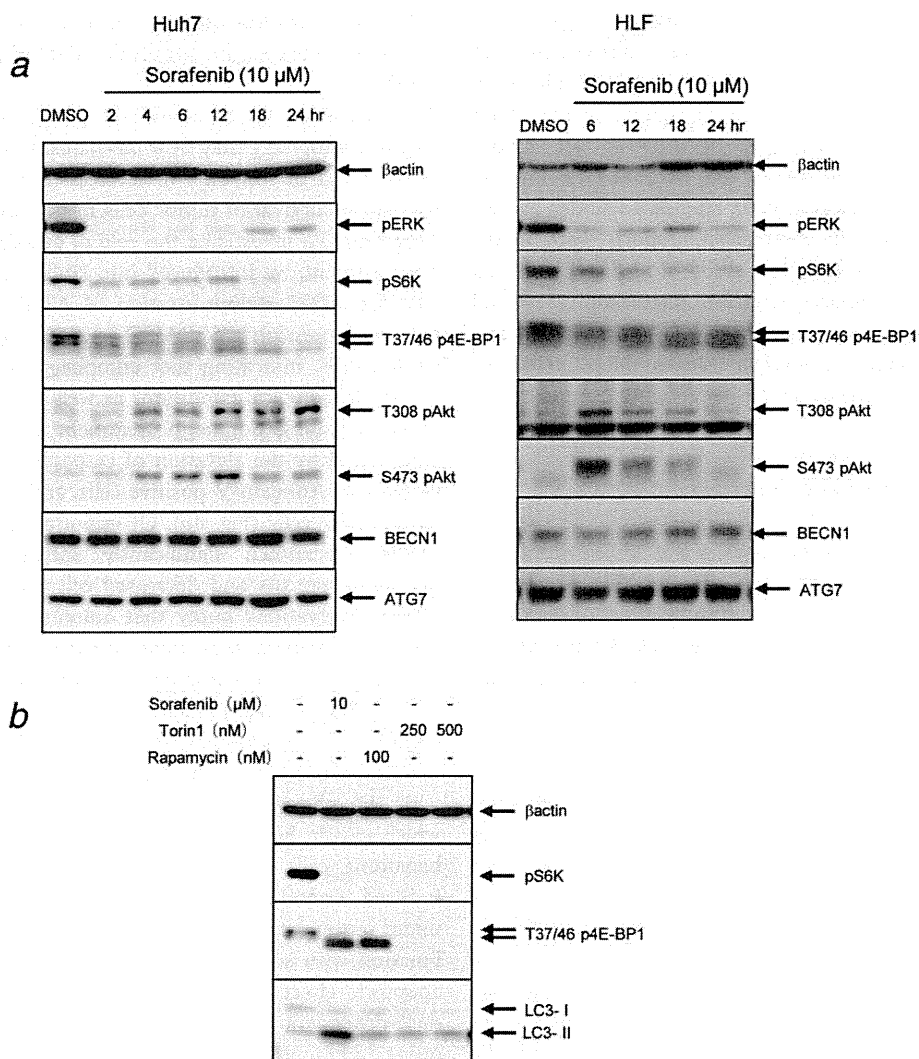


Figure 3. Raf/MEK/ERK and Akt/mTOR/S6K pathways in hepatoma cells treated with sorafenib. (a). Western blot showing decrease in ERK, S6K and 4E-BP1 phosphorylation, increase in Akt phosphorylation and stable expression of Beclin 1 and ATG7 in Huh7 cells and HLF cells after treatment with 10 μ M sorafenib. (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 μ M, 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. 6b). 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 radiation²⁵ 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

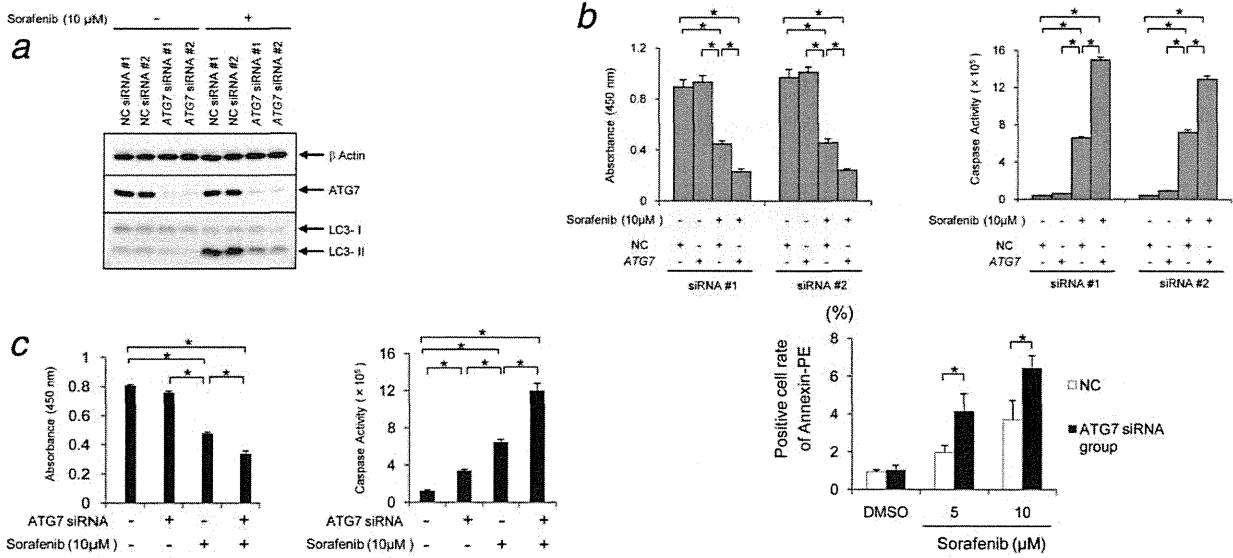


Figure 4. Genetic ablation of autophagy increases sensitivity of hepatoma cells to sorafenib. (a,b). Huh7 cells were transfected with two different sets of *ATG7* siRNA (no. 1 and 2) or control siRNA (no. 1 and 2) for 48 hr and then treated with the indicated concentration of sorafenib or vehicle for 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](http://www.interscience.wiley.com).]

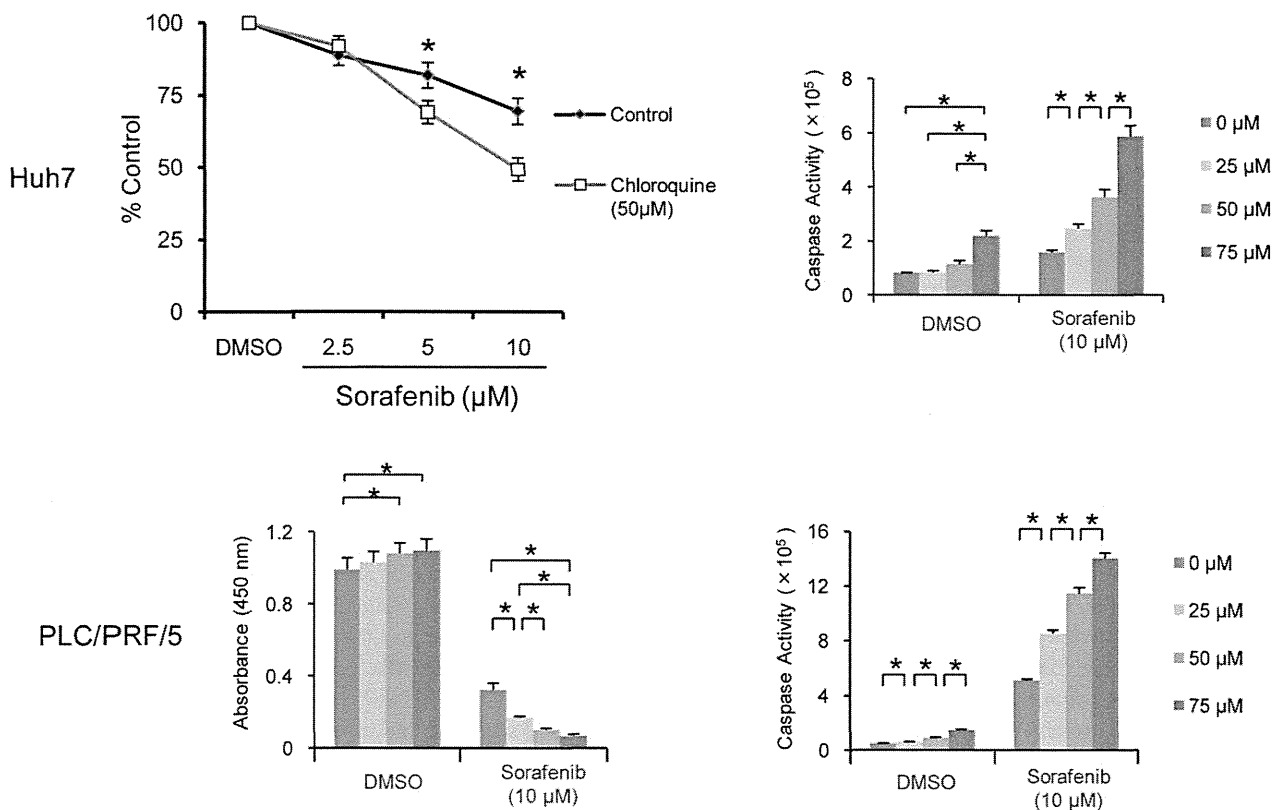


Figure 5. Pharmacological inhibition of autophagy increases sensitivity of hepatoma cells to sorafenib. Huh7 cells or PLC/PRF/5 cells were treated with or without the indicated concentration of sorafenib in the presence or absence of chloroquine for 18 hr. Caspase-3/7 activity was monitored in the supernatant,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

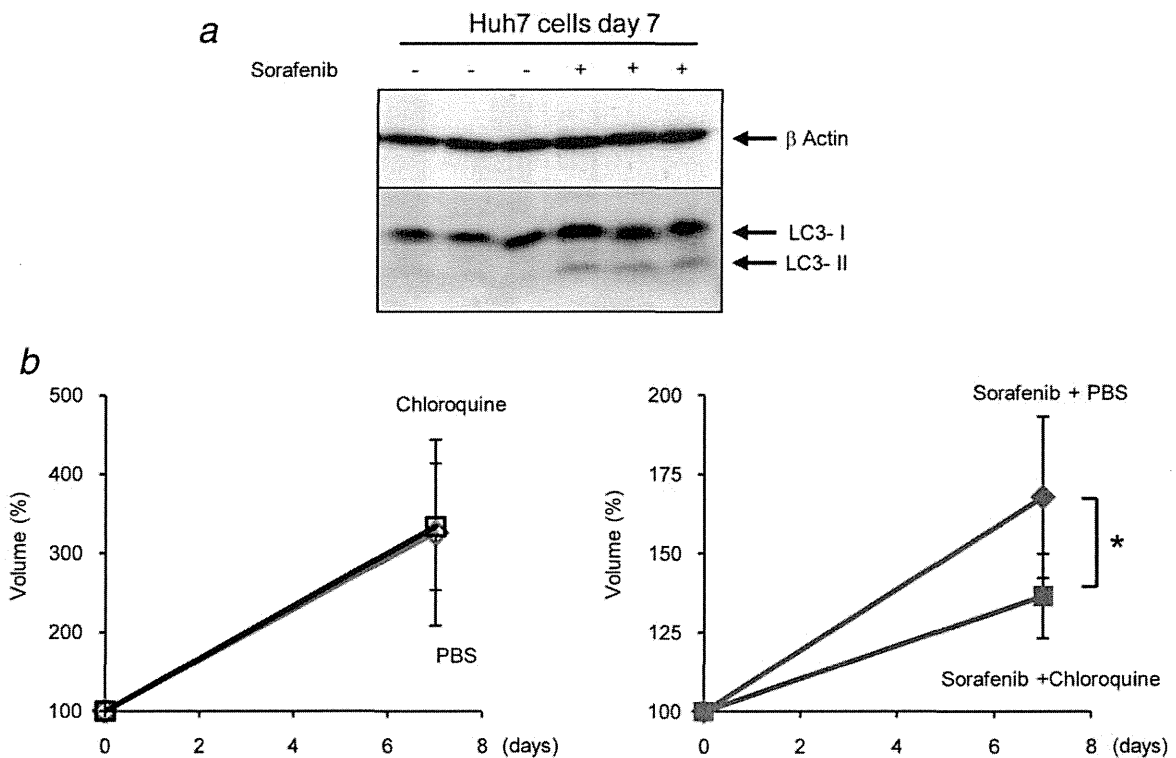


Figure 6. Inhibition of autophagy potentiates sorafenib-induced antitumor effects in Huh7 xenograft. (a). Western blot showing increase in LC3-II expression in Huh7 xenograft tumor after sorafenib therapy. Mice bearing xenograft tumor were administered sorafenib (30 mg kg^{-1}) or vehicle for 7 days ($n = 3/\text{group}$). (b). Chloroquine (60 mg kg^{-1}) itself did not affect the tumor growth of Huh7 xenograft (left panel), ($n = 7/\text{group}$), but enhanced the effect of sorafenib (30 mg kg^{-1}) in a synergistic manner (right panel), ($n = 6/\text{group}$). 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- β 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* knock-down) 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.

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Bak deficiency inhibits liver carcinogenesis: A causal link between apoptosis and carcinogenesis

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Background & Aims: Hepatocyte apoptosis is a key feature of chronic liver disease including viral hepatitis and steatohepatitis. A previous study demonstrated that absence of the Bcl-2 family protein Mcl-1 led to increased hepatocyte apoptosis and development of liver tumors in mice. Since Mcl-1 not only inhibits the mitochondrial pathway of apoptosis but can also inhibit cell cycle progression and promote DNA repair, it remains to be proven whether the tumor suppressive effects of Mcl-1 are mediated by prevention of apoptosis.

Methods: We examined liver tumor development, fibrogenesis, and oxidative stress in livers of hepatocyte-specific knockout (KO) of *Mcl-1* or *Bcl-xL*, another key antagonist of apoptosis in hepatocytes. We also examined the impact of additional KO of *Bak*, a downstream molecule of Mcl-1 towards apoptosis but not the cell cycle or DNA damage pathway, on tumor development, hepatocyte apoptosis, and inflammation.

Results: *Bcl-xL* KO led to a high incidence of liver tumors in 1.5-year-old mice, similar to *Mcl-1* KO. *Bcl-xL*- or *Mcl-1*-deficient livers showed higher levels of TNF- α production and oxidative stress than wild-type livers at as early as 6 weeks of age and oxidative DNA damage at 1.5 years. Deletion of *Bak* significantly inhibited hepatocyte apoptosis in *Mcl-1* KO mice and reduced the incidence of liver cancer, coinciding with reduction of TNF- α production, oxidative stress, and oxidative DNA damage in non-cancerous livers.

Conclusions: Our findings strongly suggest that chronically increased apoptosis in hepatocytes is carcinogenic and offer genetic evidence that inhibition of apoptosis may suppress liver carcinogenesis in chronic liver disease.

Keywords: Bcl-xL; Mcl-1; 8-OHdG.

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Abbreviations: HCC, hepatocellular carcinoma; ALT, alanine aminotransferase; RT-PCR, reverse-transcription PCR; HO-1, heme oxygenase-1; NQO1, NAD(P)H:quinone oxidoreductase 1; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling.

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Introduction

Apoptosis of epithelial cells, as well as infiltration of inflammatory cells or deposits of fibers, is frequently observed in the chronic diseased liver, which is a high-risk condition for hepatocellular carcinoma (HCC) [1]. For example, Fas-mediated hepatocyte apoptosis is a mechanism of cell death in chronic hepatitis C virus infection and hepatitis B virus infection [2,3]. Hepatocyte apoptosis shows correlation with inflammation and fibrosis in non-alcoholic steatohepatitis [4]. Cytokeratin 18 neopeptide, a well-established marker of caspase activity in serum, is elevated and associated with liver injury in chronic viral hepatitis and non-alcoholic steatohepatitis [5–7]. Although viral factors and overt organ inflammation linked to liver cancer development have been extensively studied [8,9], less information is available on the involvement of hepatocyte apoptosis in liver cancer development.

Bcl-xL and *Mcl-1* are among the anti-apoptotic members of the Bcl-2 family, which antagonizes the pro-apoptotic function of *Bak* and/or *Bax* at the mitochondrial outer membrane. We previously reported that hepatocyte-specific *Bcl-xL* or *Mcl-1* knockout (KO) mice showed persistent apoptosis of hepatocytes in the adult liver and mild fibrotic responses [10,11]. A very recent study by Weber *et al.* [12] demonstrated that hepatocyte-specific *Mcl-1* KO mice developed liver tumors in old age. This observation raised the important possibility that apoptosis in hepatocytes could lead to the development of liver cancer. However, as *Mcl-1* has been reported to possess functions other than anti-apoptosis, such as cell cycle inhibition [13,14] and DNA damage repair [15,16], it is difficult to conclude that the phenotypes observed in *Mcl-1* KO are simply ascribable to apoptosis. Indeed, *Mcl-1* KO mice showed not only increased apoptosis but also increased regeneration in the liver [12]. In the present study, we demonstrated that hepatocyte-specific *Bcl-xL* KO mice also develop liver cancer in old age and that deficiency of *Bak*, a downstream effector molecule of *Mcl-1* towards the



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Table 1. Incidence of liver tumors in KO mice.

Age (yr)	Genotype	Tumor incidence
1.5	<i>Bcl-xL</i> ^{+/+}	0% (0/10)
	<i>Bcl-xL</i> ^{-/-}	88% (7/8)*
1	<i>Bcl-xL</i> ^{+/+}	0% (0/4)
	<i>Bcl-xL</i> ^{-/-}	27% (3/11)
1.5	<i>Mcl-1</i> ^{+/+}	0% (0/22)
	<i>Mcl-1</i> ^{-/-}	100% (16/16)*
1	<i>Mcl-1</i> ^{-/-} <i>Bak</i> ^{+/+}	64% (14/22)
	<i>Mcl-1</i> ^{-/-} <i>Bak</i> ^{-/-}	0% (0/7)*

*p <0.05 vs. control.

mitochondrial pathway of apoptosis, clearly suppresses hepatocyte apoptosis and liver carcinogenesis in *Mcl-1* KO mice. We also considered possible mechanisms involving oxidative stress that underlie elevated malignant transformation in the apoptosis-prone liver. The present study offers strong support for the hypothesis that chronically increased apoptosis in hepatocytes is carcinogenic. It also provides genetic evidence that inhibition of apoptosis may suppress liver carcinogenesis in chronic liver disease.

Materials and methods

Mice

Conditional *Bcl-xL* KO mice (*bcl-x*^{fllox/flox} *Alb-Cre*) and *Mcl-1* KO mice (*mcl-1*^{fllox/flox} *Alb-Cre*) were previously described [11]. We purchased *Bak* KO mice (*bak*^{-/-}) from the Jackson Laboratory (Bar Harbor, ME). We generated hepatocyte-specific *Bak*/*Mcl-1* double KO mice (*bak*^{-/-} *mcl-1*^{fllox/flox} *Alb-Cre*) by mating the strains. They were maintained in a specific pathogen-free facility and treated with humane care with approval from the Animal Care and Use Committee of Osaka University Medical School. Measurement of serum alanine aminotransferase (ALT) level, caspase-3/7 activity and histological analyses have been previously described [11].

Western blot analysis

For immunodetection, the following antibodies were used: anti-*Bcl-xL* antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-*Mcl-1* antibody (Rockland, Gilbertsville, PA), anti-*Bak* antibody (Millipore, Billerica, MA), anti-*Bax* antibody, anti-ERK antibody, anti-phospho-ERK antibody, anti-p38 antibody, anti-phospho-p38 antibody, anti-JNK antibody, anti-phospho-JNK antibody, anti-PCNA antibody (Cell Signaling Technology, Danvers, MA), and anti-beta-actin antibody (Sigma-Aldrich, Saint Louis, MO).

Real-time reverse-transcription PCR (RT-PCR)

The following TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) were used: mouse-AFP (Mm00431715_m1), mouse-glypican-3 (Mm00516722_m1), mouse-IL-6 (Mm00446190_m1), mouse-TNF- α (Mm00443258_m1), mouse-MCP-1 (Mm00441242_m1), mouse-CD68 (Mm03047343_m1), mouse-CD4 (Mm00442754_m1), mouse-CD8 (Mm01182108_m1), mouse-heme oxygenase-1 (HO-1) (Mm00516005_m1), mouse-NAD(P)H:quinone oxidoreductase 1 (NQO1) (Mm00500821_m1), and mouse-Beta actin (Mm00607939_s1). All expression levels were corrected with the quantified expression level of beta actin.

Immunohistochemistry

8-Hydroxy-2'-deoxyguanosine (8-OHdG), cleaved caspase-9, PCNA, and ki-67 were labeled in paraffin-embedded liver sections using anti-8-OHdG antibody (Nikken Seil, Tokyo, Japan), anti-cleaved caspase-9 antibody, anti-PCNA antibody (Cell Signaling Technology), and anti-ki-67 antibody (Dako, Tokyo, Japan), respectively. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) was performed according to a previously reported procedure [17].

Statistical analysis

Data are presented as mean \pm SD. Differences between two groups were determined using the Student's *t*-test for unpaired observations. Carcinogenesis rates were analyzed using the Chi-square test. Multiple comparisons of *Bak*/*Mcl-1* double KO mice were performed by ANOVA followed by Scheffe *post hoc* correction. Fisher *post hoc* correction was used for the other multiple comparisons. A *p* <0.05 was considered statistically significant.

Results

Bcl-xL KO mice develop liver tumors in old age

We previously reported that hepatocyte-specific *Bcl-xL* KO mice developed spontaneous hepatocyte apoptosis by the mitochondrial pathway (Supplementary Fig. 1A) at as early as 1 month of age with a gradual increase in the liver fibrotic response from 3 to 7 months [10]. To examine the phenotypes at later time points, we sacrificed *Bcl-xL* KO mice and their control littermates at 1 and 1.5 years of age. Macroscopic tumors had developed in the liver of 27% and 88% of the KO mice, respectively, but not in the control littermates (Fig. 1A and Table 1). Most of the *Bcl-xL* KO mice had multiple tumors and the liver body-weight ratio for *Bcl-xL* KO mice was significantly higher than that of the control mice (Fig. 1B and C). Tumors were histologically defined as well-differentiated HCCs (Fig. 1D). To find out whether the *bcl-x* gene is really targeted in the tumors, we performed Western blot analysis for the expression of the Bcl-2 family proteins (Fig. 1E and Supplementary Fig. 2A). The tumors were confirmed to be deficient for *Bcl-xL*, excluding the possibility that transformed cells arising from hepatocytes in which the *bcl-x* gene was not deleted had expanded to form tumors. Interestingly, most of these tumors showed apparently higher levels of *Mcl-1* expression than the wild-type liver or the non-cancerous surrounding tissues. Reciprocal overexpression of *Mcl-1* may explain the possible survival advantage of these tumors. Tumors in *Bcl-xL* KO mice expressed higher levels of α -fetoprotein (Fig. 1F) and frequently showed activation of ERK and JNK (Fig. 1G), which are observed in human HCC [18,19].

Liver tumors in Mcl-1 KO mice show similar characteristics to human HCC

We have previously reported phenotypes of hepatocyte-specific *Mcl-1* KO mice, which display spontaneous hepatocyte apoptosis by the mitochondrial pathway (Supplementary Fig. 1B) and liver fibrotic responses at an early age [11]. Since our *Mcl-1* floxed mice differed from those of Weber *et al.* [12] in origin, we next examined the development of liver tumors in our hepatocyte-specific *Mcl-1* KO mice. All the *Mcl-1* KO mice, but none of the control littermates, developed liver tumors at 1.5 years of age, with a significant increase of liver body-weight ratio (Fig. 2A-C