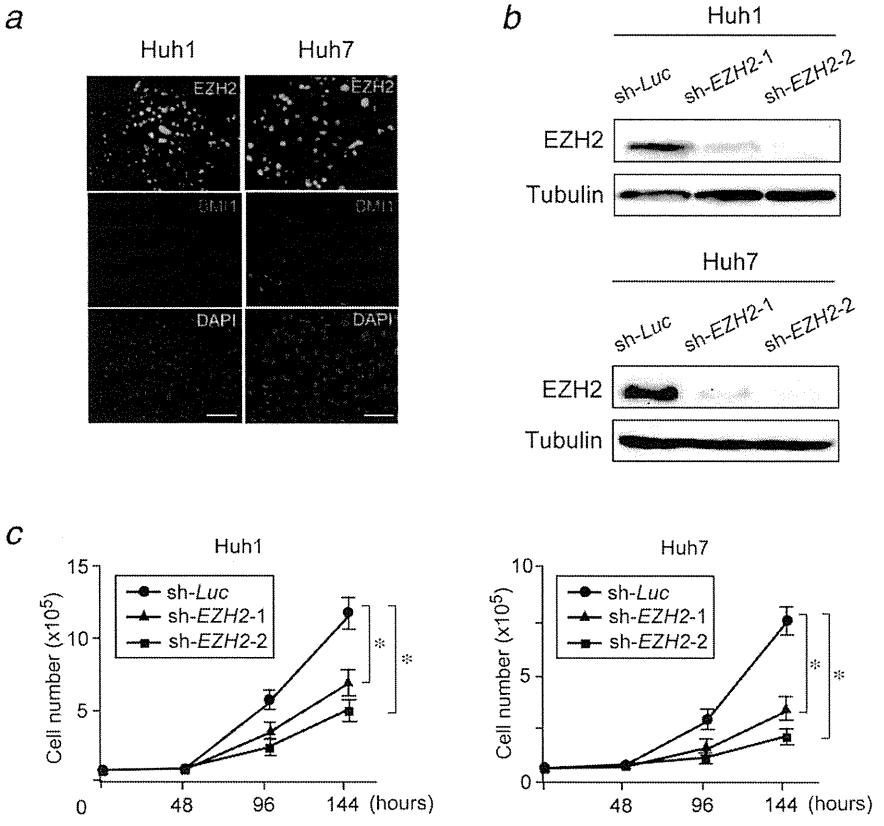

Figure 1. Basal expression and knockdown of *EZH2* in HCC cells. (*a*) Immunocytochemical analyses of EZH2 (green) and BMI1 (red) expression in Huh1 and Huh7 cells. Nuclear DAPI staining (blue) is also shown. Scale bar = 200 μm. (*b*) Cells transduced with indicated lentiviruses were selected by cell sorting for EGFP expression, and subjected to Western blot analysis using anti-EZH2 and anti-tubulin (loading control) antibodies. (*c*) Inhibition of proliferation in *EZH2* knockdown HCC cells. *Statistically significant ($p < 0.05$).

weekly. For the analyses of xenograft tumors, subcutaneous tumors were removed and minced in sterile PBS on ice. The small pieces of tumors were put in DMEM containing 5 mg/ml collagenase type II (Roche) and digested. The cell suspension was centrifuged on Ficoll (IBL, Gunma, Japan) to remove dead cells and debris. Harvested cells were subjected to flow cytometric analyses and sphere formation assays. These experiments were performed in accordance with the institutional guidelines for the use of laboratory animals.

Statistical analysis

Data are presented as the mean ± SEM. Statistical differences between 2 groups were analyzed using the Mann-Whitney U test. p values less than 0.05 were considered significant.

Results

Stable knockdown of *EZH2* in HCC cells

To investigate the role of EZH2 in HCC cells, we first examined the basal expression of EZH2 in the Huh1 and Huh7 HCC cell lines. Immunocytochemical analyses demonstrated that EZH2, as well as the PRC1 protein BMI1, were highly expressed in the nuclei of both cell lines (Fig. 1*a*). We next conducted loss-of-function analyses of EZH2 *in vitro*. We achieved the stable knockdown of *EZH2* in Huh1 and Huh7 cells with lentivirus-mediated shRNA against *EZH2* using EGFP as a marker for infection. A lentiviral vector expressing shRNA against *luciferase* was used as a control. Two different shRNAs, sh-*EZH2*-1 and sh-*EZH2*-2, both markedly repressed EZH2 protein expression and inhibited the growth of both cell lines (Fig. 1*b* and 1*c*). Because knockdown of *EZH2* and growth inhibition were more prominent with sh-*EZH2*-2 than with sh-*EZH2*-1, we used sh-*EZH2*-2 for most of the subsequent experiments. Apoptotic cell death, assessed using an anti-CASP3 antibody, was increased by *EZH2* knockdown in Huh1 and Huh7 cells approximately fourfold compared to the corresponding control cells (Supporting Information Fig. 1).

Reduced tumorigenic activity in *EZH2*-knockdown HCC cells

To examine whether *EZH2* knockdown affects the tumorigenic ability of HCC cells, we conducted a soft agar colony formation

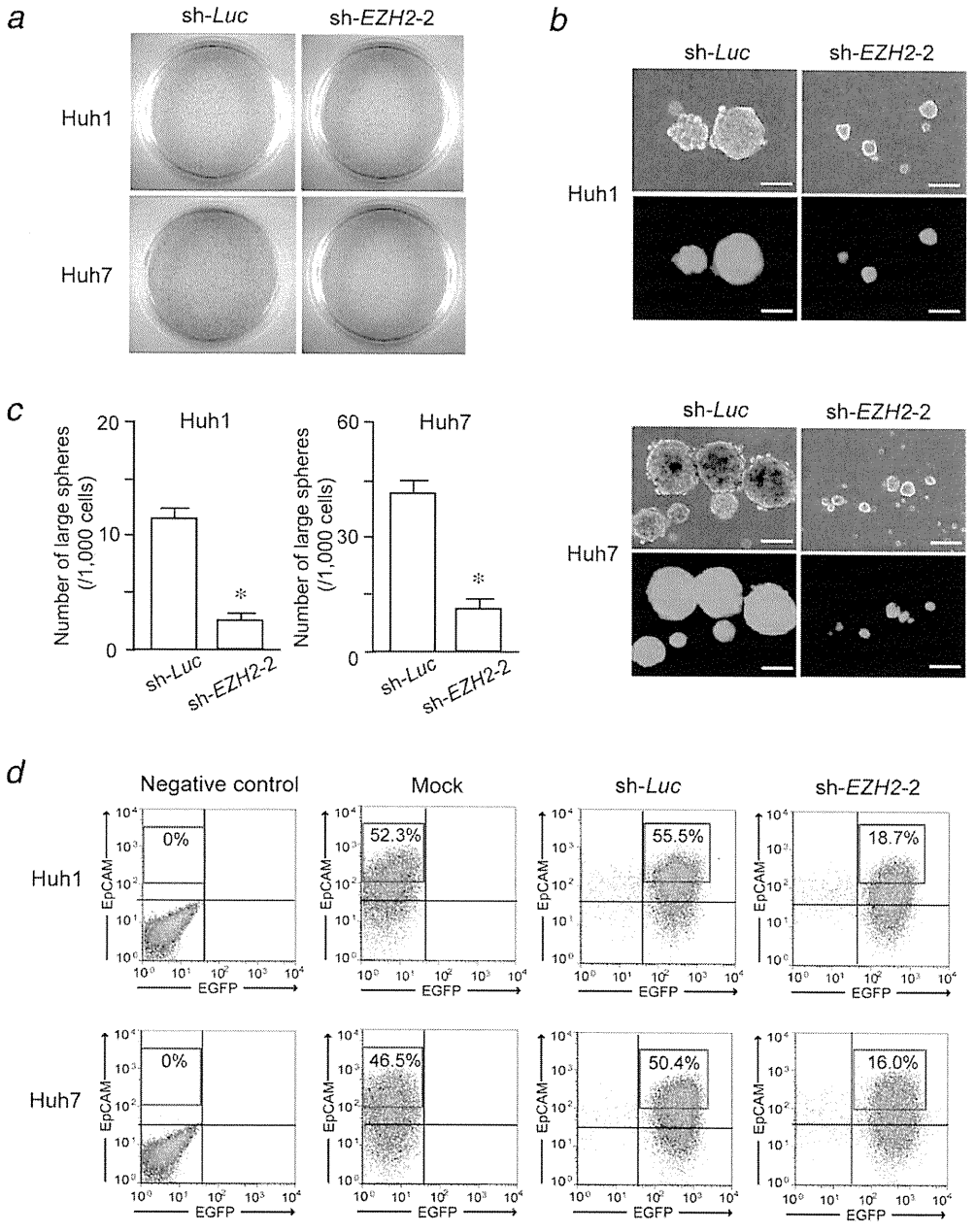

Figure 2. *In vitro* assays of HCC cells with sh-*EZH2*-2-induced *EZH2* knockdown. (*a*) Soft agar colony formation at day 21 of culture. (*b*) Non-adherent sphere formation assay at day 14 of culture. Bright-field (upper panels) and fluorescence (lower panels) images are shown. Scale bar = 100 μm. (*c*) Number of large spheres generated from 1,000 HCC cells transduced with indicated viruses. *Statistically significant ($p < 0.05$). (*d*) Flow cytometric analysis of *EZH2*-knockdown HCC cells. Flow cytometric profiles in Huh1 and Huh7 cells after the stable knockdown of *EZH2*. The percentages of $EpCAM^{high}$ fraction are shown as the mean values for three independent analyses.

assay. Reduced colony formation was clearly observed in both cell lines following *EZH2* knockdown (Fig. 2*a*). In addition, we performed a non-adherent sphere assay, a standard assay for evaluating the stem cell activity of both normal stem cells and CSCs. Consistent with the results of the soft agar assay, sphere-forming capacity was significantly impaired in *EZH2* knockdown cells compared to the control cells (Fig. 2*b* and 2*c*). It has been documented that $EpCAM^{+}$ cells and $CD133^{+}$cells

function as TICs in HCC cells, including Huh1 and Huh7 cells.[7,8] We then examined the expression of EpCAM in view of EZH2 expression using flow cytometry. Knockdown of *EZH2* decreased the EpCAM^{high} fraction from 55.5% to 18.7% in Huh1 cells and from 50.4% to 16.0% in Huh7 cells (Fig. 2*d*). Likewise, knockdown of *EZH2* in Huh7 cells decreased the CD133^{high} fraction from 40.8% to 14.3% (Supporting Information Fig. 2). In clear contrast, overexpression of *Ezh2* promoted both cell growth and sphere formation in Huh7 cells moderately but significantly (Supporting Information Fig. 3*a*–3*d*). Correspondingly, flow cytometric analyses showed an increase in the EpCAM^{high} and CD133^{high} fractions (Supporting Information Fig. 3*e*). Together, these results indicate that EZH2 expression is strongly associated with the frequency of tumor-initiating HCC cells.

Impact of EZH2 depletion on tumor-initiating HCC cells

To confirm that EZH2 directly regulates a tumorigenic subpopulation, we purified the EpCAM^{+} tumor-initiating fraction from Huh1 and Huh7 cells by flow cytometry and conducted a non-adherent sphere assay. The sphere-forming ability of EpCAM^{+} cells was significantly impaired by *EZH2* knockdown compared to that of the control cells (Supporting Information Fig. 4*a*). The secondary sphere number was also decreased by *EZH2* knockdown, indicating that EZH2 plays an important role in the maintenance of self-renewal capability in TICs (Supporting Information Fig. 4*b*). Real-time reverse transcription-polymerase chain reaction (RT-PCR) analyses of purified EpCAM^{+} Huh1 and Huh7 cells demonstrated that *EZH2* knockdown induced the down-regulation of *α-fetoprotein (AFP)*, a marker of the immature phase of hepatocytes. In clear contrast, various differentiation markers such as albumin (*ALB*) and cytochrome P450, subfamily 1, polypeptode 2 (*CYP1A2*), lipid metabolizing enzymes such as apolipoprotein C3 (*APOC3*), and enzymes involved in gluconeogenesis such as phosphoenolpyruvate carboxykinase (*PEPCK*) were upregulated to varying extents (Supporting Information Fig. 4*c*).

We next performed xenograft transplantations of sh-*EZH2*-2-expressing EpCAM^{+} cells using NOD/SCID mice (Supporting Information Fig. 4*d*). Prior to transplantation, the cells were purified by magnetic cell sorting and purity (>90%) was confirmed by flow cytometric analyses of EpCAM expression (data not shown). Importantly, and as expected, the implantation of 2×10^6 *EZH2*-knockdown EpCAM^{+} cells resulted in delayed tumor development and slower tumor growth compared with sh-*Luc* expressing control cells (Supporting Information Fig. 4*e*). Taken together, these results imply that EZH2 depletion impairs the tumorigenicity of tumor-initiating HCC cells partially through the activation of differentiation programs.

Inhibited H3K27 trymethylation by *EZH2* knockdown and DZNep treatment

Ezh2 is a histone methyltransferase and catalyzes the addition of methyl groups to H3K27. A S-adenosylhomocysteine hydrolase inhibitor, DZNep, has been reported to inhibit S-adenosylhomocysteine hydrolase and cause the retention of S-adenosylhomocysteine, thereby inhibiting S-adenosyl-L-methionine-dependent methyltransferases including EZH2. Although DZNep is not a specific inhibitor targeting EZH2, it efficiently inhibits EZH2 function.[20] DZNep also reportedly depletes EZH2 protein.[21] To examine H3K27me3 levels in *EZH2*-knockdown or DZNep-treated HCC cells, the cells were subjected to Western blotting. As expected, *EZH2* knockdown resulted in reduced levels of H3K27me3 in both HCC cells (Fig. 3*a*). Similarly, DZNep-treated HCC cells showed a significant reduction in levels of EZH2 and H3K27me3 (Fig. 3*b*).

DZNep inhibits growth and sphere formation of HCC cells

We first examined the effect of 5-FU, a widely used anti-cancer agent, on HCC cells. 5-FU efficiently inhibited the growth of HCC cells in a dose-dependent manner (Supporting Information Fig. 5*a*). Nonetheless, the effect of 5-FU to suppress non-adherent sphere formation was relatively mild compared with its effect on cell growth (Supporting Information Fig. 5*b*). Importantly, 5-FU treatment rather increased the EpCAM^{high} fraction in both Huh1 (55.9 to 83.5%) and Huh7 (45.3 to 79.1%) cells (Supporting Information Fig. 5*c*). Likewise, Huh7 cells treated with 5-FU showed an increase in the proportion of the CD133^{high} fraction from 39.0 to 85.4% (Supporting Information Fig. 6*a*). These findings indicate that tumor-initiating HCC cells were resistant to 5-FU and greatly enriched after 5-FU treatment.

Next, we examined the effect of DZNep on HCC cells *in vitro* assays. DZNep treatment inhibited growth and non-adherent sphere formation in both cell lines in a dose-dependent manner (Fig. 4*a* and 4*b*). Flow cytometric analyses revealed that the DZNep (10 μM) treatment efficiently decreased the EpCAM^{high} fraction from 49.0% to 12.5% in Huh1 cells and from 44.4% to 11.6% in Huh7 cells (Fig. 4*c*). Likewise, the CD133^{high} fraction in Huh7 cells decreased from 37.2% to 9.4% after DZNep (10 μM) treatment (Supporting Information Fig. 6*b*). These results highlighted that the biological effect of DZNep is quite different from that of 5-FU.

It has been shown that BIX01294 selectively inhibits G9a HMT activity and the generation of di-methylated H3K9.[22] To examine whether G9a inhibitor exhibits similar effect to DZNep, we conducted *in vitro* assays of Huh7 cells treated with BIX01294. Although basal level of H3K9me2/3 was comparatively high in Huh7 cells, BIX01294 treatment apparently reduced the level of H3K9me2 but not H3K9me3 (Supporting Information Fig. 7*a* and 7*b*). BIX01294 inhibited the growth and sphere formation in a dose-dependent manner (Supporting Information Fig. 7*c* and 7d). Flow cytometric analyses showed that the BIX01294 (10 μM) treatment efficiently decreased the EpCAM^{high} fraction as well as the CD133^{high} fraction in Huh7 cells (Supporting Information

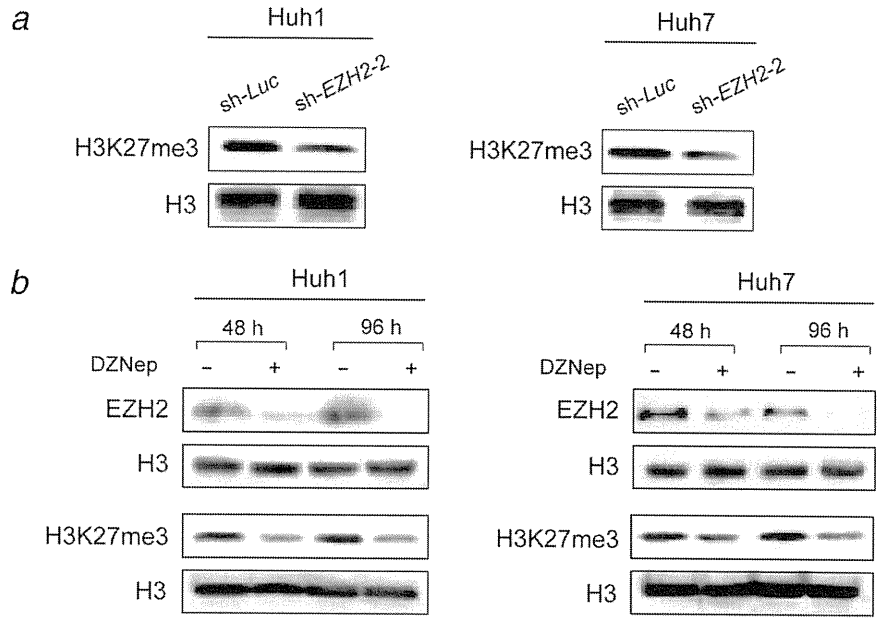

Figure 3. Changes in the trimethylated H3K27 level after EZH2 depletion. (*a*) *EZH2*-knockdown cells were subjected to Western blot analysis using anti-H3K27 and anti-H3 (loading control) antibodies. (*b*) Cells treated with DZNep (10 μM) for 48 or 96 hr were subjected to Western blot analysis using anti-EZH2, anti- trimethylated H3K27, and anti-H3 antibodies.

Fig. 7*e*). Together, depletion of H3K9me2 by BIX01294 might exert biological effects similar to DZNep.

Effect of DZNep on tumor-initiating Huh7 cells

To evaluate directly the action of DZNep towards TICs, we purified EpCAM$^+$ cells from Huh7 cells by cell sorting and conducted sphere formation assays. DZNep markedly impaired primary sphere formation and even more severely impaired secondary sphere formation (Fig. 5*a* and 5*b*). These results indicate that DZNep inhibits self-renewal of tumor-initiating HCC cells. The immunostaining of CASP-3 showed that DZNep treatment induced apoptotis dose-dependently (Fig. 5*c*). The percentage of apoptotic cells among EpCAM$^+$ Huh7 cells treated with DZNep (10 μM) was approximately eight-fold higher than among control cells (Fig. 5*d*).

Subsequently, we determined the ability of DZNep to eradicate TICs using xenograft NOD/SCID mouse models. After the implantation of 2×10^6 Huh7 cells into NOD/SCID mice, and either 5-FU (every 2 weeks) or DZNep (twice a week) was administered intraperitoneally to recipient mice. Tumor initiation and growth were apparently suppressed by both 5-FU and DZNep treatment in a dose-dependent manner. However, DZNep was more effective than 5-FU (Fig. 6*a* and *b*). Flow cytometric analyses of xenograft tumors showed that 5-FU treatment subsequently enriched tumor-initiating EpCAM^{high} cells as observed in *in vitro* analyses (Fig. 6*c*). In clear contrast, DZNep administration resulted in a drastic decrease in tumor-initiating EpCAM^{high} cells (Fig. 6*c*). We next purified EpCAM$^+$ cells derived from xenograft tumors and performed sphere formation assays. EpCAM$^+$ cells treated with DZNep gave rise to significantly fewer spheres in both primary and secondary cultures than those treated with 5-FU (Supporting Information Fig. 8*a* and 8*b*).

Together, these results indicate that DZNep suppresses tumor growth by directly affecting the growth and self-renewal of TICs.

Discussion

Accumulating evidence implies that the overexpression of EZH2 and deregulation of H3K27 methylation play an important role in a variety of cancers.[23] For example, *EZH2* expression is highly upregulated in prostate cancer, and frequent genomic loss of *microRNA-101* targeting the *EZH2* mRNA has been proposed as one mechanism responsible for the upregulation.[24] We and others reported that the level of EZH2 expression is closely associated with the progression and prognosis of HCC.[25,26] All of these findings highlight the importance of EZH2 in hepatocarcinogenesis and implicate EZH2 in regulation of the self-renewal capacity of tumor-initiating HCC cells.

In this study, we first conducted the loss-of-function assays in non-purified Huh1 and Huh7 cells. Lentiviral knockdown of *EZH2* significantly reduced both anchorage-independent colony formation and sphere formation by Huh1 and Huh7 cells in culture. Importantly, flow cytometric analysis showed a significant decrease in the percentage of

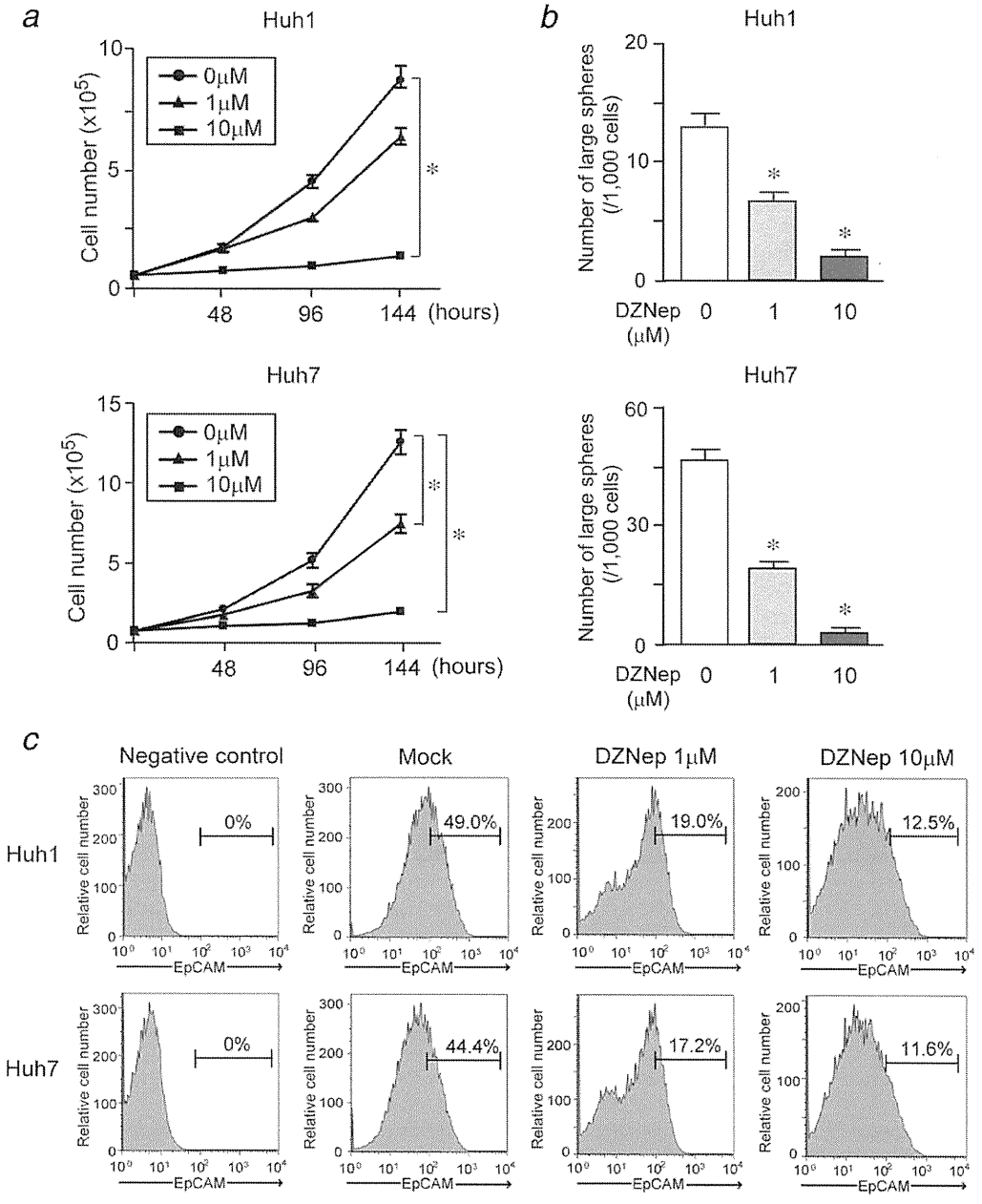

Figure 4. *In vitro* assays of HCC cells treated with DZNep. (*a*) Dose-dependent inhibition of proliferation in DZNep-treated HCC cells. *Statistically significant ($p < 0.05$). (*b*) Number of large spheres generated from 1,000 HCC cells at day 14 of culture. *Statistically significant ($p < 0.05$). (*c*) Flow cytometric profiles of HCC cells treated with DZNep (1 or 10 μM) for 144 hr. The percentages of $EpCAM^{high}$ fraction are shown as the mean values for three independent analyses.

$EpCAM^{high}$ cells following *EZH2* knockdown. Furthermore, $EpCAM^{+}$ cells purified from Huh1 and Huh7 cells exhibited reduced tumorigenicity in a xenograft transplantation assay when EZH2 was depleted. In clear contrast with the knockdown assays, overexpression of *Ezh2* in Huh7 cells enhanced their sphere forming ability and increased the number of $EpCAM^{high}$ and $CD133^{high}$ cells. These results implicated that EZH2 directly regulates a tumor-initiating subpopulation.

Of interest, *EZH2* knockdown cells also showed reduced expression of AFP, a hepatic stem/progenitor cell marker,

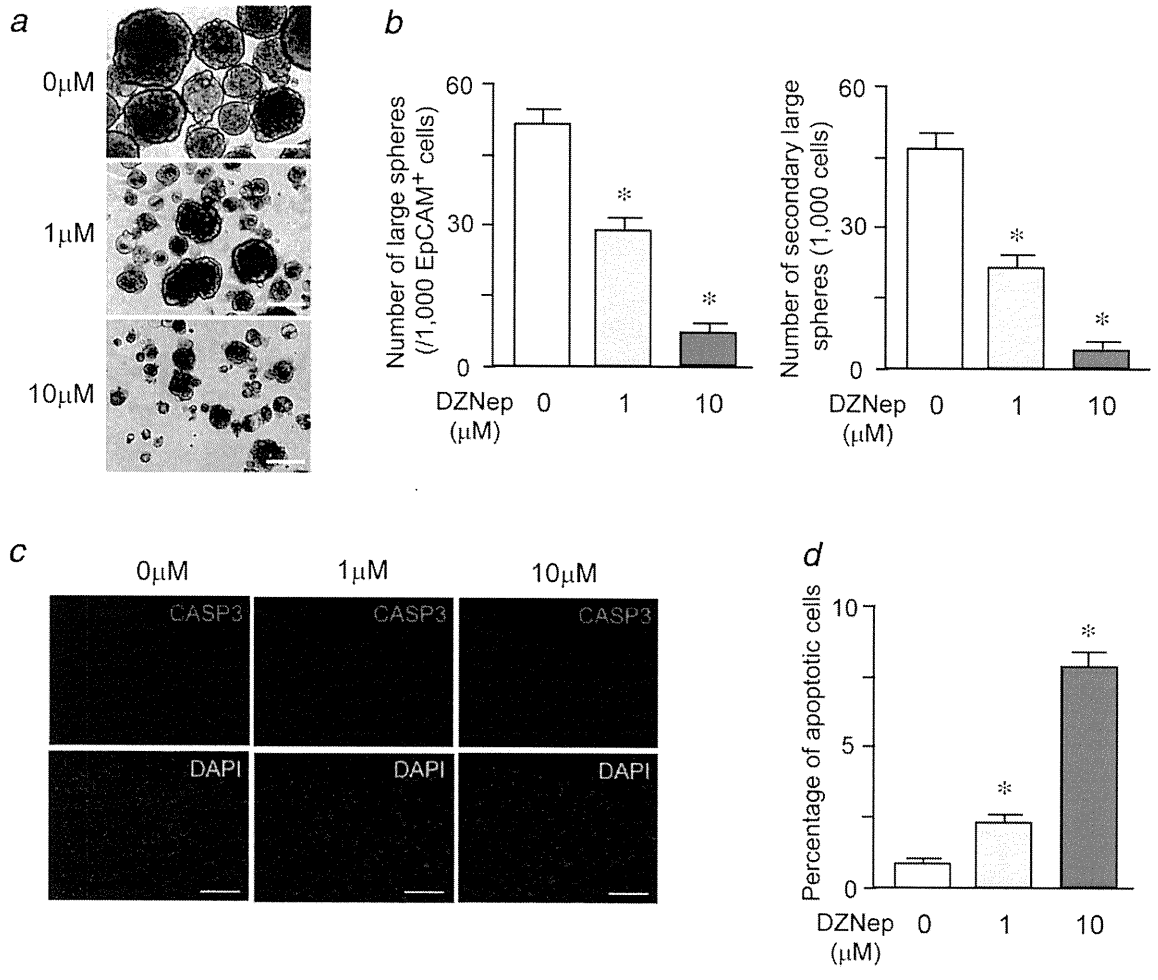

Figure 5. Effect of DZNep in tumor-initiating EpCAM$^+$ cells. (*a*) Bright-field images of non-adherent spheres on day 14 of culture. Scale bar = 100 μm. (*b*) Number of original spheres generated from 1,000 EpCAM$^+$ cells at day 14 of culture and secondary spheres 14 days after replating. *Statistically significant ($p < 0.05$). (*c*) Detection of apoptotic cell death by immunostaining of active caspase-3 (CASP3). Scale bar = 200 μm. (*d*) Quantification of the percentage of apoptotic cells is indicated at the right. *Statistically significant ($p < 0.05$).

and enhanced expression of various differentiation markers of hepatocytes. Analysis of transgenic mice in which Myc expression could be conditionally regulated revealed that multiple HCCs induced by overexpression of Myc lose their neoplastic properties and differentiate into hepatocytes and cholangiocytes upon inactivation of Myc, followed by a reduction in tumor volume and prolonged survival of the hosts.[27] Overexpression of hepatocyte nuclear factor 4α, a well-known liver-enriched transcription factor, reportedly impairs the tumorigenic activity of HCC cells by promoting their differentiation.[28] Given that the tumorigenicity of TICs is closely associated with their immature state in terms of differentiation, the induction of differentiation programs in TICs is a promising approach for targeting TICs.[29,30]

It has recently been reported that the S-adenosylhomocysteine hydrolase inhibitor DZNep depletes cellular levels of PRC2 components including EZH2, SUZ12, and EED and selectively inhibits the trimethylation of H3K27.[21] Intriguingly, DZNep more effectively induces apoptosis in transformed cells than normal cells.[21] Moreover, DZNep treatment has been demonstrated to be effective in abrogating the self-renewal and tumorigenicity of glioblastoma TICs at levels comparative to *EZH2* knockdown.[31]

As expected, our results showed that HCC cells treated with DZNep showed reduced levels of EZH2 and trimethylated H3K27. To elucidate whether DZNep has an inhibitory effect on tumor-initiating HCC cells, we performed *in vitro* assays and xenograft transplantation assays. Sphere formation

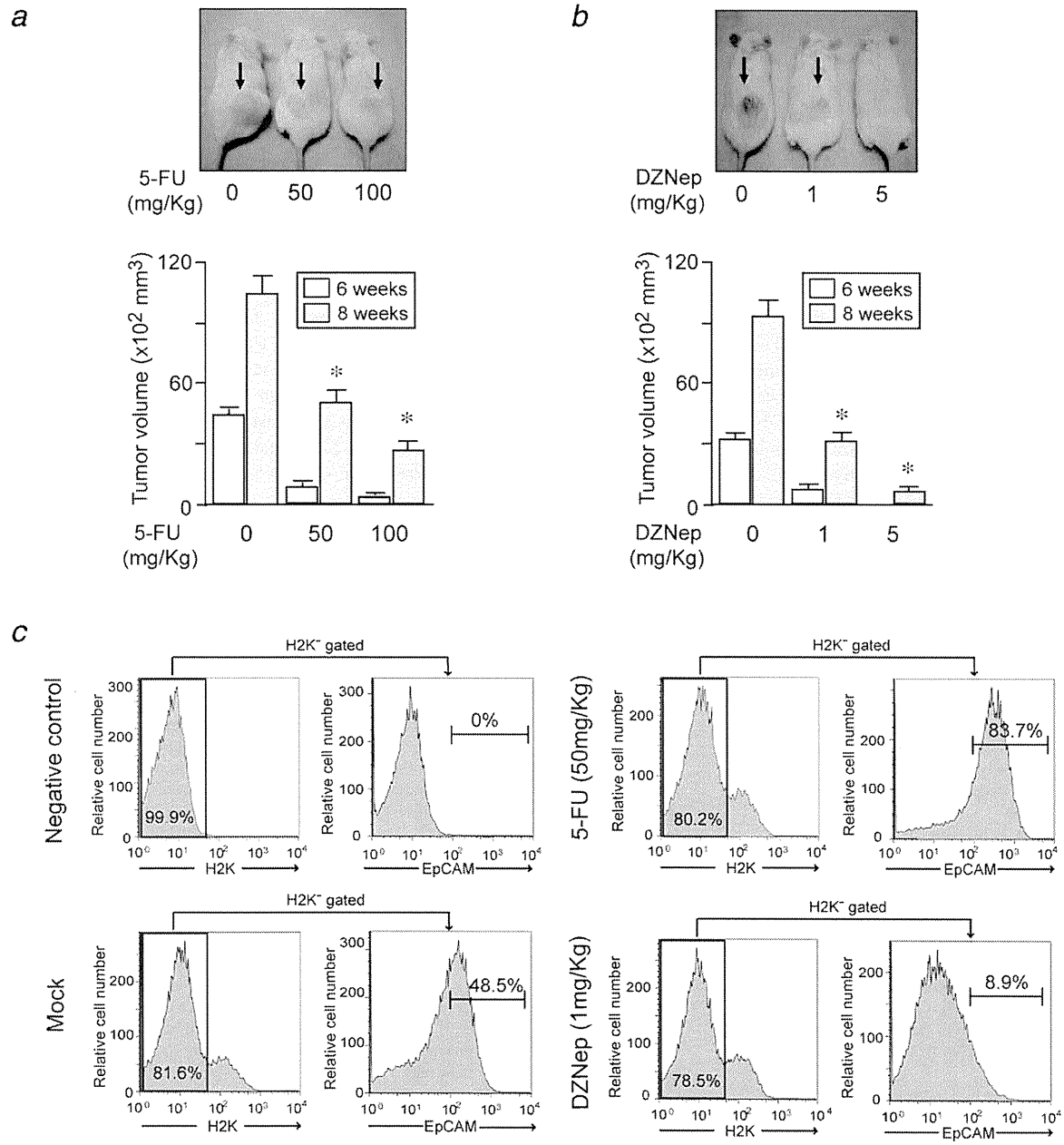

Figure 6. Transplantation and reanalysis of xenograft tumors. (*a*) A total of 2×10^6 Huh7 cells were transplanted into the subcutaneous space of NOD/SCID mice. Growth of subcutaneous tumors (arrows) was apparently suppressed by 5-FU treatment in a dose-dependent manner 6 weeks after transplantation (left panel). Subcutaneous tumor volume was determined at 6 and 8 weeks after transplantation (right panel). *Statistically significant ($p < 0.05$). (*b*) A total of 2×10^6 Huh7 cells were transplanted into NOD/SCID mice. Tumor growth (arrows) was obviously suppressed by DZNep in a dose-dependent manner 6 weeks after the transplantation (left panel). Tumor volume was determined at 6 and 8 weeks after transplantation (right panel). *Statistically significant ($p < 0.05$). (*c*) Flow cytometric profiles of xenograft tumor cells treated with 5-FU or DZNep. The expression of EpCAM was assessed in $H2K^-$ donor tumor cells. The percentages of $EpCAM^{high}$ fraction are shown as the mean values for three independent analyses. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

assays showed that DZNep suppressed more severely the formation of spheres originated from HCC cells than did 5-FU treatment. Subsequent analyses for secondary sphere formation after replating showed similar results. These results indicate that DZNep directly affects the growth and self-renewal of tumor-initiating HCC cells. In addition, although both 5-FU and DZNep suppressed the growth of subcutaneous tumors in xenograft transplantation experiments, flow cytometric analyses of xenograft tumors clearly revealed that DZNep significantly reduced the number of tumor-initiating HCC cells, whereas 5-FU treatment inversely enriched these cells. Importantly, the effects of DZNep were augmented dose-dependently. Taken together, DZNep could be of therapeutic value for the eradication of TICs in HCC.

Transcriptional silencing of tumor suppressor genes by DNA methylation is frequently observed in a variety of cancer.[32] It has been believed that a DNA demethylating agent, 5-aza-2′-deoxycytidine (5-aza-dC), inhibits the growth of cancer cells through the reactivation of these tumor suppressor genes, although 5-aza-dC has only shown limited efficacy against solid tumors. PcG-mediated trimethylation on H3K27 reportedly pre-marks genes for *de novo* methylation in colon cancer cells.[33] Although DZNep or *EZH2* knockdown is not effective in reactivating genes silenced by DNA methylation, it reactivates developmental genes not silenced by DNA methylation in cancer cells.[20,34] The manner of gene silencing might depend on the gene locus and cell-type. Further analysis is needed to understand the preference for DNA methylation or PcG-mediated histone modifications. Considering that the disruption of EZH2 contributes to the prevention of resilencing after the removal of 5-aza-dC,[35] combined use of DZNep and 5-aza-dC might be of therapeutic benefit.

In conclusion, we have successfully demonstrated that both *EZH2* knockdown and pharmacological ablation of EZH2 significantly reduced the number and tumorigenic potential of tumor-initiating HCC cells. This effect might be attributed to the impaired self-renewal capability of tumor-initiating HCC cells caused by interference with EZH2.

However, further analysis will definitely be necessary to determine the effort of EZH2 interference in primary tumor-initiating HCC cells. Although the exploration of potential therapies targeting TICs has just begun, compounds targeting PcG proteins such as EZH2 and HMT inhibitors might be of use for the eradication of TICs in HCC.

Acknowledgement

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ORIGINAL ARTICLE

Safety and tolerance of sorafenib in Japanese patients with advanced hepatocellular carcinoma

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Abstract

Purpose Sorafenib provides a survival benefit for patients with advanced hepatocellular carcinoma (HCC). However, there has been little experience with it in Japan. This study evaluated the safety and tolerance of sorafenib in Japanese patients with HCC.

Methods Clinical data for patients given sorafenib for advanced HCC were captured from eight institutions. All patients were classified as Child-Pugh A and the treatment was started at 400 mg twice daily. We recorded adverse events, treatment duration, and survival retrospectively. Adverse events were graded using Common Terminology Criteria, version 3.0; tumor response was assessed according to Response Evaluation Criteria in Solid Tumor, version 1.1.

Results Of the 54 patients treated, their median age was 69 years (range 48–82), 91% were males, 52% had HCV infection, and 22% had HBV infection. The most common drug-related adverse events were hand–foot skin reactions (HFSR) (72%), aspartate transaminase elevation (55%), alanine aminotransferase elevation (52%), rash (50%), fatigue (41%), and diarrhea (32%). Liver failure occurred in 19%. The median time to treatment failure was 2 months. Dose reduction was required in 83% of the patients, and this occurred within 2 weeks in 44%. The median overall survival was 6.9 months.

Conclusions These data suggest that sorafenib is generally tolerated in Japanese patients with HCC. Nevertheless, the majority needed a dose reduction. Adverse events including HFSR, rash, and liver failure occurred more frequently in our patients than those reported elsewhere. Careful attention must be paid to these adverse events during sorafenib administration.

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide [1]. HCC develops mostly in patients with liver cirrhosis, which is typically caused by hepatitis C virus (HCV) infection, hepatitis B virus (HBV) infection, or alcohol [2]. The annual incidence of HCC in HCV-positive liver cirrhosis and chronic hepatitis is 6–7% and 1–2%, respectively [2]. The risk of cancer developing from chronic hepatitis or cirrhosis depends on the degree of fibrosis [3]. The hepatocarcinogenesis in the patients with hepatitis viruses differs between HCV and HBV. HCC occurs frequently in the cirrhotic livers of patients with HCV-positive liver disease. By contrast, HCC often develops in chronic HBV infection in the absence of cirrhosis. HCC developing from HBV infection has a lower cirrhosis complication rate than does HCC developing from HCV infection.

The etiology of HCC varies regionally [4]. In the Asia-Pacific region, except Japan, 70% of HCC is HBV-related and 20% is HCV-related [5]. In contrast, in Japan, 71–75% of HCC is HCV-related [2, 6]. The incidence of HCV infection is also increasing in the USA and Europe, as is the incidence of HCC [7].

Both surgical resection and local ablation therapy, including radiofrequency ablation, are considered curative for HCC [8–10]. Transarterial chemoembolization (TACE) has been applied to patients with advanced incurable HCC [11, 12]. However, the majority of patients experience recurrence or metastasis after these treatments. Although systemic therapy is available for advanced HCC, the prognosis remains poor. No standard systemic therapy that prolongs survival had been indentified before sorafenib was approved.

Sorafenib, an oral multikinase inhibitor, blocks tumor cell proliferation by targeting Raf/MEK/ERK signaling at the level of Raf kinase, and exerts an antiangiogenic effect by targeting vascular endothelial growth factor receptor-beta (VEGFR-β, PDGF-β) tyrosine kinases [13]. The Sorafenib HCC Assessment Randomized Protocol (SHARP) and Asia-Pacific studies demonstrated a significant survival benefit and good tolerance in patients with advanced HCC, making sorafenib the new reference standard for systemic therapy of patients with advanced HCC [14, 15]. In the SHARP study, approximately 90% of the patients were enrolled from Europe [14], and the Asia-Pacific study was conducted in China, Taiwan, and South Korea [15], but not Japan. The sorafenib groups in the SHARP and Asia-Pacific studies reflected the geographic patient pools, including HCV infection (29 vs. 10.7%) and HBV infection (19 vs. 70.7%) [14, 15]. In both studies, baseline disease characters differed from those of Japanese HCC patients. HCV-related HCC is most common in Japan, as mentioned above, and most of these patients have hepatitis or cirrhosis due to HCV.

In Japan, a phase I study evaluated the pharmacokinetics, safety, and preliminary efficacy of sorafenib in HCC patients [16]. Then, based on the results of the SHARP and Asia-Pacific studies, together with the phase I study in Japanese HCC, the use of sorafenib to treat HCC patients was approved by the Japanese Ministry of Health, Labour, and Welfare in May 2009 [14–16]. However, the phase I study included few patients (six Child-Pugh A patients and eight Child-Pugh B patients receiving 400 mg twice daily) [16]. Thus, little is, in fact, known about the safety and tolerance profile of sorafenib in Japanese HCC patients. In this study, we evaluated the safety and tolerance of sorafenib in Japanese HCC patients.

Materials and methods

HCC patients treated with sorafenib between May 2009 and December 2009 at eight medical centers in Japan were analyzed retrospectively. Patients were required to meet the following criteria at baseline: (1) diagnosis of HCC based on the European Association for the Study of Liver Disease/American Association for Liver Disease criteria or liver histology [8]; (2) Eastern Cooperative Oncology Group Performance Status (ECOG-PS) 0, 1, or 2; (3) classified as Child-Pugh A; (4) required to have adequate renal, hematological, and hepatic function (platelet count $\geq 50 \times 10^9$/L, hemoglobin concentration $\geq$8.5 g/L, albumin concentration $\geq$2.8 g/L, total bilirubin concentration $\leq$3.0 mg/dL, alanine aminotransferase (ALT) concentration $\leq$5 times the upper limit of normal (ULN), serum creatinine concentration $\leq$1.5 times the ULN, and prothrombin time-international normalized rate (INR) $\leq$2.3. Patients who received 400 mg sorafenib twice daily as an initial dose were selected, and treatment interruptions and dose reductions (first to 400 mg once daily, and then to 400 mg once every other day) were allowed for the toxicity study. Dose reduction and treatment discontinuation were based on the package insert and were required for drug-related toxicities. For grade 3/4 toxicities, patients received a lower dose when the toxicity improved to grade 2 or better, but therapy was discontinued if the recovery time was 30 days or longer. Dose reduction was introduced for grade 3 non-hematologic toxicities until the toxicity was grade 2 or better; patients were then treated at one dose

level lower, and therapy was discontinued if the recovery time was 30 days or longer. Treatment was discontinued for patients with drug-related grade 4 non-hematologic toxicities. However, a modified scale resulting from a phase II trial was used for skin toxicity [17].

We recorded demographics, prior therapy, plasma α-fetoprotein (AFP) level, existence of microvascular invasion, or extrahepatic spread of HCC, Barcelona Clinic Liver Cancer (BCLC) score, tumor response, survival data, and relevant toxicities.

Adverse events were recorded according to the Common Terminology Criteria for Adverse Events, version 3.0 (CTCAE v3.0). Based on contrast-enhanced computed tomography (CT) or contrast-enhanced magnetic resonance imaging (MRI), performed at baseline and 1–3 months after treatment, the tumor response was evaluated using the Response Evaluation Criteria in Solid Tumors criteria version 1.1 (RECIST v1.1). The duration of treatment and survival were estimated using the Kaplan–Meier method.

Results

Patient baseline characteristics

In total, 54 patients were included in this retrospective study. Their median age was 69 years (range 48–82), and 49 patients (91%) were males. Most had good performance status (ECOG-PS was 0 in 81% and 1 in 15% of patients). At baseline, 28 patients (52%) had HCV infection and 12 patients (22%) had HBV infection. Of the patients, 38 (70%) were classified as BCLC stage C and 28 patients (52%) had extrahepatic metastases. Before receiving sorafenib therapy, 50 patients (93%) had been treated with surgery, local ablation, or TACE (Table 1).

Table 1 Baseline demographics and disease characteristics of the enrolled patients

Number of patients	54
Sex, no. (%)	
Male	49 (91)
Female	5 (9)
Age (years)	
Median (range)	69 (48–82)
Body weight (kg)	
Median (range)	60.8 (43.6–81.3)
Body surface area ($m^{2)}$	
Median (range)	1.66 (1.32–1.93)
ECOG PS, no. (%)	
0	44 (81)
1	8 (15)
2	2 (4)
Child-Pugh score, no. (%)	
5	36 (67)
6	18 (33)
Hepatitis virus status, no. (%)	
HCV infection	28 (52)
HBV infection	12 (22)
Alcohol	8 (15)
Other	6 (11)
BCLC stage, no. (%)	
B (intermediate)	16 (30)
C (advanced)	38 (70)
Macroscopic vascular invasion, no. (%)	12 (22)
Extrahepatic spread, no. (%)	
Any	28 (52)
Lymph nodes	8 (15)
Lung	14 (26)
Bone	6 (11)
Prior treatment, no. (%)	
Any	50 (93)
Surgery	27 (50)
Local ablation	25 (46)
Transarterial chemoembolization	43 (80)
Biochemical analysis, median (range)	
Platelets/mm^3	133,500 (50,000–296,000)
Albumin (g/dL)	3.7 (2.8–4.9)
Total bilirubin (mg/dL)	0.8 (0.2–1.9)
Aspartate aminotransferase (AST) (IU/L)	51 (18–176)
Alanine aminotransferase (ALT) (IU/L)	40 (11–162)
Alpha fetoprotein (AFP) (ng/mL)	246.6 (2.8–184,100.0)

Safety and tolerability

The overall incidence of drug-related adverse events of any grade was 98% and 36 patients (68%) experienced grade 3/4 adverse events (Table 2). HFSR occurred in 39 patients (72%) and was grade 3/4 in 14 patients (26%). Rash occurred in 27 patients (50%) and was grade 3/4 in 7 patients (13%). Fatigue, diarrhea, and hypertension occurred in 22 (41%), 17 (32%), and 14 patients (26%), respectively; none of these toxicities was grade 3/4. Liver failure under treatment, defined as encephalopathy, massive ascites, or jaundice, occurred in ten patients (19%). The median average daily dose was 450 mg (range 182–800 mg). Dose reduction was required in 45 patients (83%) (Table 3). The most common adverse events leading to dose reduction were HFSR ($n = 21$, 38%), aspartate transaminase (AST)/ALT elevation ($n = 8$, 15%), rash ($n = 7$, 13%), and liver failure ($n = 4$, 7%). Treatment was discontinued in 17 patients (31%) for sorafenib intolerance (Table 4). The most frequent adverse events leading to

Table 2 Drug-related adverse events

	Any	Grade 3/4
Overall incidence	53 (98)	36 (68)
Hematological		
Hemoglobin	1 (2)	0
Leukocytes	4 (8)	0
Platelets	14 (26)	3 (6)
Dermatologic events		
Hand–foot skin reaction	39 (72)	14 (26)
Rash	27 (50)	7 (13)
Alopecia	9 (17)	
Gastrointestinal events		
Anorexia	12 (22)	4 (7)
Diarrhea	17 (32)	0
Vomiting	3 (6)	1 (2)
Fatigue	22 (41)	0
Voice changes	2 (4)	0
Hypertension	14 (26)	0
Abdominal pain not otherwise specified	5 (9)	0
Bleeding	4 (8)	2 (4)
Laboratory		
AST	30 (55)	13 (24)
ALT	28 (52)	8 (15)
Bilirubin	15 (28)	6 (11)
Amylase	15 (28)	3 (6)
Liver failure	10 (19)	

Liver failure is defined as encephalopathy, massive ascites, or jaundice

Table 3 Adverse events causing dose reduction

	Number of patients (%)
Patients requiring dose reduction	45 (83)
Hand–foot skin reaction	21 (38)
AST/ALT	8 (15)
Rash	7 (13)
Liver failure	4 (7)
Anorexia	2 (4)
Bleeding	2 (4)
Vomiting	1 (2)
Time to dose reduction	
<2 weeks	24 (44)
≥2 weeks to <4 weeks	12 (22)
≥4 weeks	9 (17)

treatment discontinuation were liver failure ($n = 4$, 7%), HFSR ($n = 4$, 6%), fatigue ($n = 3$, 6%), and abdominal pain not otherwise specified ($n = 3$, 6%). The median time to treatment failure (TTF; defined as the period from first treatment to discontinuation of sorafenib treatment, progression, or death) was 2 months (Fig. 1).

Table 4 Adverse events leading to treatment discontinuation

	Number of patients (%)
Any adverse events	17 (31)
Liver failure	4 (7)
Hand–foot skin reaction	3 (6)
Fatigue	3 (6)
Abdominal pain not otherwise specified	3 (6)
Anorexia	2 (4)
Rash	2 (4)

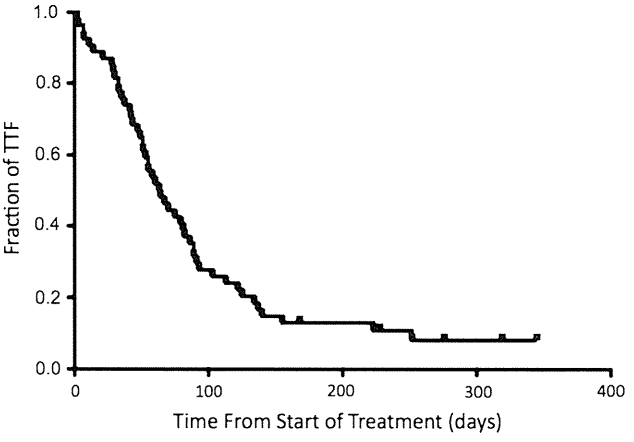

Fig. 1 Kaplan–Meier analysis of time to treatment failure (TTF). The median TTF was 2 months

Efficacy

According to RECIST version 1.1, one patient (2%) had a partial response, 25 patients had stable disease (57%), and the disease control rate (DCR; defined as no disease progression for ≥4 weeks) was 34% (Table 5).

At the time of analysis, with a median follow-up of 5.7 months (range 0.5–13.3), 49 patients had discontinued treatment (92%) and 28 patients were dead (52%). The overall median survival was 6.9 months (Fig. 2)

Discussion

The SHARP and Asia-Pacific studies, large, multicentre, phase III, randomized, double-blind, placebo-controlled trials of sorafenib, revealed a survival benefit and the tolerability of sorafenib in advanced HCC patients. However, considering the varying etiologies and treatment strategies for HCC in different regions [4], it is unclear whether these results apply to Japanese HCC patients. In Japan, high-risk groups for HCC, such as cirrhosis or hepatitis patients, undergo ultrasonography every 3–4 months and CT or MRI every 6–12 months for the early detection of HCC. Because we find HCC when it is earlier, Japanese HCC

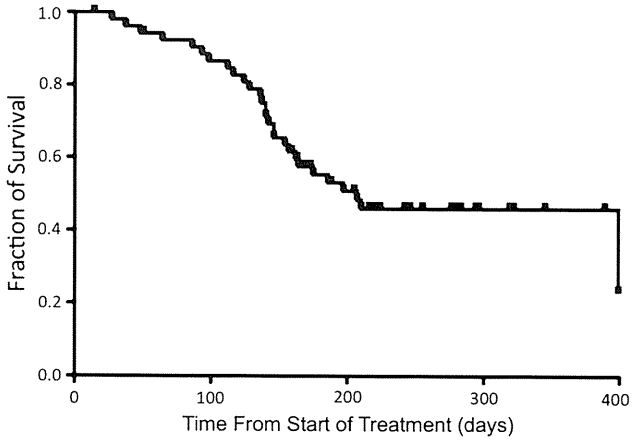

Fig. 2 Kaplan-Meier analysis of overall survival (OS). The median OS was 6.9 months

Table 5 Response rates using the response evaluation criteria in solid tumors

Response ($n = 44$)	Number of patients (%)
Complete response	0
Partial response	1 (2)
Stable disease	25 (57)
Progressive disease	18 (41)
DCR	15 (34)

DCR is the disease control rate, defined as the proportion of patients who had a best response rating of a complete response, partial response, or stable disease that was maintained for ≥4 weeks from the first manifestation of the rating

patients are often able to undergo surgery, local ablation, and TACE. Despite the efficacy of these procedures, patients frequently develop recurrence or disease progression after these treatments. In contrast, in much of the rest of Asia, the majority of patients are present with advanced disease, with large tumors, multiple tumors, and portal tumor thrombosis. These patients are less likely to receive curative treatment [18]. Furthermore, the liver function of HBV-related HCC patients tends to be better than that of HCV-related HCC patients. Shiratori et al. [2] reported that 38.6, 39.3, and 22.1% of cases presented as Child-Pugh A, B, and C when the severity of cirrhosis was classified in Japanese HCV-related HCC patients. By contrast, among the HBV-related HCC patients, 65.2, 26.1, and 8.7% cases presented as Child-Pugh A, B, and C. Additionally, liver function might worsen with the repetition of local therapies because sorafenib was only given to Child-Pugh A patients. Fewer HCV-related HCC patients (52%) were included in the present analysis compared with the general HCC prevalence in Japan (71–75%) [2, 6].

In the SHARP study, common drug-related adverse events were diarrhea (39%), fatigue (22%), HFSR (21%), rash (16%), alopecia (14%), anorexia (14%), and nausea (11%) [14]. Dose reduction due to adverse events was needed in 26% of subjects. The most common adverse events leading to dose reduction were diarrhea (8%), HFSR (5%), and rash (3%) [14]. Treatment was discontinued because of adverse events in 38%. The most frequent adverse events leading to sorafenib discontinuation were gastrointestinal events (6%), fatigue (5%), and liver dysfunction (5%) [14]. In comparison, in the Asia-Pacific study, the common drug-related adverse events were HFSR (45.0%), diarrhea (25.5%), alopecia (24.8%), fatigue (20.1%), rash (18.8%), hypertension (18.8%), and anorexia (12.8%) [15]. Dose reduction due to adverse events was needed in 30.9%, and treatment was discontinued due to adverse events in 19.5% [15]. The most common drug-related adverse events resulting in dose reduction were HFSR (11.4%) and diarrhea (7.4%) [15]. Compared with these studies, we observed a higher incidence of adverse events, especially HFSR, rash, hypertension, and liver failure.

The incidence of HFSR and rash in the Asia-Pacific study was higher than in the SHARP study [14, 15]. In a phase I study of a small population of Japanese patients with HCC, five of the six patients experienced HFSR and four experienced rash; these patients were Child-Pugh A receiving 400 mg twice daily [16]. In a phase II study of Japanese patients with advanced renal cell carcinoma [19], HFSR occurred in 55% and rash occurred in 37.4%. Asian patients, particularly Japanese, frequently develop HFSR. Although it is possible that the physiological difference is partly associated with race, prevention and management of HFSR are required in Japanese patients.

Regarding hypertension, Wu et al. [20] reported a 23.4% (95% CI 16.0–32.9%) overall incidence from a systemic review and meta-analysis of nine studies of renal cell cancer or other solid tumor. Hypertension was experienced by 14 patients (26%) in our study; no case was grade 3/4. Varying rates of hypertension have been reported, with a 5% incidence in the SHARP study and an 18.8% incidence in the Asia-Pacific study. In our study, the incidence of hypertension was comparable with that reported by Wu et al., although it was slightly higher compared with that reported in the SHARP and Asia-Pacific studies.

Liver failure occurred in ten patients (19%), while it was uncommon in the SHARP and Asia-Pacific studies. Nevertheless, Ozenne et al. [21] reported that seven (21%) French patients with Child-Pugh A experienced liver failure. The SHARP and Asia-Pacific studies showed the efficacy of sorafenib in carefully selected patients with advanced HCC. Liver failure may occur with the use of sorafenib in an unselected cirrhotic population. In our study, the median time to experience liver failure was 33 days (range 7–115); liver failure can happen in the

early days of treatment. Furthermore, a common adverse event leading to treatment discontinuation was liver failure (7%).

In our study, 43 patients required dose reduction due to adverse events (83%). This was more frequent than in either the SHARP or Asia-Pacific studies. The most common adverse event leading to dose reduction was HFSR (43%) [12, 13]. Our patients suffered more HFSR than those in the SHARP and Asia-Pacific studies [12, 13]. The cause may be differences, such as age or race. Nevertheless, treatment discontinuation due to HFSR was required in only 6% of the patients; in the majority of the patients, it could be controlled by dose reduction. This concurred with the finding that two of seven patients with Child-Pugh A experienced HFSR when they took 400 mg daily in the Japanese phase I study [16].

In our series, 44% of the patients required dose reduction within 2 weeks and the median daily dose was 450 mg (range 182–800), demonstrating that it is difficult for Japanese patients to continue sorafenib treatment at 400 mg twice daily. Treatment was discontinued because of adverse events in 31% of our patients, which was similar to the rate in the SHARP study, but higher than in the Asia-Pacific study. Adverse events could be managed by dose reduction in the majority of patients. Therefore, careful follow-up is recommended.

The median overall survival was 10.7 months in the SHARP trial and 6.5 months in the Asia-Pacific trial. The differences in survival time might have been caused by differences in patient background. Patients in the Asia-Pacific study displayed more extrahepatic spread, more hepatic tumors, a worse ECOG-PS, and increased concentrations of AFP compared with patients in the SHARP study [14, 15]. The median survival time was 9.2 months in a phase II study [17] and 15.6 months in a Japanese phase I study [16], although Child-Pugh B patients were included in both of these studies. More recently, two retrospective studies from Europe showed that the median survival times for Child-Pugh A patients were 8.9 [21] and 8.3 months [22]. The median overall survival in our series was 6.9 months, although the survival benefits cannot be directly compared, as this was a retrospective study. Our study included many patients with higher serum AFP levels, suggesting the inclusion of highly advanced cases in the present study.

In summary, the present study demonstrated that sorafenib was generally tolerated in Japanese HCC patients because the probability of treatment discontinuation due to adverse events was acceptable, although most patients needed dose reduction. The overall safety profile of sorafenib was similar to that seen in previous studies in patients with HCC, except for the higher rates of HFSR, rash, and liver failure.

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Review Article

Management of hepatitis B: Consensus of the Japan Society of Hepatology 2009

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Recently, much progress has been made in the field of hepatitis B, such as natural history of the disease in relation to the amount of hepatitis B virus (HBV) DNA, genotypes of HBV influencing the natural course and treatment effects, mutations of HBV influencing the severity of the disease and development of hepatocellular carcinoma, and antiviral treatment such as nucleos(t)ide analogues and pegylated interferon. To make the consensus for the diagnosis, management and treatment of hepatitis B, a meeting was held during 45th annual meeting of Japan Society of Hepatology (JSH) in June 2009. In the meeting, recommendations and informative statements were discussed on the following subjects: (i) natural history of HBV infection; (ii) clinical implication of HBV genotypes; (iii) HBV mutations and their potential impact on pathogenesis of HBV infection; (iv) indications for antiviral treatment of chronic hepatitis B; (v) nucleos(t)ide analogues for chronic hepatitis B; and (vi) interferon therapy for chronic hepatitis B. The presenters reviewed the data on these subjects and proposed the consensus statements and recommendations. These statements were discussed among the organizers and presenters, and were approved by the participants of the meeting. In the current report, the relevant data were reviewed and the 12 consensus statements and nine recommendations on chronic hepatitis B were described.

Key words: genotype, hepatitis B virus, interferon, mutation, natural history, nucleotide analogue

Hepatitis B virus (HBV) is one of the most distributed viruses which infect humankind. More than 3 billion people, one half of the world's population, have been exposed to HBV during their life.[1] Acute infection in adults is self-limited in general whereas infection during early childhood will develop into persistent chronic infection in most individuals.[2] More than 400 million people worldwide are chronically infected with HBV and are at risk of developing life-threatening complications including liver cirrhosis and hepatocellular carcinoma (HCC).[1] HBV is a major public health problem worldwide especially in East Asia and Africa. In Japan, approximately 1.5 million people are infected with HBV and it is one of the major causes of HCC and chronic hepatic failure. Other complications of HBV infection include fulminant hepatitis and acute liver failure.

The consensus meeting for diagnosis, management and treatment for hepatitis B was held during the 45th annual meeting of the Japan Society of Hepatology (JSH) in June 2009 (Congress President: M Kudo), where the recommendations and informative statements were discussed. Although the JSH consensus meeting of hepatitis B had been held four times so far, recommendations were hitherto published only in Japanese and this is the first report in English. Established

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information for pathogenesis and contributing factors for disease progression which was agreed by the organizers and presenters are shown as "consensus statements", and clinically useful consensus are shown as "recommendations". The quality of recommendations or informative statements are required to show a "level" (assessing strength or certainty) of evidence and "grading" of recommendations or assessment according to a standard reporting system of clinical guidelines.[3]

NATURAL HISTORY OF HBV INFECTION

AN EVALUATION OF studies on the natural history of HBV infection was done using the scoring system proposed by MacMahon *et al.*[4] in the present analysis because scoring systems for treatment studies cannot always be applied directly to those using natural history. The proposed scoring system consists of levels 1 (1a, 1b), 2 (2a, 2b, 2c), and 3. Level 1a is defined as a population-based longitudinal cohort study with a hepatitis B surface antigen (HBsAg) negative comparison group. Level 1b is identical to level 1a, but with no comparison group. Level 2a is defined as a clinic-based longitudinal cohort study, level 2b is a population-based or clinic-based cohort nested case–control study, and level 2c is a cross-sectional clinic-based study. Level 3 is defined as an observation study case series.

The natural history of chronic HBV infection can be classified into several phases based on levels of alanine aminotransferase (ALT), hepatitis B e-antigen (HBeAg) status, amounts of HBV DNA, and estimated immunological states.[4–9] A representative classification of these phases is shown in Table 1. In the immune tolerance phase, HBeAg is positive, serum levels of ALT are normal, histological activities of hepatitis are absent or minimal, and levels of HBV DNA are elevated. The immune tolerance phase is thought to occur most frequently in individuals who are infected through perinatal transmission, and this phase usually lasts until adolescence or young adulthood.[10–12]

The chronic hepatitis B phase is characterized by elevated ALT and HBV DNA levels. In this phase, the host's immune system recognizes HBV as being foreign and initiates an immune response that results in hepatitis. In cases who are HBeAg positive, active hepatitis can be prolonged and may result in cirrhosis. However, chronic hepatitis B eventually transitions into an inactive phase with a loss of HBeAg positivity in the majority of patients. Seroconversion to anti-HBe and the fall of serum HBV DNA to low levels result in the disappearance of disease activity, despite persisting HBsAg and low levels of HBV DNA.[13–16] Seroconversion rates range 7–16% per year according to reports with higher evidence levels (levels 1b, 2a).[16–19] Factors associated with seroconversion are age (level 1b),[20] ALT levels (level 1b), occurrence of acute exacerbation of hepatitis (level 1b),[19,21] and genotype (level 2c).[22,23]

The seroconversion of HBeAg results in the transition from hepatitis phase to inactive carrier phase, which is generally thought to be a benign course for HBV carrier, but sometimes hepatitis can be reactivated spontaneously.[24] Patients experiencing reactivation undergo another transition, with increases in HBV DNA and ALT levels and disease activity without reappearance of HBeAg.[24] This phase is referred to as HBeAg negative chronic hepatitis B. Occasional severe hepatitis B flare-ups with middle range HBV DNA levels (3–8 log copies/mL) occur in this phase.[8,25] HBeAg negative chronic hepatitis B is caused by mutant strains of HBV unable to produce HBeAg,[25,26] and tends to develop into cirrhosis and complicate HCC more than HBeAg positive chronic hepatitis B.[27–30]

Table 1 Phases in the natural history of HBV carriers (modified from [4])

Phase	Hepatitis	Blood			Liver
		DNA	HBeAg	HBsAg	cccDNA
Immune tolerance	–	8–11	+	+	+
HBeAg positive Chronic hepatitis	Usually Persistent	6–10	+	+	+
HBeAg negative Chronic hepatitis	Often Fluctuating	3–8	–	+	+
Inactive carrier	–	<4	–	+	+
Recovery	–	–	–	–	+

HBV DNA: log copies/mL. cccDNA, covalently close circular DNA; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus.

Many factors that are associated with the development of HCC have been reported so far. Higher age (level 1a), male sex (level 1a), presence of cirrhosis (level 2a) and familial cluster of carriers (level 2c) are reported as host factors.[31,32] Viral factors include high viral load (level 1b),[33–36] existence of pre-core and core promoter mutations (level 2a), genotype C and high ALT levels (level 1b). High viral load should be considered as a factor in patients over 35–40 years of age. Co-infection with hepatitis C virus, hepatitis D virus or HIV (level 2a), drinking habit (level 2c) and exposure to aflatoxin (level 2c) are reported as social and environmental factors.[37–39] Other lifestyle-related factors, such as smoking habit, obesity and complications from diabetes mellitus, have been documented as well.

Consensus 1
In patients with chronic hepatitis B, seroconversion of HBeAg usually results in the transition from hepatitis phase to inactive carrier phase, which generally has low HBV replication and normal ALT levels. However, reactivation of chronic hepatitis can spontaneously occur without the reappearance of HBeAg. At this point, active hepatitis continues and the risk of complicating cirrhosis and HCC is high in patients with HBeAg negative chronic hepatitis B. (Level 1b.)

In the inactive carrier phase, HBV replication is continuously suppressed as a result of predominantly host immunological pressure against HBV. Patients in the inactive carrier phase generally have a benign course because active hepatitis subsides and the risk of HCC decreases.[19,20,24,40] However, regular follow up is required because reactivation of HBV sometimes occurs spontaneously or as a result of immunosuppressive therapy.[19,24]

Hepatitis B surface antigen is known to fall to undetectable levels in some inactive carriers. This HBsAg negative phase, referred to as the recovery phase, has no hepatitis and a low risk of HCC. Still, caregivers must be aware that patients who are old or cirrhotic have a relatively higher risk of HCC.[41,42] Disappearance of HBsAg in the recovery phase does not indicate complete eradication of HBV because the HBV genome remains as covalently close circular DNA (cccDNA) in the nucleus of hepatocytes.

Consensus 2
2-1 HBV can not be completely eradicated using any currently existing treatment measures. (Level 2a.)
2-2 Patients in the inactive carrier or recovery phase have a benign clinical course. However, regular follow up of such patients is required because reactivation of hepatitis B and ensuing HCC can occur. (Level 1b, 2a.)

Clinicians have to consider two types of hepatitis B reactivation: one during the inactive carrier phase and the other in the recovery phase.[4] Both types of reactivation have been attributed with increasing incidence to strong immunosuppressive therapies. De novo hepatitis B, a reactivation of hepatitis B in the recovery phase, tends to develop into fulminant hepatitis, which has a very high mortality rate.[43–46] Thus, establishment of effective measures to prevent reactivation of hepatitis B is necessary.

Consensus 3
3-1 Reactivation of hepatitis B can occur during the inactive carrier or recovery phases and stems mainly from strong immunosuppressive treatment courses. (Level 2a.)
3-2 Recent advances in medical care have increased the use of immunosuppressive agents and thus the incidence of hepatitis B reactivation. (Level 2a.)
3-3 Reactivation of hepatitis B tends to develop into fulminant hepatitis. (Level 2a.)

Recommendation 1
In addition to the loss or seroconversion of HBeAg, a substantial decrease in HBV viral load and subsequent disappearance of hepatitis are the primary targets in the treatment of patients with chronic hepatitis B. (Level 1b.)

Recommendation 2
The main goals of HBV carrier treatment are patients in the inactive carrier and recovery phases. However, caregivers should be aware that reactivation of hepatitis B and complication of HCC can occur even in these benign phases. (Level 1b.)

Recommendation 3
Reactivation of hepatitis B due to immunosuppressive therapy tends to develop into severe hepatitis, thus requiring the establishment of effective preventative measures. (Level 2a.)

CLINICAL IMPLICATION OF HBV GENOTYPES

DISTINCT CLINICAL AND/OR virological characteristics of the HBV infection have been reported in different geographical parts of the world and are increasingly associated with host factors, environmental factors and the genetic diversity of the infecting virus.[47] HBV is classified into at least eight genotypes (A–H) based on an intergroup divergence of 8% or more in the complete nucleotide sequence and a number of subgenotypes (Aa/A1, Ae/A2, Bj/B1, Ba/B2, Cs/C1, Ce/C2, D1, D2, and so forth) that are currently known to have distinctive association with ethnic and/or geographical distribution.[48]

Association between HBV genotype and clinical manifestation

Acute hepatitis

The universal vaccination program against HBV has significantly reduced the number of new infection cases in most countries with levels of endemicity estimated from intermediate to high.[49] However, efficiency of universal vaccination in countries with a low level of endemicity still remains controversial. Japan is one of the countries with a low level of endemicity and mainly vertical (mother to baby) transmission route.[50] In Japan, HBV vaccination in combination with HBV immunoglobulin treatment is the only recommended measure for infants born to HBsAg positive mothers. Studies in Japan indicated genotype C (subgenotype Ce/C2) to be the major type in the country and genotype B (subgenotype Bj/B1) is the second distributed. Surveillance studies have shown a recent trend toward increase in number of acute hepatitis B infection among young adults mainly through sexual contacts.[51,52] Although most cases are associated with genotype C infection, there is a continuous trend toward increase in prevalence of genotype A among acute hepatitis cases.[51,53–56] Patients infected with genotype C have been known to be rarely associated with development of chronic persistence after acute infection in immune competent adults in Japan (1%) in contrast to the higher rates of those infected with genotype A (6–23%).[53,54] A recent multicenter study in Japan indicated a trend among chronic hepatitis B patients toward increase in prevalence of genotype A (from 1.7% in 2002 to 3.5% in 2006), whereas other genotypes remained stable at their prevalence during the same period.[57] The shift in genotype prevalence with the increase of genotype A among chronically infected carriers can be explained by higher risk of genotype A to develop persistence. This is consistent with higher rates of chronic persistence after acute infection in adults in European countries where genotype A is prevalent (10%).[48,58] This is also consistent with results of *in vitro* and *in vivo* comparisons of different genotype strains showing different dynamics of replication: slow for genotype A and rapid by genotype C.[59,60] The surveillance study indicated that all patients treated with lamivudine (LVD) recovered from acute hepatitis, whereas none of the three patients who developed a chronic outcome had received antiviral treatment during their acute phase of infection, indicating that LVD might be able to prevent the chronic outcome.[54] Cumulatively, these data indicate the clinical importance of routine genotyping for acute hepatitis B patients.

Fulminant hepatitis

One of the most serious complications of acute HBV infection is fulminant hepatitis. In Japan, the annual number of fulminant hepatitis reported was approximately 400 cases, with approximately half of these caused by HBV infection. Despite its rather low incidence, fulminant hepatitis is a national problem because the mortality rate is extremely high.[61] It is important to understand factors predisposing for development of fulminant hepatitis. Viral factors associated with the development of fulminant hepatitis are mutations in the core promoter (T1762/A1764)[62] and the pre-core region (A1896).[54,63,64] However, these findings were not consistent with studies in Europe and the USA.[65–67] A large-scale cross-sectional study in Japan revealed association between genotype B (subgenotype Bj/B1) infection and development of fulminant hepatitis; on the other hand, no cases of fulminant hepatitis were registered among those infected with genotype A (subgenotype Ae/A2).[54] Differences in genotypes circulating in Asia and Europe/USA may indicate that distinct viral factors are playing roles in manifestation of infection by different genotype.

Chronic hepatitis

Chronic HBV infection is the most common cause of HCC in Asia.[68] Efficient surveillance and early diagnosis of development of this life complication requires risk stratification of chronic hepatitis B patients. Older age, male sex and liver cirrhosis are well recognized factors associated with increased risk of HCC.[69,70] In addition, recent large-scale population-based and clinical case–control studies carried out in Asia, have shown that infecting virus factors associated with a high risk of HCC, include HBV DNA levels,[71,72] HBV basal core promoter mutations,[35] genotype C (vs B),[22,36,73,74] and sub-