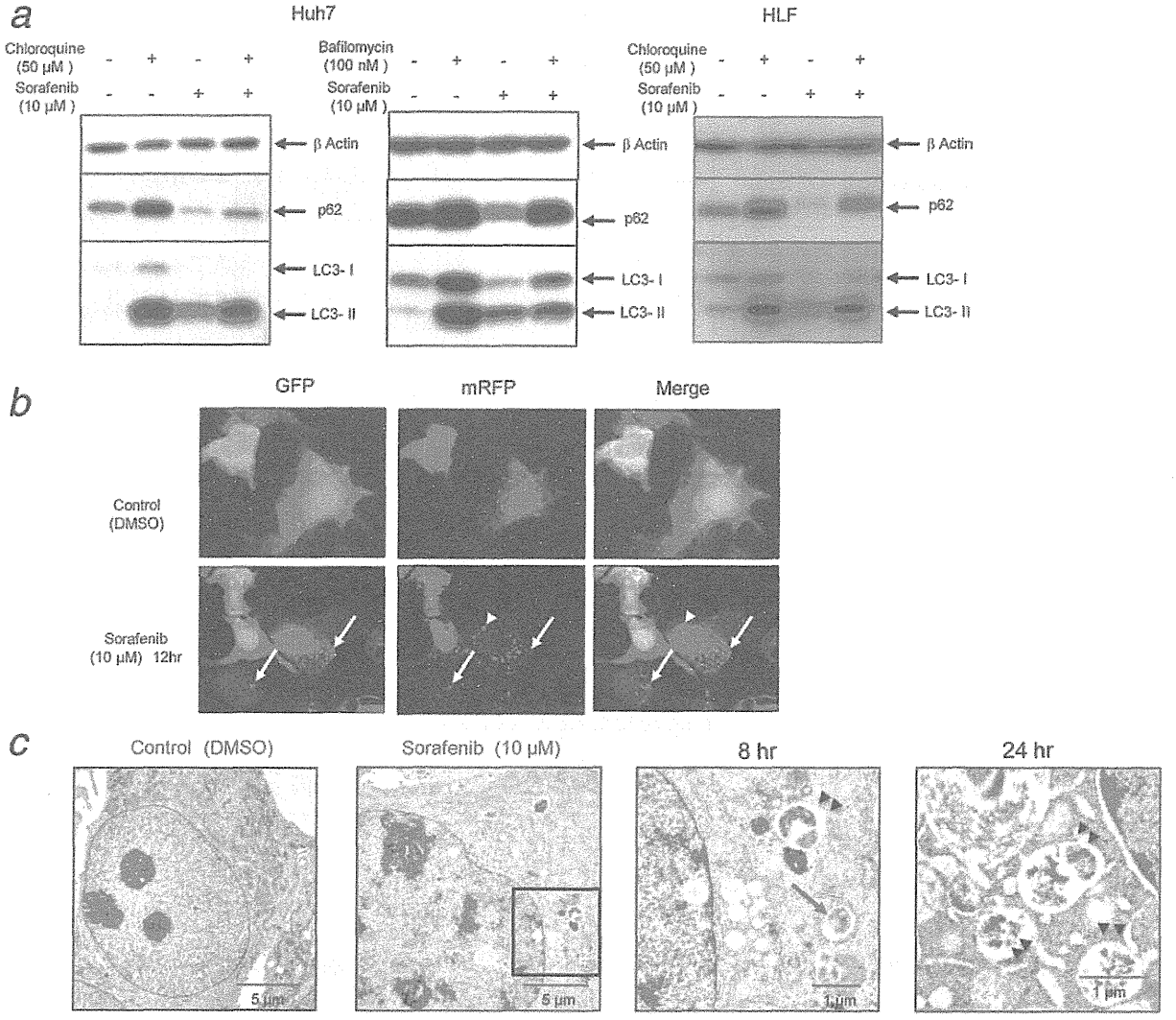

Figure 2. Sorafenib activates autophagic flux in hepatoma cells. (*a*). Western blot showing p62 degradation and LC3 lipidation in Huh7 cells and HLF cells treated with sorafenib and/or lysosomal inhibitors. Huh7 cells or HLF cells were treated with or without 10 μM sorafenib in the presence or absence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

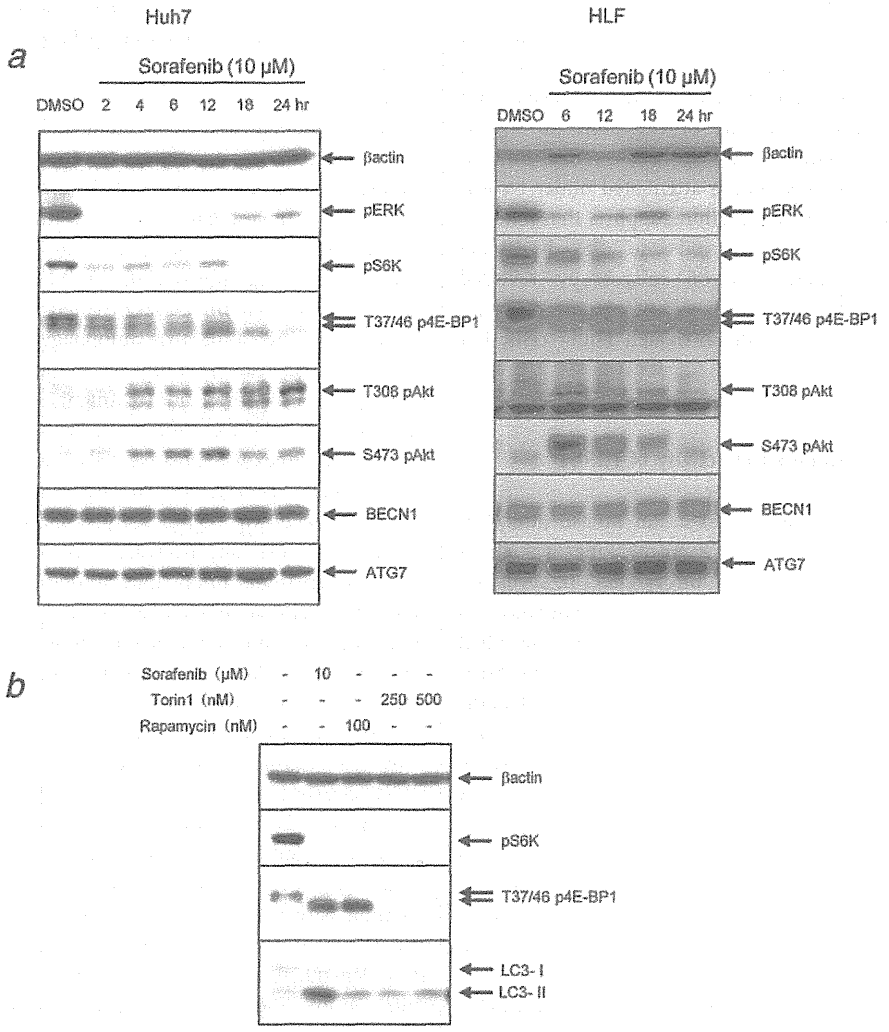

Figure 3. Raf/MEK/ERK and Akt/mTOR/S6K pathways in hepatoma cells treated with sorafenib. (*a*). Western blot showing decrease in ERK, S6K and 4E-BP1 phosphorylation, increase in Akt phosphorylation and stable expression of Beclin 1 and ATG7 in Huh7 cells and HLF cells after treatment with 10 μM sorafenib.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

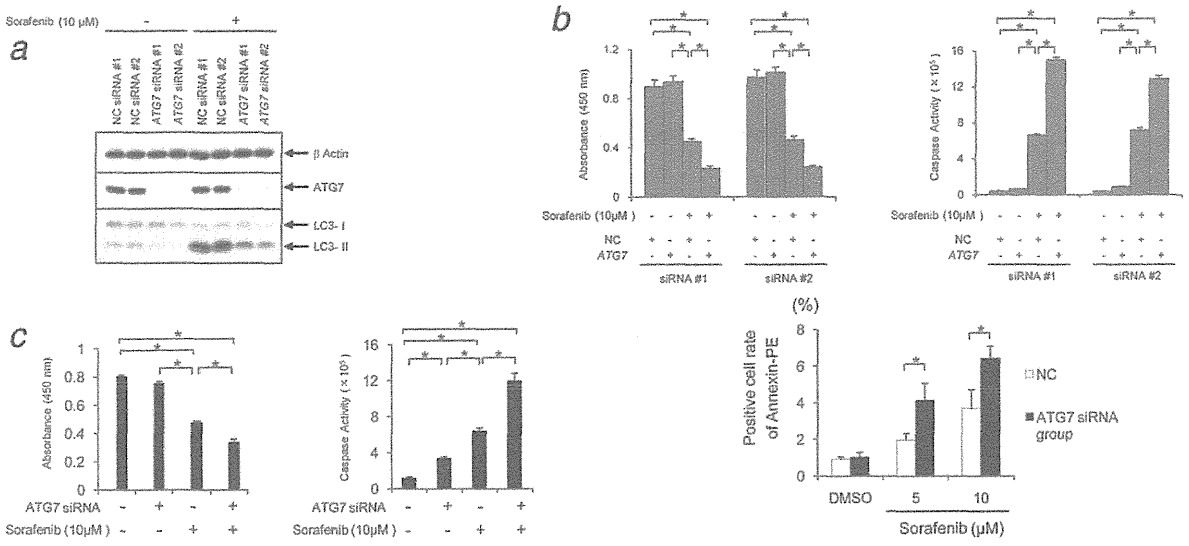

Figure 4. Genetic ablation of autophagy increases sensitivity of hepatoma cells to sorafenib. (*a*,*b*). Huh7 cells were transfected with two different sets of *ATG7* siRNA (no. 1 and 2) or control siRNA (no. 1 and 2) for 48 hr and then treated with the indicated concentration of sorafenib or vehicle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

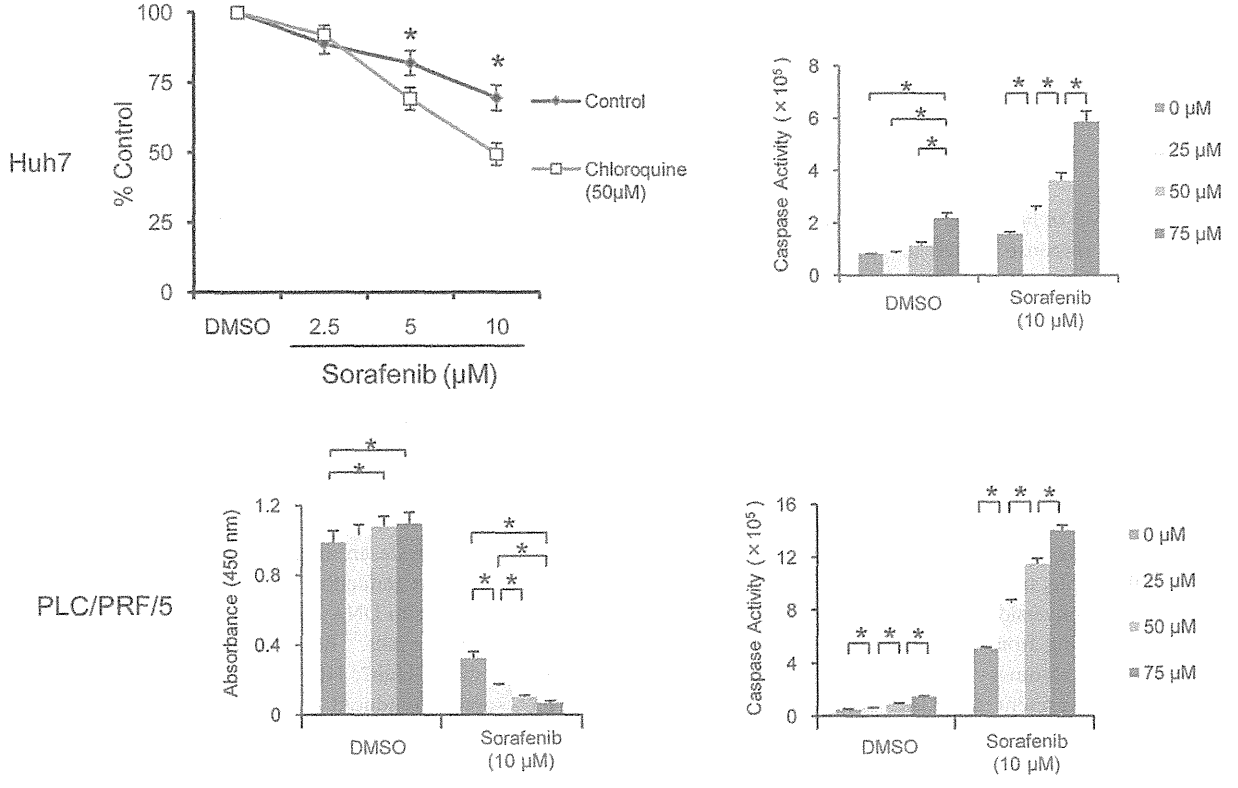

Figure 5. Pharmacological inhibition of autophagy increases sensitivity of hepatoma cells to sorafenib. Huh7 cells or PLC/PRF/5 cells were treated with or without the indicated concentration of sorafenib in the presence or absence of chloroquine for 18 hr. Capsase-3/7 activity was monitored in the supernatant,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

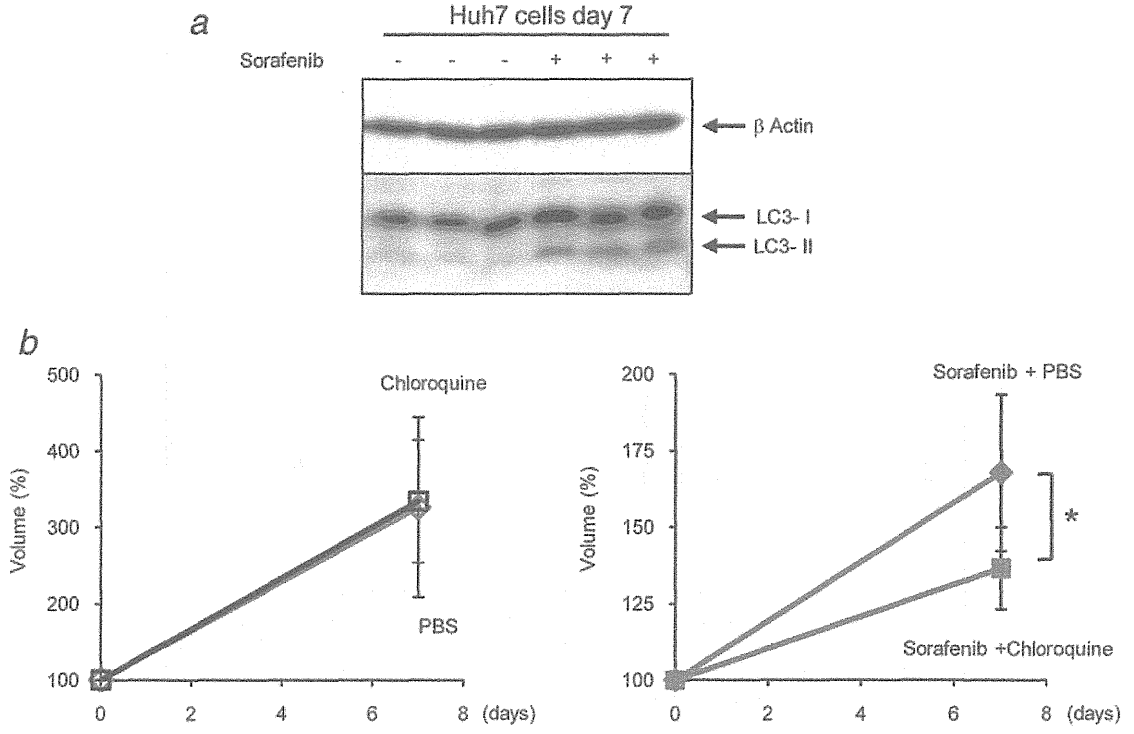

Figure 6. Inhibition of autophagy potentiates sorafenib-induced antitumor effects in Huh7 xenograft. (*a*). Western blot showing increase in LC3-II expression in Huh7 xenograft tumor after sorafenib therapy. Mice bearing xenograft tumor were administered sorafenib (30 mg kg^{-1}) or vehicle for 7 days ($n = 3$/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.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Reducing Peg-IFN doses causes later virologic response or no response in HCV genotype 1 patients treated with Peg-IFN alfa-2b plus ribavirin

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Abstract
Background The timing to the first undetectable hepatitis C virus (HCV) RNA level is strongly associated with sustained virologic response in pegylated interferon (Peg-IFN) plus ribavirin combination therapy for patients with chronic hepatitis C (CH-C) with genotype 1. This study was conducted to clarify the impact of drug exposure to Peg-IFN on the timing of HCV RNA negativity in Peg-IFN plus ribavirin combination therapy for CH-C patients with genotype 1.

Methods A total of 1409 patients treated with Peg-IFN alfa-2b plus ribavirin were enrolled and classified into four categories according to the Peg-IFN dosage. Furthermore, 100 patients were extracted from each Peg-IFN dosage category to adjust for characteristic factors, using the propensity score method.

Results Peg-IFN exposure was dose-dependently associated with the timing of HCV RNA negativity ($p \leq 0.001$). The HCV RNA negative rate at week 4 decreased from 12% with a Peg-IFN dose of >1.5 μg/kg/week to 1–3% with a dose of <1.5 μg/kg/week ($p \leq 0.001$), and at week 12 the rate had decreased from 44% with a dose of

T. Oze and N. Hiramatsu contributed equally to this work.

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≥1.2 μg/kg/week to 18% with a dose of <1.2 μg/kg/week ($p = 0.001$). Treatment failure (patients without a 1-log decrease of HCV RNA at week 4 or a 2-log decrease of HCV RNA at week 12, or positive at week 24) was found in 54–66% of patients given <1.2 μg/kg/week ($p \leq 0.001$), and these patients accounted for 64% of the non-responders.

Conclusions The timing of HCV RNA negativity depends significantly on the Peg-IFN dose. Reducing the Peg-IFN dose can induce a later virologic response or non-response in HCV genotype 1 patients treated with Peg-IFN plus ribavirin.

Keywords Chronic hepatitis C · Pegylated interferon plus ribavirin · Drug adherence · HCV RNA negativity · Propensity score matched study

Introduction

The timing to the first undetectable hepatitis C virus (HCV) RNA level during pegylated interferon (Peg-IFN) plus ribavirin combination therapy for patients with chronic hepatitis C (CH-C) genotype 1 is strongly associated with a sustained virologic response (SVR), defined as undetectable HCV RNA at 24 weeks after the finishing of the treatment. The SVR rate was 87–100% in patients with undetectable HCV RNA at week 4, 73–81% at week 12, and 14–44% between weeks 12 and 24 in patients receiving the standard 48-week treatment [1–8]. These results suggest that HCV RNA negativity should be achieved as soon as possible during treatment in order to attain a higher SVR rate.

Previous studies have revealed that many factors affect the complete virologic response (c-EVR) and SVR, such as age, gender, degree of liver fibrosis, HCV genotype, HCV viral load, and the amount of drug exposure [1–3, 9–16]. Of these factors, only the amount of drug exposure can be controlled in order to try to improve the antiviral effect, as the other factors are fixed for individual patients. Also, recently, the single-nucleotide polymorphisms (SNPs) of the *IL28B* gene have been revealed to be associated with the antiviral effects of pegylated interferon-alpha and ribavirin therapy [17–19].

Peg-IFN has been reported to be dose-dependently correlated with c-EVR [13]. Patients' characteristic factors can be related to drug adherence, as aged patients, female patients, and patients with progression of liver fibrosis have a tendency to show low drug adherence. This suggests that patients with low drug adherence could be those who are difficult to treat. Therefore, patients with similar characteristic factors should be compared in order to precisely assess the actual impact of drug exposure on the timing to the first undetectable HCV RNA level.

Only a few randomized controlled trials (RCTs) have examined the relationship between drug dose reduction and antiviral effect with Peg-IFN plus ribavirin combination therapy [1–3, 20–23], and the findings are controversial. Manns et al. [1] reported that the SVR rate was significantly lower in patients given 0.5 μg/kg/week of Peg-IFN than in those given 1.5 μg/kg/week of Peg-IFN (34 vs. 42%, $p < 0.05$). McHutchison et al. [3] reported that the SVR rate did not differ between groups given 1.0 and 1.5 μg/kg/week of Peg-IFN (38 vs. 40%, $p = 0.20$). No detailed study of the relationship between dose reduction and delay of HCV RNA negativity or the relationship between dose reduction and an increase of non-responders to the treatment has been reported, and the real impact of drug exposure on the anti-viral effect remains unclear.

In this present work, we conducted a matched study in which characteristic factors other than drug exposure were adjusted using propensity scores. We investigated the impact of drug exposure to Peg-IFN on the timing to the first undetectable HCV RNA level.

Patients and methods

Patients

The present study was a retrospective, multicenter trial conducted by Osaka University Hospital and other institutions participating in the Osaka Liver Forum. A total of 1409 Japanese patients with CH-C treated with a combination of Peg-IFN alfa-2b plus ribavirin were enrolled in this study between December 2004 and July 2008.

Patients eligible for this study were those who were infected with HCV genotype 1 and had a viral load of $\geq 10^5$ IU/ml, but were negative for hepatitis B surface antigen and anti-human immunodeficiency virus. Patients were excluded from this study if they had decompensated cirrhosis or other forms of liver disease (alcoholic liver disease, autoimmune hepatitis). Informed consent was obtained from each patient included in this study, which was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki.

Treatment

All patients received Peg-IFN alfa-2b (PEGINTRON; Schering-Plough, Kenilworth, NJ, USA) plus ribavirin (REBETOL; Schering-Plough). Peg-IFN alfa-2b was given subcutaneously once weekly at a dosage of 60–150 μg based on body weight (body weight 35–45 kg, 60 μg; 46–60 kg, 80 μg; 61–75 kg, 100 μg; 76–90 kg, 120 μg; 91–120 kg, 150 μg) and ribavirin was given orally twice a day at a total dose of 600–1000 mg/day based on body weight

(body weight <60 kg, 600 mg; 60–80 kg, 800 mg; >80 kg, 1000 mg), according to the standard treatment protocol for Japanese patients.

Dose reduction

As a rule, dose modification, which was performed according to the intensity of the adverse hematologic effects, was done by following the manufacturer's drug information. The dose of Peg-IFN alfa-2b was reduced to 50% of the assigned dose if the white blood cell (WBC) count declined to $<1500/mm^3$, the neutrophil count declined to $<750/mm^3$, or the platelet (Plt) count declined to $<8 \times 10^4/mm^3$, and was discontinued if the WBC count declined to $<1000/mm^3$, the neutrophil count declined to $<500/mm^3$, or the Plt count declined to $<5 \times 10^4/mm^3$. Ribavirin was also reduced from 1000 to 600 mg, or from 800 to 600 mg, or from 600 to 400 mg if the hemoglobin (Hb) level decreased to <10 g/dl, and was discontinued if the Hb level decreased to <8.5 g/dl.

Virologic assessment and definition of virologic response

Serum HCV RNA level was quantified using the COBAS AMPLICOR HCV MONITOR test, version 2.0 (detection range 6–5000 KIU/ml; Roche Diagnostics, Branchburg, NJ, USA) and qualitatively analyzed using the COBAS AMPLICOR HCV test, version 2.0 (lower limit of detection 50 IU/mL). The HCV RNA level was evaluated every 4 weeks during treatment. A rapid virologic response (RVR) was defined as undetectable serum HCV RNA at week 4, a c-EVR as undetectable serum HCV RNA at week 12, and a late virologic response (LVR) as detectable HCV RNA at week 12 but undetectable at week 24. Patients with <a 1-log decrease in the HCV RNA level at week 4 or <a 2-log decrease at week 12 compared with the baseline or detectable HCV RNA at week 24 were considered to have experienced treatment failure (non-response, NR) and had to stop treatment. If patients discontinued the treatment due to adverse events, without HCV RNA negativity being attained, they were also regarded as having had treatment failure.

Assessment of drug exposure

The amounts of Peg-IFN alfa-2b and ribavirin actually taken were evaluated by reviewing the medical records and calculating the amount taken from the start until the timing of the first undetectable HCV RNA level for the patients achieving HCV RNA negativity, and calculating the amount taken throughout the treatment for the patients not attaining HCV RNA negativity. For patients who discontinued the treatment, if their HCV RNA had become negative before discontinuation, the drug amount data were calculated from the start of treatment until the timing of the first undetectable HCV RNA level, and if HCV RNA had not become negative before discontinuation, the data throughout the treatment before discontinuation were used. The amounts of both drugs were divided individually on the basis of body weight at baseline as the average: Peg-IFN alfa-2b was expressed as μg/kg/week and ribavirin as mg/kg/day.

Evaluation of impact of drug exposure on HCV RNA negativity

We evaluated the relationship between the exposure to both drugs and HCV RNA negativity at week 24 by univariate and multivariate analyses for the patients who completed 24 weeks of treatment, using the mean administration doses of both drugs during the first 24 weeks and the characteristic factors other than drug exposure at baseline.

The patients were divided into four categories according to the Peg-IFN dose: up to 0.9 μg/kg/week of Peg-IFN; from 0.9 to less than 1.2 μg/kg/week; from 1.2 to less than 1.5 μg/kg/week; and from 1.5 μg/kg/week. The propensity score matching method was used to adjust the patients' characteristic factors among these categories. This score was calculated for each patient by logistic regression analysis, with four patient characteristic factors as independent variables; age, gender, Plt values, and history of IFN treatment. We then performed 1:1 nearest neighbor matching within a caliper of 0.15 standard deviation of the propensity score: one patient in each group with 0.9–1.2 μg/kg/week, 1.2–1.5 μg/kg/week, and ≥1.5 μg/kg/week to one patient with <0.9 μg/kg/week, and extracted 100 patients from each category.

Statistical analysis

Baseline data for various demographic, biochemical, and virologic characteristics of the patients were expressed as means ± SD or median values. Factors associated with HCV RNA negativity at week 24 were assessed by univariate analysis using the Mann–Whitney *U*-test or the χ^2 test, and by multivariate analysis using logistic regression analysis. To analyze the difference between baseline data among the four Peg-IFN groups, analysis of variance (ANOVA) or the χ^2 test was performed. The significance of trends in values for the timing to the first undetectable HCV RNA level was determined with the Mantel–Haenszel χ^2 test. A two-tailed p value of <0.05 was considered significant. Statistical analysis was conducted with SPSS version 15.0J (SPSS, Chicago, IL, USA).

Table 1 Baseline characteristics of patients before matching

Factor	All patients	<0.9 μg/kg/week of Peg-IFN	0.9–1.2 μg/kg/week of Peg-IFN	1.2–1.5 μg/kg/week of Peg-IFN	≥1.5 μg/kg/week of Peg-IFN	*p* value
Number	1409	153	159	670	427	
Age (years)	56.3 ± 10.4	58.0 ± 9.9	57.3 ± 10.2	55.9 ± 10.6	56.3 ± 10.4	0.069
Sex: male/female	722/687	70/83	69/90	376/294	207/220	0.004
History of IFN treatment: naïve/experienced	862/547	98/55	96/63	408/262	260/167	0.894
White blood cells (/mm^3)	5060 ± 1532	4325 ± 1419	4566 ± 1394	5246 ± 1562	5215 ± 1456	<0.001
Neutrophils (/mm^3)	2578 ± 1073	2129 ± 1049	2258 ± 949	2699 ± 1080	2667 ± 1052	<0.001
Red blood cells (×10^4/mm^3)	440 ± 46	424 ± 42	429 ± 45	445 ± 46	441 ± 45	<0.001
Hemoglobin (g/dl)	14.0 ± 1.4	13.6 ± 1.2	13.7 ± 1.5	14.1 ± 1.4	14.1 ± 1.4	<0.001
Platelets (×10^4/mm^3)	16.3 ± 5.6	11.9 ± 3.9	13.2 ± 4.8	17.4 ± 5.7	17.4 ± 5.2	<0.001
ALT (IU/l)	78 ± 61	93 ± 64	87 ± 68	75 ± 58	73 ± 60	0.001
Serum HCV RNA (KIU/ml)[a]	1450	1300	1900	1700	1900	0.176
Histology (METAVIR)[b]						
Fibrosis, 0–2/3–4 (%)[c]	810/186 (17%)	78/34 (30%)	70/30 (30%)	392/77 (16%)	270/45 (14%)	<0.001
Activity, 0–1/2–3	527/468	43/67	38/62	259/210	187/129	<0.001

ALT alanine aminotransferase, *HCV* hepatitis C virus, *IFN* interferon, *Peg-IFN* pegylated interferon

[a] Data shown are median values

[b] Data missing for 413 patients

[c] Percent of patients with 3–4

Results

Clinical characteristics of all patients according to Peg-IFN dosage before matching

A total of 1409 patients were enrolled in this study, and the baseline characteristics of the patients are shown in Table 1. Based on the Peg-IFN dosage, these patients were classified into four categories. With the decrease of Peg-IFN dosage, the ratio of female-to-male patients increased, the peripheral blood cell count decreased, and the number of patients with progression of liver fibrosis (METAVIR fibrosis score 3 or 4) increased significantly ($p < 0.001$). Patients with a lower Peg-IFN dosage tended to be older ($p = 0.07$).

Next, we analyzed the factors associated with HCV RNA negativity at week 24 for the 1226 patients who completed 24 weeks of treatment, using the baseline characteristic variables, excluding liver histology, shown in Table 1 and the mean doses of both drugs during the first 24 weeks. The HCV RNA negative rate at week 24 was 68% (829/1226). The results of univariate analysis are shown in Table 2. The factors evaluated by multivariate analysis were those for which the p value was <0.10 by univariate analysis for HCV RNA negativity at week 24: age, gender, history of IFN treatment, WBC, neutrophils, red blood cells (RBC), Hb, Plt, alanine aminotransferase, and the mean doses of Peg-IFN and ribavirin during the first 24 weeks. By the multivariate analysis, in addition to the RBC value ($p = 0.02$), Plt value ($p < 0.001$), and history of IFN treatment ($p = 0.04$), the factor of Peg-IFN exposure was an independent factor for HCV RNA negativity at week 24 ($p < 0.001$) (Table 3). The mean dose of ribavirin did not show a significant correlation with HCV RNA negativity at week 24 ($p = 0.07$).

Table 2 Univariate analysis of factors associated with HCV RNA negativity at week 24

Factor	Negative	Positive	*p* value
Number	829	397	
Age (years)	55.1 ± 10.5	57.6 ± 10.1	<0.001
Sex: male/female	437/392	189/208	0.094
History of IFN treatment: naïve/experienced	523/306	229/168	0.069
White blood cells (/mm^3)	5175 ± 1498	4566 ± 1394	<0.001
Neutrophils (/mm^3)	2665 ± 1087	2429 ± 1059	<0.001
Red blood cells (×10^4/mm^3)	445 ± 44	434 ± 47	<0.001
Hemoglobin (g/dl)	14.1 ± 1.4	13.9 ± 1.4	0.004
Platelets (×10^4/mm^3)	17.2 ± 5.6	14.8 ± 5.3	<0.001
ALT (IU/l)	73 ± 55	83 ± 63	0.001
Serum HCV RNA (KIU/ml)	1750	1800	0.673
Mean Peg-IFN dose (μg/kg/week)	1.39 ± 0.24	1.25 ± 0.32	<0.001
Mean ribavirin dose (mg/kg/day)	10.6 ± 1.8	9.7 ± 2.2	<0.001

ALT alanine aminotransferase, *HCV* hepatitis C virus, *IFN* interferon, *Peg-IFN* pegylated interferon

Clinical characteristics of patients extracted from each Peg-IFN dosage category after matching

Patients in the four Peg-IFN categories were matched by the propensity score method and 100 patients were extracted from each category. The c-statics for the propensity score model between the patients with <0.9 μg/kg/week of Peg-IFN and those given different levels of Peg-IFN were 0.62 for 0.9–1.2 μg/kg/week of Peg-IFN, 0.82 for 1.2–1.5 μg/kg/week of Peg-IFN, and 0.82 for ≥1.5 μg/kg/week of Peg-IFN.

Table 3 Multivariate analysis of factors associated with HCV RNA negativity at week 24

Factor	Category	Odds ratio	95% CI	*p* value
Age	1 year	–	–	NS
Sex	Male/female	–	–	NS
History of IFN treatment	Naïve/experienced	0.756	0.581–0.984	0.037
White blood cells	$1 \times 10^3/mm^3$	–	–	NS
Neutrophils	$1 \times 10^3/mm^3$	–	–	NS
Red blood cells	$1 \times 10^4/mm^3$	1.004	1.001–1.007	0.02
Hemoglobin	1 g/dl	–	–	NS
Platelets	$1 \times 10^4/mm^3$	1.054	1.026–1.083	<0.001
ALT	1 IU/l	–	–	NS
Mean Peg-IFN dose	0.1 μg/kg/week	1.096	1.045–1.149	<0.001
Mean ribavirin dose	1 mg/kg/day	1.060	0.994–1.130	0.074

ALT alanine aminotransferase, *CI* confidence interval, *IFN* interferon, *NS* not significant, *Peg-IFN* pegylated interferon

The baseline characteristics of the patients extracted according to the Peg-IFN dosage category are shown in Table 4. There was no significant difference among the four Peg-IFN categories in any of the factors, indicating that the extracted cohort of 400 patients was well matched according to propensity score methods.

Timing to the first undetectable HCV RNA level according to Peg-IFN dosage

We evaluated the relationship between the virologic response during the treatment and the drug exposure to Peg-IFN using our matched cohort of 400 patients (Fig. 1). Of the 400 patients, 23 had discontinued treatment due to adverse events by week 24 (<0.9 μg/kg/week, $n = 5$; 0.9–1.2 μg/kg/week, $n = 4$; 1.2–1.5 μg/kg/week, $n = 5$; ≥1.5 μg/kg/week, $n = 9$). The proportion of patients with treatment failure increased according to the decrease in the dose of Peg-IFN: 66% among patients with <0.9 μg/kg/week of Peg-IFN, 54% among those with 0.9–1.2 μg/kg/week of Peg-IFN, 35% among those with 1.2–1.5 μg/kg/week of Peg-IFN, and 32% among those with ≥1.5 μg/kg/week of Peg-IFN ($p < 0.001$). Additionally, the timing to the first undetectable HCV RNA level tended to shift to an earlier time

Table 4 Baseline characteristics of patients after matching

Factor	All patients	<0.9 μg/kg/week of Peg-IFN	0.9–1.2 μg/kg/week of Peg-IFN	1.2–1.5 μg/kg/week of Peg-IFN	>1.5 μg/kg/week of Peg-IFN	*p* value
Number	400	100	100	100	100	
Age (years)	56.9 ± 9.6	57.4 ± 10.0	56.6 ± 9.9	56.8 ± 9.5	56.7 ± 9.1	0.941
Sex: male/female	190/210	47/53	47/53	46/54	50/50	0.948
History of IFN treatment: naïve/experienced	286/114	70/30	71/29	73/27	72/28	0.970
White blood cells ($/mm^3$)	4557 ± 1344	4331 ± 1310	4532 ± 1372	4642 ± 1399	4725 ± 1280	0.186
Neutrophils ($/mm^3$)	2261 ± 955	2070 ± 855	2200 ± 883	2416 ± 1068	2357 ± 972	0.054
Red blood cells ($\times 10^4/mm^3$)	429 ± 43	423 ± 40	427 ± 42	432 ± 44	434 ± 46	0.300
Hemoglobin (g/dl)	13.8 ± 1.5	13.7 ± 1.3	13.7 ± 1.6	13.9 ± 1.4	14.0 ± 1.5	0.300
Platelets ($\times 10^4/mm^3$)	12.1 ± 3.7	11.8 ± 3.9	12.0 ± 3.8	12.1 ± 3.5	12.4 ± 3.6	0.625
ALT (IU/l)	89 ± 67	98 ± 65	92 ± 70	86 ± 61	80 ± 69	0.254
Serum HCV RNA (KIU/ml)[a]	1700	1400	1800	1700	1750	0.742
Histology (METAVIR)[b]						
Fibrosis, 0–2/3–4 (%)[c]	186/89 (32%)	47/22 (32%)	46/22 (32%)	48/21 (30%)	45/24 (35%)	0.958
Activity, 0–1/2–3	115/159	25/42	26/42	32/37	32/38	0.585

ALT alanine aminotransferase, *HCV* hepatitis C virus, *IFN* interferon, *Peg-IFN* pegylated interferon

[a] Data shown are median values

[b] Data missing for 125 patients

[c] Percent of patients with 3–4

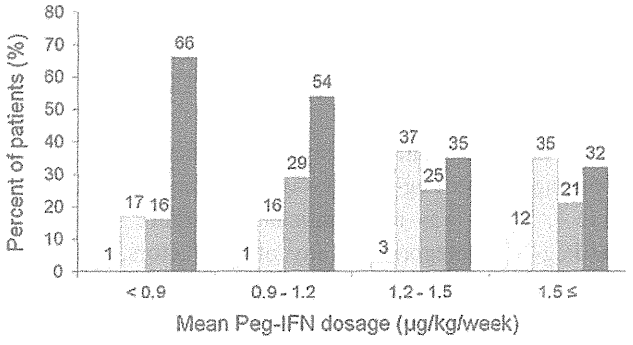
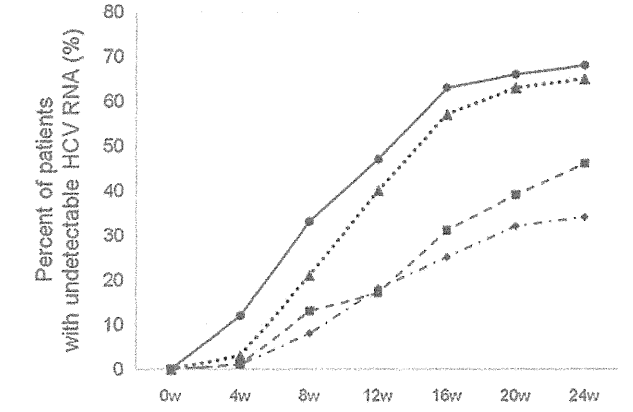


Fig. 1 Timing to the first undetectable hepatitis C virus (HCV) RNA level according to pegylated interferon (*Peg-IFN*) dosage. *Light gray bars* patients with undetectable HCV RNA at week 4. *Medium gray bars* patients with undetectable HCV RNA during 5 to 12 weeks. *Dark gray bars* patients with undetectable HCV RNA during 13–24 weeks. *Black bars* patients with treatment failure (patients with less than a 1-log decrease in HCV RNA level at week 4 or less than a 2-log decrease at week 12 compared with the baseline or detectable HCV RNA at week 24 and those with treatment discontinuance without HCV RNA negativity). Peg-IFN exposure was dose-dependently associated with the timing of HCV RNA negativity ($p \leq 0.001$)



Peg-IFN (μg/kg/week), %						
< 0.9	1	8	18	25	43	34
0.9 ≤ < 1.2	1	13	17	31	39	46
1.2 ≤ < 1.5	3	21	40	57	63	66
1.5 ≤	12	33	47	63	66	68

Fig. 2 Longitudinal negative HCV RNA rates from the start to 24 weeks of the treatment. *Filled circles* Peg-IFN ≥1.5 μg/kg/week, *filled triangles* Peg-IFN 1.2–1.5 μg/kg/week, *filled squares* Peg-IFN 0.9–1.2 μg/kg/week, *filled diamonds* Peg-IFN <0.9 μg/kg/week. The HCV RNA negative rate at week 4 was significantly higher among the patients with Peg-IFN ≥1.5 μg/kg/week than among those with Peg-IFN <1.5 μg/kg/week ($p \leq 0.001$). The HCV RNA negative rates at weeks 12 and 24 were significantly higher among the patients with Peg-IFN ≥1.2 μg/kg/week than among those with Peg-IFN <1.2 μg/kg/week ($p = 0.001$, $p = 0.002$, respectively). *w* week

during the treatment according to the increase in the Peg-IFN dose ($p \leq 0.001$).

Figure 2 shows the longitudinal data of the HCV RNA negative rate. The data for patients with treatment failure were included until the end of each patient's treatment. The percentage of patients with undetectable HCV RNA at week 4 decreased from 12 to 1–3% if they were given <1.5 μg/kg/week of Peg-IFN ($p \leq 0.001$). As for the HCV RNA negative rates at week 12 and week 24, there was no significant difference between patients with 1.2–1.5 μg/kg/week and those with >1.5 μg/kg/week of Peg-IFN. The two groups with <1.2 μg/kg/week of Peg-IFN showed significantly lower HCV RNA negative rates than the other two groups given ≥1.2 μg/kg/week of Peg-IFN (week 12, 18 vs. 44%, $p = 0.0001$, week 24, 40 vs. 67%, $p = 0.0002$). The patients with <0.9 μg/kg/week tended to show a decreased HCV RNA negative rate at week 24 compared to the patients given 0.9–1.2 μg/kg/week (34 vs. 46%, $p = 0.08$).

Figure 3 shows the proportion of patients, according to Peg-IFN exposure, among those with undetectable HCV RNA at week 4 ($n = 17$) and at week 12 ($n = 122$), as well as the proportion of patients with detectable HCV RNA at week 24 ($n = 213$). The patients given ≥1.5 μg/kg/week of Peg-IFN accounted for 70% of the patients with undetectable HCV RNA at week 4, and those given ≥1.2 μg/kg/week of Peg-IFN accounted for 71% of the patients with undetectable HCV RNA at week 12. On the other hand, the patients given <1.2 μg/kg/week of Peg-IFN accounted for 64% of the patients with detectable HCV RNA at week 24.

Discussion

The association between drug exposure and HCV RNA negativity has been reported [9–13]. However, most studies have shown only the fixed-point relationship at week 12, and it remains unclear whether dose modification accelerates or delays the timing to the first undetectable HCV RNA level. The present study is the first to clarify this. Induction regimens in which a high dose (360 μg/week) of Peg-IFN alfa-2a was administered for the first 12 weeks failed to improve SVR rates compared to treatment with a standard dose of Peg-IFN in the CHARIOT [22] and PROGRESS [23] RCTs. In contrast, the adherence study of McHutchison et al. revealed that patients who received ≥80% of the planned dose of Peg-IFN and ribavirin for ≥80% of the full 48 weeks of treatment had a significantly higher SVR rate (51%) than those who received <80% of the planned dose of one or both drugs for ≥80% of the full 48 weeks of treatment (34%) ($p = 0.011$) [10]. These apparently paradoxical results for the relationship between drug dosage and antiviral effect imply that the dose-dependent increase of the antiviral effect was observed up to a certain dose and the antiviral effect then reached a plateau above the regular dose. This paradoxical effect could explain the impact of Peg-IFN reduction from a regular dose on the timing to the first undetectable HCV

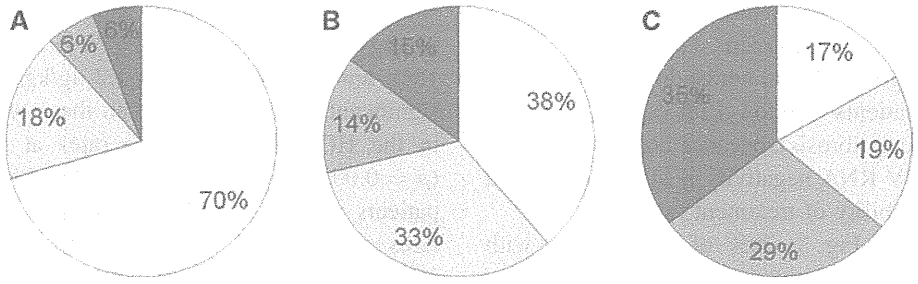


Fig. 3 Proportions of patients according to Peg-IFN exposure among patients with undetectable HCV RNA at weeks 4 and 12 and those with detectable HCV RNA at week 24. **a** Week 4 ($n = 17$), **b** week 12 ($n = 122$), **c** week 24 ($n = 213$). *Light gray segments* Peg-IFN <0.9 μg/kg/week. *Medium gray segments* Peg-IFN 0.9–1.2 μg/kg/week. *Dark gray segments* Peg-IFN 1.2–1.5 μg/kg/week. *Black segments* Peg-IFN ≥1.5 μg/kg/week

RNA level in the present study differing from that of the induction therapy with a high Peg-IFN dose in the above two studies.

In the present study, characteristic matched patients were extracted from a large retrospective cohort to examine the impact of Peg-IFN dosage on viral dynamics. The reason for using a matched cohort was that performing an RCT according to Peg-IFN doses poses an ethical problem, because a low dose of Peg-IFN is known to show little efficacy. The reason for our focusing on Peg-IFN dosage was based on the finding that ribavirin was indeed a significant factor for HCV RNA negativity at week 24 on univariate analysis, but not on multivariate analysis, and Peg-IFN was significantly correlated with HCV RNA negativity at week 24 in an independent manner in this study cohort. Our previous report that Peg-IFN, but not ribavirin, was correlated with c-EVR supports this [13].

To calculate the propensity score, we chose four covariates as candidates for adjustment: age, gender, Plt values, and history of IFN treatment, because there was a need to match universal features such as age, gender, and factors associated with HCV RNA negativity at week 24, such as Plt values and the history of IFN treatment. As shown in Table 4, the baseline characteristic factors in the different Peg-IFN patient categories were well matched after propensity score adjustment. That is, c-statics, the hallmark of application to logistic regression analysis, was regarded as adequate for random assignment. Only the c-statics for the patients given <0.9 μg/kg/week of Peg-IFN and the patients given 0.9–1.2 μg/kg/week of Peg-IFN showed a low value (0.62), because the number of patients in the Peg-IFN category of 0.9–1.2 μg/kg/week ($n = 153$) was not very large. However, the patient characteristic factors in two categories after extraction were well matched and were considered to be adequate for further analysis. In this study, the populations extracted after matching were composed of patients with relatively advanced liver fibrosis compared to the original population; the mean Plt value was lower and the proportion of patients with progression of liver fibrosis (METAVIR fibrosis score 3 or 4) was higher in the extracted population than in the original one (mean Plt value 12.1 × 10^4/mm^3 vs. 16.3 × 10^4/mm^3, proportion of patients with progression of liver fibrosis, 32 vs. 19%, respectively). The patients with <0.9 μg/kg/week of Peg-IFN, which was the smallest population among the four Peg-IFN categories and included more patients with advanced liver fibrosis, were used as the control for the propensity score matching.

Recently, the usefulness of extended therapy has been revealed for patients with LVR, defined as HCV RNA negativity between week 12 and week 24 (or week 36). In addition, we have reported that, even with extended treatment of 72 weeks, the timing of HCV RNA disappearance showed a strong correlation with relapse after treatment [24]. Accordingly, at present, it is necessary to verify how reducing drug doses affects the delay of the timing to the first undetectable HCV RNA level or treatment failure, and in the present study we demonstrated the appropriate dose of Peg-IFN required to attain HCV RNA negativity by 24 weeks. As shown in Fig. 1, Peg-IFN dose-dependently affected the timing to the first undetectable HCV RNA level during the treatment. These results indicate that dose reduction of Peg-IFN can cause a shift from c-EVR to LVR and a shift from LVR to HCV RNA-positivity at week 24. The proportion of patients with treatment failure among those given <0.9 μg/kg/week of Peg-IFN (66%) was decreased by half among the patients given ≥1.2 μg/kg/week of Peg-IFN (32–35%). Considering that the effectiveness of extended treatment for patients with LVR is obvious, if patients without a c-EVR were to attain HCV RNA negativity by 24 weeks, those patients would have the potential to attain an SVR with extended treatment. However, if patients do not attain HCV RNA negativity, those patients must discontinue the treatment. Therefore, causing patients to shift from HCV RNA negativity by week 24 to being HCV RNA-positive at week 24 would be missing the chance to obtain SVR even with extended treatment. As shown in Fig. 2, the longitudinal negative

rate of HCV RNA was dose-dependently affected by Peg-IFN at all points during the treatment. Therefore, a marked dose reduction of Peg-IFN should not be done at the start of treatment even for patients with lower Plt values (which are indicative of advanced fibrosis), because dose reduction of Peg-IFN before HCV RNA negativity is attained can lead to an increased possibility of treatment failure.

Next, as shown in Fig. 3, 70% of the patients with undetectable HCV RNA at week 4 were given ≥1.5 μg/kg/week of Peg-IFN, 71% of those with undetectable HCV RNA at week 12 were given ≥1.2 μg/kg/week, and 64% of those with detectable HCV RNA at week 24 were given ≤1.2 μg/kg/week. Therefore, in HCV genotype 1 patients treated with Peg-IFN plus ribavirin, the treatment goal for c-EVR or non-NR should be to maintain a Peg-IFN dose of ≥1.2 μg/kg/week, and that for RVR should be to maintain a Peg-IFN dose of ≥1.5 μg/kg/week. Using granulocyte–macrophage colony-stimulating factor for patients who develop a severe decrease of blood cells and are forced to decrease Peg-IFN can be beneficial, as long as HCV RNA is positive.

A limitation of the present study is that the actual SVR rate could not be compared among the four Peg-IFN categories because some patients with LVR were treated for 72 weeks and some were treated for 48 weeks; actual SVR rates were 20% in patients with Peg-IFN <0.9 μg/kg/week, 18% in those with 0.9–1.2 μg/kg/week, 36% in those with 1.2–1.5 μg/kg/week, and 48% in those ≥1.5 μg/kg/week. On the assumption that the SVR rate for patients with RVR is 90%, the SVR rate for those with c-EVR without RVR is 75% for 48-week treatment, and the SVR rate for those with LVR is 60% for 72-week treatment, the SVR rate of response-guided therapy was calculated to be 23% for patients given <0.9 μg/kg/week of Peg-IFN, 30% for those given 0.9–1.2 μg/kg/week of Peg-IFN, 45% for those given 1.2–1.5 μg/kg/week of Peg-IFN, and 50% for those given ≥1.5 μg/kg/week of Peg-IFN in the matched cohort in the present study. Thus, dose reduction of Peg-IFN can reduce the SVR rate even if response-guided therapy is done. Another limitation of this study is that the *IL28B* SNP, which is known to be a host factor affecting the antiviral effect, could not be examined in all cases, because the characteristic matched patients were extracted from a large retrospective cohort. However, we had the result of the *IL28B* SNP (rs8099917) for 290 patients; 214 patients had TT and 76 had TG or GG. The proportions of patients with the *IL28B* SNP TT were similar among the four Peg-IFN categories (≤0.9 μg/kg/week, 76%, 31/41; 0.9–1.2 μg/kg/week, 71%, 27/38; 1.2–1.5 μg/kg/week, 67%, 99/147; ≥1.5 μg/kg/week, 77%, 57/74, $p = 0.853$). Therefore, it would appear that there was no bias for any cases. Among the patients with the *IL28B* SNP TT, the HCV negative rates at weeks 4, 12, and 24 were 0% (0/58), 33% (19/58), and 69% (40/58) among the patients with <1.2 μg/kg/week of Peg-IFN and 4% (7/156), 62% (97/156), and 82% (128/156) among those with ≥1.2 μg/kg/week. There were significant differences between these two Peg-IFN groups in the HCV RNA negative rates at weeks 12 and 24 ($p = 0.002$, $p = 0.04$, respectively). Similarly, among the patients with *IL28B* SNP TG or GG, the HCV negative rates at weeks 4, 12, and 24 were 0% (0/21), 0% (0/21), and 10% (2/21) among the patients with <1.2 μg/kg/week of Peg-IFN and 2% (1/55), 9% (5/55), and 27% (15/55) among those with ≥1.2 μg/kg/week. The HCV RNA negative rates at weeks 12 and 24 tended to be higher in the patients with ≥1.2 μg/kg/week of Peg-IFN ($p = 0.06$, $p = 0.13$, respectively). From the above-mentioned results, it appears that the dose-dependent effect of Peg-IFN on the timing of HCV RNA negativity could be considered regardless of the *IL28B* SNP.

In conclusion, this matched study has demonstrated that, in patients with CH-C with genotype 1 receiving Peg-IFN plus ribavirin combination therapy, Peg-IFN dose-dependently affects the timing to the first undetectable HCV RNA level and the failure to attain HCV RNA negativity. Dose reduction of Peg-IFN to <1.2 μg/kg/week before HCV RNA negativity is attained delays HCV RNA clearance dose-dependently and increases the rate of treatment failure. Maintaining the Peg-IFN dose at ≥1.2 μg/kg/week, and preferably at ≥1.5 μg/kg/week, can accelerate the timing to the first undetectable HCV RNA level for CH-C genotype 1 patients treated with Peg-IFN plus ribavirin.

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Long-term effect of lamivudine treatment on the incidence of hepatocellular carcinoma in patients with hepatitis B virus infection

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Abstract
Background Nucleotide analogues have recently been approved for the treatment of patients with hepatitis B virus (HBV) infection. However, it is still controversial whether the decrease of HBV-DNA amount induced by treatment with nucleotide analogues can reduce the risk of hepatocellular carcinoma (HCC) development in HBV patients.

Methods A total of 293 HBV patients without HCC who were treated with lamivudine (LAM) were enrolled in a multicenter trial. The incidence of HCC was examined after the start of LAM therapy, and the risk factors for liver carcinogenesis were analyzed. The mean follow-up period was 67.6 ± 27.4 months.

Results On multivariate analysis for HCC development in all patients, age ≥50 years, platelet count $<14.0 \times 10^4/mm^3$, cirrhosis, and median HBV-DNA levels of ≥4.0 log copies/ml during LAM treatment were significant risk factors. The cumulative carcinogenesis rate at 5 years was

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3% in patients with chronic hepatitis and 30% in those with cirrhosis. For the chronic hepatitis patients, the log-rank test showed the significant risk factors related to HCC development to be age ≥50 years, platelet count $<14.0 \times 10^4/mm^3$, and hepatitis B e antigen negativity, but median HBV-DNA levels of <4.0 log copies/ml (maintained viral response, MVR) did not significantly suppress the development of HCC. In cirrhosis patients, however, the attainment of MVR during LAM treatment was revealed to reduce the risk of HCC development.

Conclusions These results suggest that the incidence of HCC in HBV patients with cirrhosis can be reduced in those with an MVR induced by consecutive LAM treatment.

Keywords Lamivudine · Chronic hepatitis B · Cirrhosis · Hepatocellular carcinoma · HBV-DNA level

Abbreviations

HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
LAM	Lamivudine
ADV	Adefovir
ETV	Entecavir
Hbs Ag	Hepatitis B surface antigen
PCR	Polymerase chain reaction
TMA	Transcription-mediated amplification
IVR	Initial viral response
MVR	Maintained viral response
HBe Ag	Hepatitis B e antigen
CT	Computed tomography
MRI	Magnetic resonance imaging
ALT	Alanine aminotransferase

Introduction

More than 350 million people worldwide suffer from chronic infection with hepatitis B virus (HBV) [1–3]. Chronic HBV infection eventually leads to the development of cirrhosis and hepatocellular carcinoma (HCC), and raises the risk of hepatic disease-related death [4–6]. In Japan, up to 15% of HCC patients are diagnosed with HBV-related liver disease [7].

HCC is one of the most common malignancies in Japan and its incidence has been increasing over the past 30 years. Recently, various treatments such as transcatheter arterial embolization/chemoembolization, radio-frequency ablation, and hepatic resection have been reported to yield significant improvements in overall patient survival [8–11]. However, HCC relapse has thus far been observed in a majority of treated patients due to its highly malignant potential. In this regard, successful treatment of chronic HBV infection should prevent the patient's liver from progressing to cirrhosis and reduce the risk of HCC development. In recent years, the treatment of chronic hepatitis has changed greatly with the development of various antiviral therapies with nucleoside/nucleotide analogues such lamivudine (LAM), adefovir (ADV), and entecavir (ETV) [12–15]. LAM has long been used against chronic hepatitis, and many reports have demonstrated that LAM is effective in stabilizing inflammatory activity, suppressing HBV-DNA replication, and improving liver histological findings in chronic hepatitis patients [16, 17] and in HBV-related cirrhosis patients [18]. Furthermore, LAM has been reported to reduce the incidence of HCC in patients with chronic hepatitis B [19]. However, it is still controversial whether or not treatment using nucleotide analogues can reduce the risk of HCC development in HBV-infected patients [20, 21], and the relationship between the effect of HBV suppression and HCC development during LAM treatment has not yet been discussed in detail. Also, the risk factors for HCC development in HBV-infected patients who have been treated with LAM have not been sufficiently evaluated. In this study, we aimed to clarify whether the decrease of HBV-DNA amount induced by LAM therapy could reduce the incidence of HCC in HBV-infected patients.

Patients and methods

Patient selection and study design

This study was conducted at Osaka University Hospital and other institutions participating in the Osaka Liver Forum in Japan. The subjects were 293 consecutive patients with HBV infection who underwent continuous LAM therapy for more than 24 weeks from September 2000 to September 2006. All patients tested positive for hepatitis B surface antigen (HBs Ag) or had detectable levels of HBV DNA in their sera according to findings from a polymerase chain reaction (PCR)-based method or a transcription-mediated amplification (TMA) method. Exclusion criteria were patients with anti-hepatitis C antibody, anti-human immunodeficiency virus antibody, and other liver diseases (alcoholic liver disease, drug-induced liver disease, and autoimmune hepatitis). Also excluded were patients with a history of HCC and those who developed HCC within the first 24 weeks of the follow-up period after the initiation of LAM therapy (because of the possibility that microscopic HCC had been present before the initiation of treatment).

All patients were treated with 100 mg of LAM daily. Of the 293 patients, 129 underwent ADV (10 mg/day) therapy in addition to receiving ongoing LAM treatment. For 43 patients who started ETV administration in lieu of LAM, the observation period was terminated when they started

ETV. LAM resistance was confirmed by virological breakthrough and was defined as an increase in serum HBV-DNA by >1 $\log_{10}$ greater than the nadir [22]. If virological breakthrough developed and alanine aminotransferase (ALT) was elevated over the upper normal limit, the patients received add-on ADV at 10 mg/day.

In this study, all patients were examined for serum HBV-DNA level just before therapy initiation and every 6 months during treatment. The initial viral response (IVR) was defined as HBV-DNA <4.0 log copies/ml in the first 24 weeks of the follow-up period after the initiation of LAM therapy, and the maintained viral response (MVR) was defined as median HBV-DNA levels of less than 4.0 log copies/ml measured every 6 months during therapy.

This study protocol followed the ethical guidelines of the Declaration of Helsinki amended in 2008, and informed consent was obtained from each patient.

HBV testing

HBs Ag, hepatitis B e antigen (HBe Ag) and anti-hepatitis B e antibody (anti-HBe) levels were examined by chemiluminescence immunoassay or enzyme immunoassay. HBV DNA was measured by a PCR-based method (Amplicor HBV monitor; Roche Diagnostics, Tokyo, Japan) or a TMA method (TMA-HPA; Fujirebio, Tokyo, Japan), which have lower detection limits of 2.6 and 3.7 log copies/ml, respectively. The LAM-resistant YMDD mutant virus was examined by a PCR-ELMA method. Serum samples were stored frozen at −80°C.

Diagnosis of HCC and cirrhosis

Ultrasonography was carried out before LAM therapy and every 3–6 months during the follow-up period. New space-occupying lesions detected or suspected at the time of ultrasonography were further examined by computed tomography (CT), magnetic resonance imaging (MRI), or hepatic angiography. HCC was diagnosed by the presence of typical hypervascular characteristics on angiography, in addition to the findings from CT or MRI. If no typical image of HCC was observed, fine-needle aspiration biopsy was carried out with the patient's consent or the patient was carefully followed until a diagnosis was possible with definite observation by CT, MRI, or hepatic angiography. Cirrhosis was diagnosed by liver biopsy or laparoscopy, and for patients without this information, by clinical data, imaging modalities, and portal hypertension.

Statistical analysis

Quantitative variables were expressed as means ± SD. Quantitative variables at the baseline were compared among two groups, the chronic hepatitis and cirrhosis groups, using the Mann–Whitney *U*-test. Categorical data, such as gender and status of HBe Ag, were compared using Fisher's exact test. The cumulative incidence of HCC was evaluated with a Kaplan–Meiercurve and the differences between groups were analyzed by the log-rank test. For multivariate analysis to investigate factors affecting the cumulative incidence of HCC, Cox's regression analysis was carried out. A value of $p < 0.05$ (two-tailed) was considered to be statistically significant. All calculations were performed with SPSS version 15.0J (SPSS, Chicago, IL, USA).

Results

Baseline characteristics of patients

The baseline clinical features of the enrolled patients before LAM administration are shown in Table 1. The mean age of the patients was 48.0 ± 10.7 years, 214 (73%) of the entire group were male, and 163 (56%) tested positive for HBe Ag. Of the 293 patients, 205 (70%) were diagnosed as having chronic hepatitis and 88 (30%) as having cirrhosis. The median HBV-DNA level was 7.0 (range 3.0 to 8.5<) log copies/ml. At baseline, the aspartate aminotransferase (AST) level was 131 ± 151 IU/l, the ALT level was 203 ± 252 IU/l, the total bilirubin level was 1.2 ± 1.6 mg/dl, the albumin (Alb) level was 3.8 ± 0.5 g/dl, and the platelet count was 13.7 ± 5.4 × 10^4/mm^3. The mean follow-up period for all patients was 67.6 ± 27.4 months, with a range of 12–110 months from the start of LAM treatment. There were significant differences between patients with chronic hepatitis and those with liver cirrhosis in age, AST, ALT, total bilirubin, Alb, and platelet counts.

Cumulative incidence of development of HCC

Figure 1a shows the Kaplan–Meier curve of the cumulative HCC incidence for all HBV patients treated with LAM or LAM plus ADV. Of the 293 patients with HBV infection, 32 (10.9%) developed HCC and the cumulative carcinogenesis rate was 6% at 3 years, 12% at 5 years, and 15% at 7 years.

Figure 1b shows the Kaplan–Meier curve of the cumulative HCC incidence according to initial diagnosis (chronic hepatitis vs. cirrhosis). Eight (4%) of the 205 enrolled chronic hepatitis patients developed HCC and the cumulative carcinogenesis rate was 2% at 3 years, 3% at 5 years, and 5% at 7 years. On the other hand, 24 (27%) of the 88 enrolled cirrhosis patients developed HCC and the cumulative carcinogenesis rate was 15% at 3 years, 30% at 5 years, and 35% at 7 years.

Table 1 Patient characteristics

Factor	All	Chronic hepatitis	Cirrhosis	*p* value
Number of patients	293	205	88	
Age (years)	48.0 ± 10.7	46.3 ± 10.7	51.9 ± 9.8	<0.001**
Sex (male/female)	214/79	147/58	67/21	0.475
HBe Ag (positive	163 (56%)	121 (59%)	42 (48%)	0.068
HBV DNA (log copies/ml)[a]	7.0 (3.0 to 8.5<)	6.8±1.1	6.6 ± 1.1	0.162
AST (IU/l)	131 ± 151	143 ± 162	104 ± 120	0.045*
ALT (IU/l)	203 ± 252	235 ± 269	129 ± 189	<0.001**
Total bilirubin (mg/dl)	1.2 ± 1.6	0.9 ± 0.6	1.8 ± 2.7	<0.001**
Alb (g/dl)	3.8 ± 0.5	3.9 ± 0.4	3.5 ± 0.6	<0.001**
Platelets ($\times 10^4$/mm^3)	13.7 ± 5.4	15.6 ± 9.3	9.3 ± 3.8	<0.001**
Follow-up period (months)	67.6 ± 27.4	68.5 ± 26.5	65.5 ± 29.5	0.393

HBe Ag Hepatitis B e antigen, *HBV* hepatitis B virus, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *Alb* albumin

[a] Values are expressed as medians

* $p < 0.05$, ** $p < 0.001$, comparing patients with chronic hepatitis and those with liver cirrhosis using the Mann–Whitney *U*-test for quantitative variables and Fisher's exact test for categorical variables

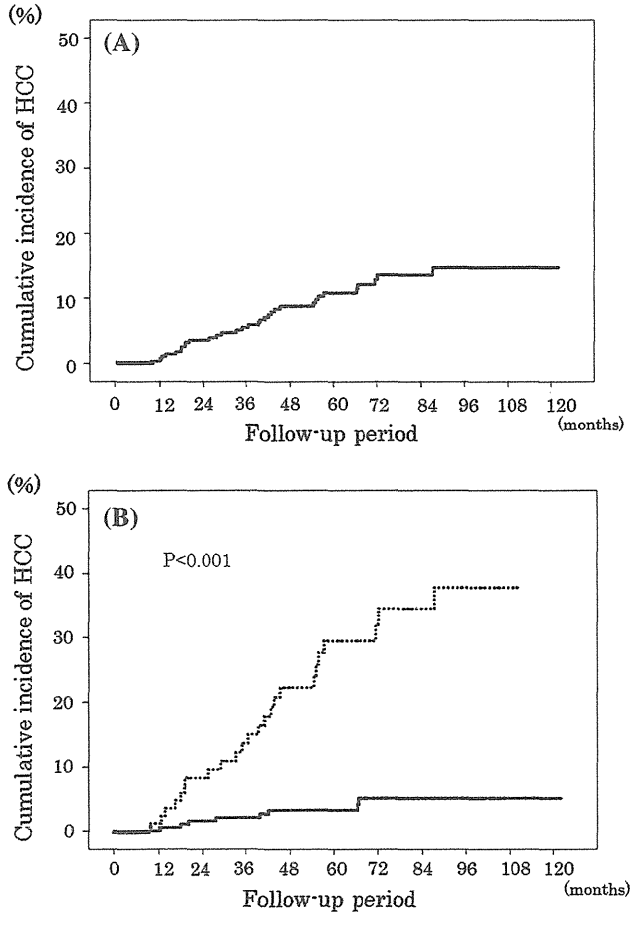

Fig. 1 Cumulative incidence of development of hepatocellular carcinoma (*HCC*) in patients with hepatitis B virus infection treated with lamivudine (LAM). **a** All cases; **b** chronic hepatitis or cirrhosis. *Solid line* Chronic hepatitis, *dotted line* cirrhosis

Risk factors for cumulative incidence of HCC development in all HBV-infected patients

Univariate analysis with the log-rank test was performed for all HBV-infected patients treated with LAM, with the results shown in Table 2. Univariate analysis with the log-rank test showed that the following were significant risk factors for the development of HCC: older age (≥50 years) ($p < 0.001$), cirrhosis ($p < 0.001$), high total bilirubin level (>1.2 g/dl) ($p = 0.004$), low Alb level (<3.8 g/dl) ($p = 0.019$), low platelet count (<14 × 10^4/mm^3) ($p < 0.001$), and non-MVR ($p = 0.035$).

Stepwise multivariate analyses of four of these variables were performed by Cox's regression analysis for all patients treated with LAM with the results shown in Table 3. The analysis indicated the following factors as independent significant risk factors related to the development of HCC: age ≥50 years [hazard ratio (HR) 3.20, 95% confidence interval [CI] 1.08–9.53, $p = 0.036$], platelet count <14.0 × 10^4/mm^3 (HR 4.76, 95% CI 0.05–0.96, $p = 0.045$), cirrhosis (HR 4.64, 95% CI 1.75–12.4, $p = 0.002$), and non-MVR (HR 2.70, 95% CI 1.09–6.56, $p = 0.032$).

Cumulative incidence of and risk factors for HCC development in patients with chronic hepatitis and cirrhosis

The results of univariate analysis with the log-rank test for the development of HCC in chronic hepatitis patients treated with LAM are shown in Table 4, and the following were significant risk factors: older age (≥50 years) ($p = 0.002$), HBe Ag negativity ($p = 0.005$), and low platelet count (<14 × 10^4/mm^3) ($p = 0.004$). Suppression of median HBV-DNA levels to <4.0 log copies/ml by LAM treatment was not associated with the development of HCC in the chronic hepatitis patients. Only non-MVR (median HBV-DNA amount ≥4.0 log copies/ml) was shown to be a significant risk factor for the development of HCC in the cirrhosis patients ($p = 0.029$), while the factors of age, HBe Ag status, and platelet count were not significant in these patients (Table 4).