

HCV Infection and Replication Assays

The production of cell culture-generated HCV JFH-1 (HCVcc) and HCV pseudovirus (HCVpp) has been reported previously^{5,15} and is described in detail in the Supporting Document. HCVpp harboring E1/E2 glycoproteins from genotypes 1a, 1b, 2a, 3a, 4a, 5a, and 6a were described previously.¹⁶ For viral internalization assay, Hep3B cells were incubated for 1 hour at 4°C to allow binding of HCVpp (pHCV7a) to cells, washed repeatedly with phosphate-buffered saline to remove unbound virus, and treated with concanamycin A (Sigma-Aldrich, St. Louis, MO) (25 nmol/L), Anti-E2 AP33 antibody¹⁷ (25 µg/mL), PS-ON (100 nmol/L), or PO-ON (100 nmol/L) overnight at 37°C for viral entry. The efficiency of infection was measured by luciferase assay 24 hours later. Transient assay of genotypes 1b (Con-1) and 2a (JFH-1) subgenomic reporter replicons have been reported previously^{18,19} and are described in detail in the Supporting Document.

HCV Binding and Fusion Assays

The HCV-like particle (LP) binding assay was performed at 4°C for 1 hour in 100 µL of TNC (50 mmol/L Tris, pH 7.4, 100 mmol/L NaCl, 1 mmol/L CaCl₂) buffer containing 1% bovine serum albumin as reported previously²⁰ and is described in detail in the Supporting Document. Both Hep3B and Huh7.5 cells were tested. Direct binding of PS-ON or PO-ON to HCV-LP was measured by a plate-binding assay and is described in the Supporting Document. For viral fusion assay, HCVpp/liposome lipid mixing assays with rhodamine-labelled liposomes were performed as previously reported²¹ and are described in the Supporting Document.

HCV Infection in Chimeric Mice

Human hepatocyte-transplanted mice generated in severe combined immunodeficient (SCID)/urokinase plasminogen activator (uPA) mice were purchased from PhenixBio (Hiroshima, Japan).²² These uPA/SCID mice stably transplanted with human hepatocytes were treated intraperitoneally with 10 mg/kg of poly C PS-ON or poly C PO-ON (40mer) on days -1, 0, 1, 3, 5, and 7. Control poly C PO-ON (40mer stabilized by 2'-O-methyl ribose modification) was also tested. A fourth group of mice did not receive any compounds (only normal saline administration). Approximately 5-15 mice were included in each group. The mice were intravenously inoculated on day 0 with HCV patient serum containing 3.9×10^3 copies of HCV genotype 1b. Serum samples were obtained on days 0 (prior to HCV inoculation), 7, 14, 21, 28, and 35 for HCV RNA, HCV core antigen, and human albumin determination. Human albumin in the blood of the chimeric mice was measured with the Alb-II Kit (Eiken Chemical, Tokyo, Japan).

Statistical Analysis

Data from at least triplicate experiments were averaged and expressed as means \pm standard deviations. Statistical analysis was performed using the Student *t* test or Welch *t* test. *P* values of less than .05 were considered statistically significant.

Results

APs Inhibit HCV Infection in a Sequence-Independent Manner

To assess whether APs can inhibit HCV infection, fully degenerate 40mer oligonucleotides that were either phosphorothioated (PS-ON) resulting in a stable amphipathic DNA polymer or that had a 2'-O-methyl modification on the ribose moiety (PO-ON) conferring stability but not altering the polyanionic nature of DNA^{14,23} were tested. Huh7.5 cells were infected with HCVcc in the presence of either PS-ON or PO-ON. At 72 hours postinfection, HCV-infected cells were assessed by immunofluorescence assay (Figure 1A) and intracellular HCV RNA quantification (Figure 1B). HCV infection was significantly inhibited by PS-ON and not PO-ON (*P* < .05). The inhibitory effect of PS-ON was also confirmed by reduced HCV core antigen and HCV RNA levels in the culture supernatant, as compared with those of the PO-ON-treated cells (*P* < .05) (Figure 1C and D). To evaluate further the efficacy of PS-ON against viral entry, HCVpp harboring genotype 1b was used to infect Hep3B. The PS-ON blocked infection of HCVpp in a similar dose-dependent manner (Figure 1E). The PO-ON exhibited some inhibitory effect at high concentration, which could be attributed to noncytotoxic inhibition of cellular adherence by the polyanion nature of PO-ON. To assess whether the PS-ON inhibitory effect is specific for HCV, retroviral pseudovirus carrying the vesicular stomatitis virus G protein (VSVGpp) was tested in the presence of PS-ON or PO-ON. Neither PS-ON nor PO-ON had any effect on VSVGpp infection (Figure 1F). Furthermore, adenoviral infection was not inhibited by PS-ON (Supplementary Figure 1).

A series of homo- and heteropolymeric APs including poly G, A, T, C, TG AC, TC, and AG PS-ONs were tested for their inhibitory activities on HCV infection in both HCVcc and the HCVpp systems. These APs had similar inhibitory activities as the degenerate PS-ON with random sequence in the HCVcc system except for poly G and poly A (Figure 2A). Similar effects were also observed on HCV core antigen and HCV RNA levels in the culture supernatant (Figure 2B and C). In the HCVpp system, these PS-ONs also had similar inhibitory effects (Figure 2D).

AP Inhibition of HCV Infection Is Dependent on Size and Amphipathicity

Different sizes of degenerate PS-ONs (6-, 10-, 20-, 30-, 40-, 50- and 80mer) were tested for their inhibitory

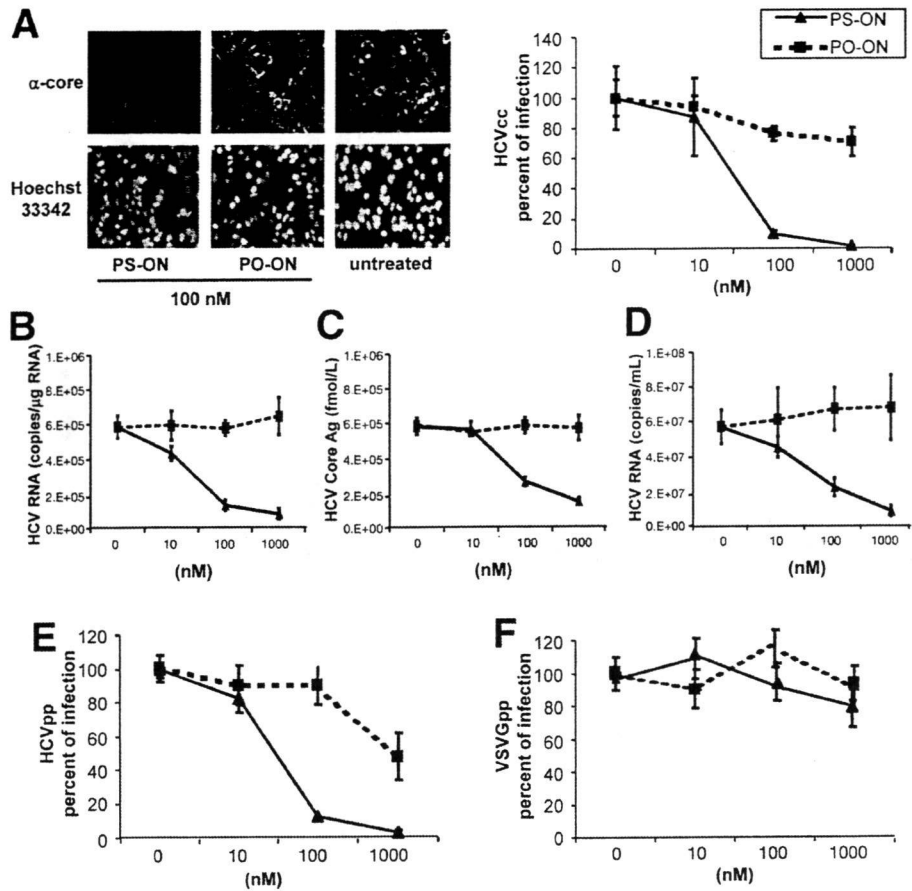


Figure 1. Effect of PS-ON on HCV infection. (A) Huh7.5 cells were infected with HCVcc in the presence of various concentrations of 40mer PS-ON or PO-ON (degenerate sequence). Two days after infection, infected cells were detected by immunofluorescence assay using anticore antibodies (left panel). Percentage of infection was determined by dividing the number of HCV-expressing cells in treated over the untreated cells (right panel). The intracellular HCV RNA levels (B) and HCV core Ag (C) and supernatant HCV RNA (D) levels in the culture medium were determined. Hep3B cells were infected with (E) HCVpp genotype 1b or (F) VSVGpp and treated with various concentrations of PS-ON and PO-ON, and luciferase activities were determined 2 days later. Results are shown as percentages of infection + standard deviations (SD).

activities in the HCVcc and HCVpp systems. Only PS-ONs with lengths of 40mer or greater potently inhibited HCV infection (Figure 2E). This result was confirmed with the poly C PS-ONs (Supplementary Figure 2). To determine the requirement of amphipathicity for antiviral activity of these compounds, additional oligonucleotide analogs that had diminished hydrophilic character were prepared and include degenerate PS-ON analogs with either the base and/or the sugar removed (Supplementary Figure 3). An additional degenerate PS-ON analog containing the 2'-O-methyl ribose modification that does not affect the amphipathicity was tested. These analogs were tested for their inhibitory activities in the HCVcc and HCVpp systems. Only analogs that retained the amphipathic properties inhibited HCV infection (Figure 2F). These observations suggest that the amphipathic nature of these PS-ONs is necessary for inhibiting HCV infection.

APs Inhibit Infection of Various Genotypes of HCV Without Affecting Replication and Cell Attachment

To study the effects of APs on various HCV genotypes, HCVpp harboring E1/E2 glycoproteins from genotypes 1a, 1b, 2a, 3a, 4a, 5a, and 6a were

tested.¹⁶ Infections by all genotypes were equally blocked by the degenerate PS-ON, whereas the degenerate PO-ON had no effect (Figure 3A). Similar observation was obtained with the poly C compounds (Supplementary Figure 2D).

The degenerate PS-ON compound was tested for its effect on viral replication in the HCV replicon system, which supports viral replication without the viral entry step. Genotype 1b and 2a subgenomic replicons were tested. Subgenomic replicon RNAs containing luciferase reporter were transfected into Huh7.5 cells, and the replication efficiency was determined in the presence of the PS-ON or PO-ON control. Neither PS-ON nor PO-ON displayed any antiviral activities in both subgenomic replicon systems (Figure 3B). To eliminate the possibility that PS-ON may induce an antiviral state with increasing time of exposure to cells, the HCV replicon assay was performed after exposure to either PS-ON or PO-ON for 24–48 hours, and no difference in replication was observed (data not shown). Furthermore, Huh7.5 cells treated with PS-ON or PO-ON did not produce any detectable levels of type I interferons.

To dissect further the effect of PS-ON on viral entry, we applied the HCV-LP binding assay, which has been

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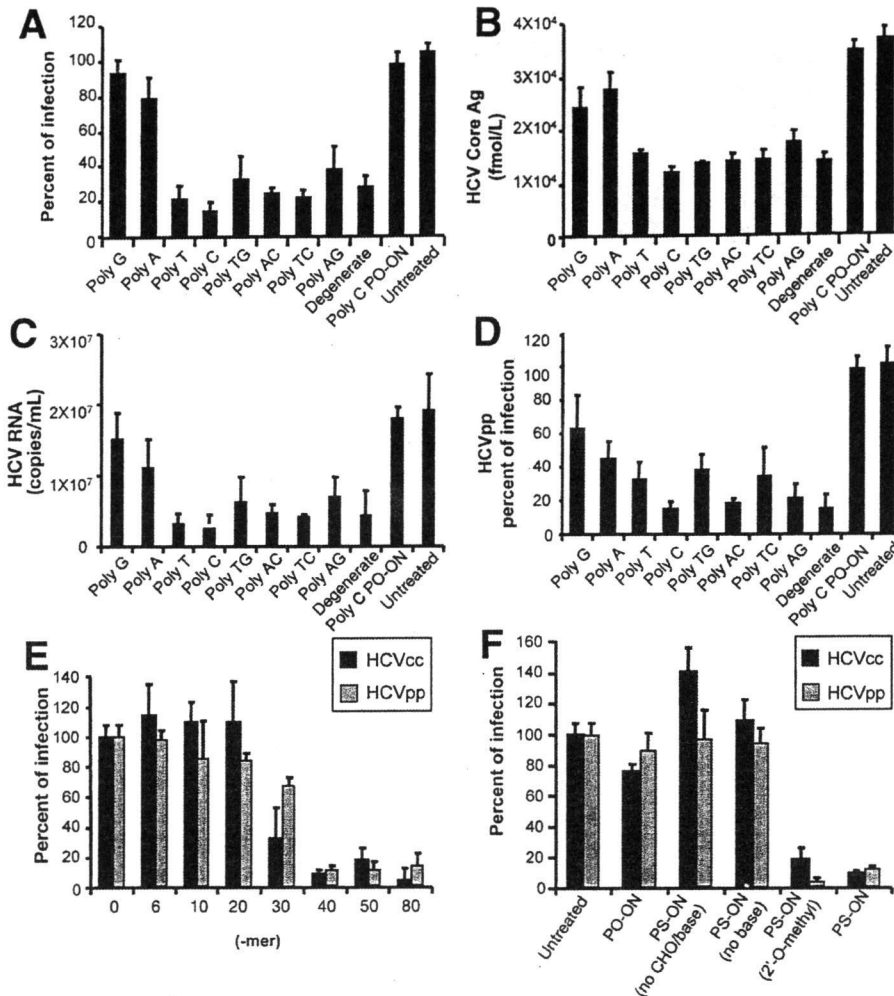


Figure 2. Sequence-independent and size- and phosphorothioation-dependent effects of PS-ON on HCV infection. A series of 40mer PS-ONs with specific sequences including poly poly G, A, T, C, TG AC, TC, and AG were tested for their inhibitory effect on HCV infection. (A) HCVcc was inoculated with Huh7.5 cells and treated with 100 nmol/L of these homo- and heteropolymeric PS-ONs. Expression of HCV core was detected by immunofluorescence assay using anticore antibodies. (B) The HCV core Ag titers and (C) HCV RNA levels in the culture medium were determined. (D) Hep3B cells were infected with HCVpp genotype 1b and treated with these various PS-ONs at 100 nmol/L, and luciferase activities were determined 2 days later. (E) Various sizes of PS-ON (10–80mers) at 100 nmol/L were tested in the HCVcc and HCVpp systems. (F) Various structures of oligonucleotides, PS-ON analogue with phosphorothioate backbone but without the sugar or base, and PS-ON analogue with 2'-O-methyl ribose modification, were synthesized. Each 40mer oligonucleotide at 100 nmol/L was tested in the HCVcc and HCVpp systems. All results are shown as percentages of infection + SD.

developed as a surrogate system to assess HCV binding to cells.^{24–26} HCV-LP were incubated in the presence of PS-ON and PO-ON for 1 hour at 4°C with Huh7.5 or Hep3B cells. Under this condition, virus attaches to the cells but does not enter. HCV patients' serum containing high-level of anti-E1/E2 antibodies was included as a control. The binding was detected with FITC-labeled mouse monoclonal anti-E2 antibodies (Figure 3C). The results showed that the anti-HCV antibodies inhibited the HCV-LP binding to the cells, whereas the PS-ON and PO-ON-treated HCV-LP did not inhibit HCV-LP binding. To validate the HCV-LP binding assay, HCVcc binding to cells was performed in the presence of PS-ON, PO-ON, or HCV serum. HCV RNA bound to the cells was quantified to determine the percentage of binding. As shown in Figure 3D, HCV antibody significantly inhibited HCVcc binding to cells (~80%), whereas PS-ON and PO-ON had minor effects (<20%). These results suggest that the target of inhibition by APs is at the postbinding, cell entry step.

To address the question of whether PS-ON binds to HCV directly to inhibit HCV infection, HCV binding

assays were performed. First, in an immunoassay format using HCV-LP as a capture antigen, neither PS-ON nor PO-ON showed any significant binding to HCV-LP (Table 1). Second, sedimentation density gradient analysis did not show a preferential cosedimentation of HCVcc with PS-ON or PO-ON in comparison with the control preparation (Figure 3E), indicating that neither PS-ON nor PO-ON binds to HCVcc to any significant extent. The amount of PS-ON in the HCVcc or the control fraction was higher than that of PO-ON, probably reflecting the different physical properties of PS-ON and PO-ON. However, it is possible that low-affinity binding of HCV and PS-ON could be present and required for subsequent inhibitory action but not detected by the currently applied assays.

APs Inhibit Viral Internalization

To determine which entry step that APs targets, the HCVpp assay was performed in the presence of concanamycin A (25 nmol/L), degenerate PS-ON (100 nmol/L), degenerate PO-ON (100 nmol/L), or AP33+ALP98

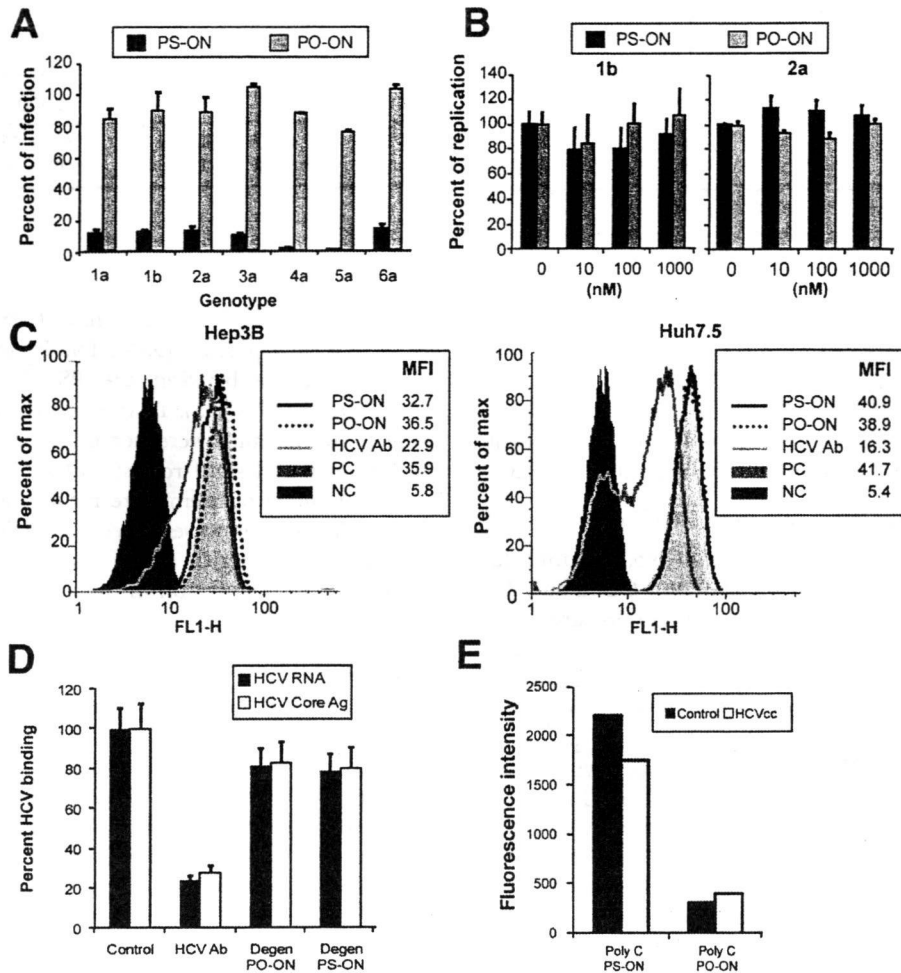


Figure 3. Effects of PS-ON on infection of various HCV genotypes, HCV replication, and cell binding. (A) HCVpp harboring E1/E2 glycoproteins from genotypes 1a, 1b, 2a, 3a, 4a, 5a, and 6a were inoculated into Hep3B cells and simultaneously treated with 100 nmol/L of degenerate PS-ON and PO-ON (40mer). Luciferase activities were determined 2 days later. (B) Subgenomic RNA of genotype 1b Con1 or 2a JFH1 were transfected into Huh7.5 cells. Four hours after transfection, a set of transfected cells was harvested as a control for transfection efficacy, and the remaining cells were treated with 100 nmol/L of PS-ON and PO-ON. Cells were then harvested at 72 hours posttransfection and luciferase activities determined. The replication level was presented as the ratio of the luciferase activity of the sample at 72 hours over that of 4 hours. Percentages of replication were determined by dividing the replication level of treated over that of untreated samples. (C) Hep3B and Huh7.5 cells were incubated with 20 μ g/mL HCV-LP and 100 nmol/L PS-ON or PO-ON at 4°C for 1 hour. The cells were washed and incubated with anti-E2 ALP98 monoclonal antibody for 30 minutes followed by FITC-labeled goat anti-mouse immunoglobulin for 30 minutes at 4°C. HCV-LP binding was analyzed by flow cytometry. The *black filled peaks* are negative controls without the anti-E2 antibody. The *gray filled peaks* are positive controls showing HCV-LP binding without any compounds. The *black solid lines* and *gray dotted lines* represent treatments with PS-ON and PO-ON, respectively. The *gray solid line* represents samples in the presence of HCV serum that has been shown previously to inhibit HCV-LP binding. The mean fluorescence intensity (MFI) of each sample is shown. (D) HCVcc was incubated with Huh7.5 cells in the presence of HCV serum PS-ON or PO-ON at 4°C for 1 hour. The unbound virus was washed off, and the bound HCVcc was determined by HCV RNA quantification and HCV core Ag assay. (E) HCVcc was incubated with Cy3-labeled degenerate PS-ON or PO-ON (40mer) and subjected to iodixanol density gradient analysis as described in the online Supporting Document. Control preparation generated the same way was used for comparison. The fluorescence intensity of the fraction where infectious HCV sedimented was determined and shown.

monoclonal anti-E2 antibodies (25 μ g/mL total concentration) at 37°C. Hep3B cells were first incubated with HCVpp at 4°C to allow binding and then at 37°C with various compounds after the inoculating HCVpp was removed. Concanamycin A is known to inhibit HCV entry by preventing acidification of endosome.¹² As shown in Figure 4A, AP33+ALP98 anti-E2 antibodies

blocked HCV binding to the cells but had no effect on HCV entry. On the other hand, both concanamycin A and the degenerate PS-ON inhibited HCV entry.

To demonstrate that APs may inhibit HCV internalization at the fusion step, a viral fusion assay was performed with HCVpp or VSVpp as control.²¹ Degenerate sequence and poly C PS-ONs and the control PO-ONs

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Table 1. Lack of Binding of PS-ON to HCV-LP

	PS-ON (nmol/L) ^a				PO-ON (nmol/L) ^a				AP33 ^b
	0	10	100	1000	0	10	100	1000	1 μ g/mL
HCV-LP	155 + 14	105 + 9	162 + 13	116 + 10	137 + 12	15 + 10	123 + 12	157 + 13	1475 + 150
Control	147 + 13	117 + 10	120 + 11	117 + 9	111 + 10	33 + 14	100 + 8	103 + 9	153 + 16

^aCy3 labeled degenerate PS-ON and PO-ON (40mer).

^bAP33 binding to HCV-LP was detected with Cy3-labeled goat anti-mouse IgG antibody.

were tested. Both PS-ON compounds showed significant inhibition of HCVpp fusion over their control PO-ON, whereas VSVGpp fusion was largely unaffected by either PS-ON or PO-ON (Figure 4B and Supplementary Figure 4). The inhibitory effect of PS-ON on fusion was evident on both the rate and maximum of fusion in the assay.

APs Inhibit HCV Infection In Vivo

To test the efficiency of APs in vivo, sodium salts of amphipathic polymers (40mers) of poly C and poly AC and their respective PO-ON controls were prepared. Degenerate oligonucleotides were avoided because they might potentially contain CpG motifs, which could induce endogenous interferons, although in vitro testing did not reveal such a possibility. Human hepatocyte-transplanted uPA/SCID mice were treated with these compounds as described and inoculated with infectious HCV genotype 1b patient serum. In this model, the production of human albumin in serum was monitored for the engraftment index of human hepatocytes. All mice showed robust and comparable human albumin concentrations that did not change significantly during the experimental period (Figure 5). Only 1 animal in the poly C PS-ON-treated group ($n = 7$) and 2 in the poly AC PS-ON-treated group ($n = 5$) were HCV positive. The remaining mice in both groups of mice were persistently negative. All 7 mice in the poly C PO-ON-treated mice

(100%) and 14 of 15 untreated mice (normal saline placebo) were HCV positive (93%). The P value was statistically significance between the PS-ON- and PO-ON-treated groups ($P < .05$). To rule out the possibility that these protected mice were not intrinsically resistant to HCV infection despite robust human hepatocytes engraftment, some of them were rechallenged with infectious HCV inoculum several weeks later. They all became infected, supporting the specific inhibitory effect of APs on de novo HCV infection in this in vivo model.

Discussion

Current therapy for hepatitis C is based on peginterferon and ribavirin. However, the therapy is only effective in approximately half of the patients, and there is little option to those who fail current therapy. Recent advances in the development of small molecule inhibitors targeting the viral-encoded enzymes showed promise,²⁷ but viral resistance to these drugs is a major clinical issue because HCV is highly variable with rapid viral proliferation and low-fidelity replication. Phosphorothiate modification of oligonucleotides was initially designed to reduce enzymatic degradation. This modification also increases the hydrophobicity of the phosphodiester backbone and thus imparts an amphipathic character to the oligonucleotide polymer.²⁸ Recent studies showed that

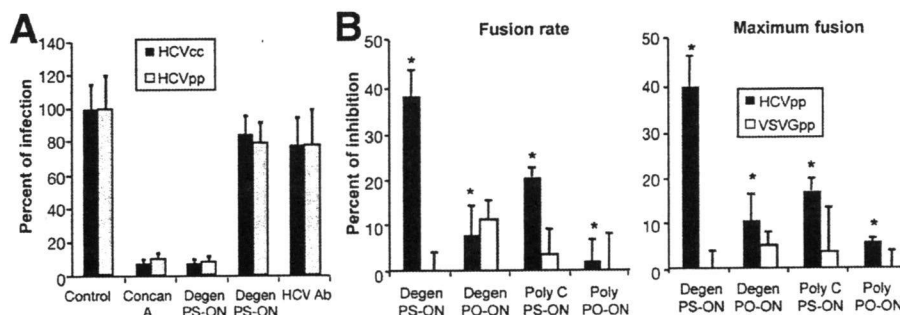


Figure 4. Effects of PS-ON on HCV viral entry. (A) Hep3B cells were incubated with HCVpp at 4°C for 1 hour to bind the virus and washed to remove the unbound virus. Cells were then incubated with fresh culture medium containing 25 nmol/L concanamycin A, 100 nmol/L PS-ON, 100 nmol/L PO-ON, or 25 μ g/mL (total concentration) AP33+ALP98 monoclonal antibodies at 37°C for 16 hours. The luciferase activities were determined 24 hours later. Results are shown as percentages of infection + SD. (B) Fusion assay was performed with HCVpp or VSVGpp in the presence of PS-ON (degenerate or poly C) or the PO-ON controls. The results are expressed as mean percentages (means + SD) of inhibition of either the fusion rate at the origin of the fusion kinetics (left panel) or the maximum fusion of the curve at 500 S (right panel) relative to incubation in the absence of the compounds. The fusion curves are shown in Supplementary Figure 4. * $P < .05$ comparing the PS-ON and the corresponding PO-ON in the HCVpp fusion assay.

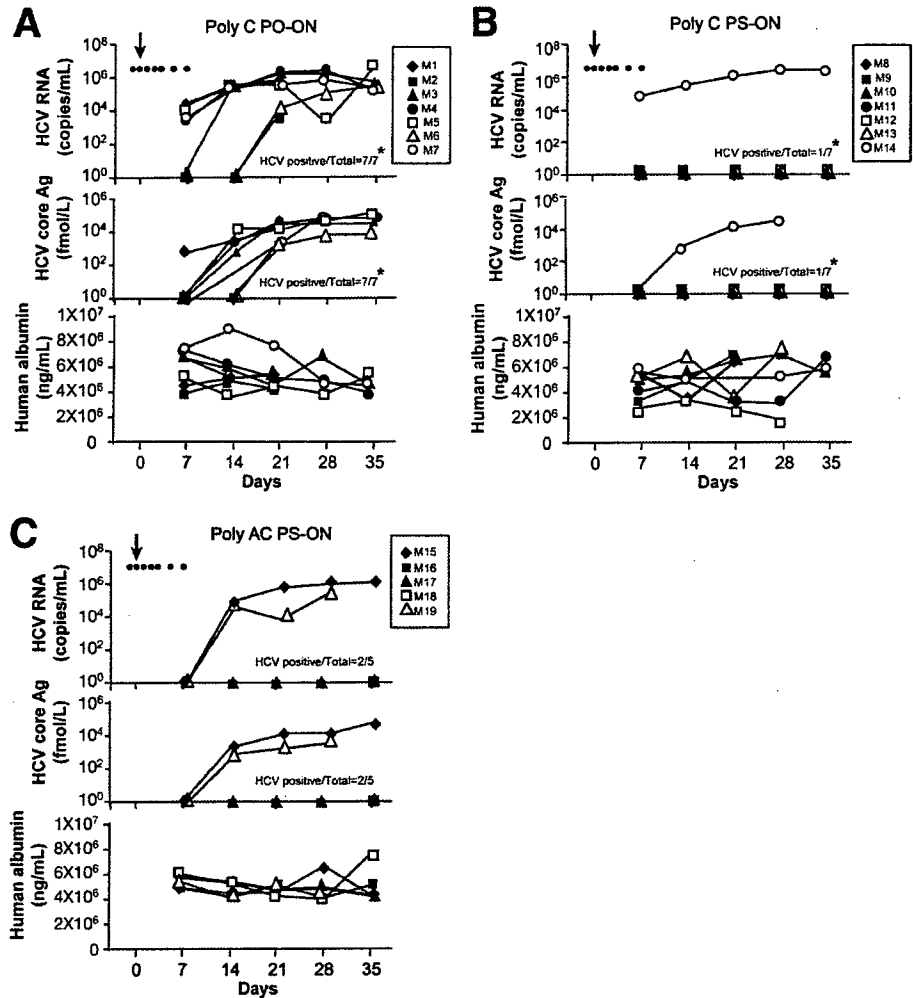


Figure 5. Effects of PS-ON on HCV infection in vivo. Human hepatocytes-transplanted uPA/SCID mice were treated intraperitoneally with 10 mg/kg of PS-ON (poly C) (n = 7) or (poly AC) (n = 5) on days -1, 0, 1, 3, 5, and 7 (indicated by dots). The corresponding control PO-ON (poly C) was also tested (n = 7). A fourth group of mice did not receive any compounds (n = 15). The mice were intravenously inoculated on day 0 with HCV patient serum containing 3.9×10^3 copies of HCV genotype 1b (indicated by arrow). Serum samples were obtained on days 0 (prior to HCV inoculation), 7, 14, 21, 28, and 35 for HCV RNA and human albumin determination. HCV core antigen was also measured and showed the same results as the HCV RNA determination.

the amphipathic PS-ONs have a sequence-independent antiviral activity against HIV-1 and other viruses,^{14,29} suggesting that these compounds may exhibit a broad-spectrum antiviral activity.

Our data showed that PS-ON blocked HCV infection in the HCVcc and HCVpp systems in a similarly dose-dependent manner, with 50% inhibitory concentration in the nanomolar range. PS-ON had no effect on infection of VSVGpp (an enveloped RNA virus with mechanism of viral entry distinct from type I and II fusion) or adenovirus (a nonenveloped DNA virus). The amphipathic nature of PS-ON is crucial for its anti-HCV property because PS-ON analogs lacking the amphipathicity are inactive. Polynucleotides are polyanions, a class of compounds that have been shown to interfere with a variety of viral infections.^{30,31} However, our data showed clearly that the polyanionic nature is not relevant to the PS-ON inhibitory activity because the control PO-ON is not active in HCV inhibition. Furthermore, the inhibitory effect of PS-ON could not be explained by the increased stability of the phosphorothioation because the control

PO-ON has the 2'-O-methyl modification that also stabilizes the oligonucleotides.^{14,23}

The inhibitory activity of APs is sequence independent but length dependent. The degenerate APs were equally effective as the homo- and heteropolymeric sequences, with the exception of poly A and G, which can form unique polypurine quartet structures in solution.³² The minimal length of PS-ON required for potent inhibitory activities is 40mer, which appears to be the same for all active PS-ON compounds. This length-specific requirement may indicate a critical structural feature of the HCV entry process that is susceptible to these compounds. Although the degenerate PS-ON may contain CpG motif, the other hetero- and homopolymer PS-ONs tested herein are devoid of CpG motifs. The comparable antiviral activity of these compounds to the degenerate PS-ON demonstrates that the antiviral activity is not mediated by the potential CpG-mediated induction of interferon. Furthermore, Huh7.5 cells express very little or none of the cell surface toll-like receptors involved in recognition of nucleic acid-based motifs,³³ and we did

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not observe any production of endogenous type I interferons in cells exposed to either PS-ON or PO-ON.

The inhibitory activity of PS-ON appears to target the postbinding and prereplication stage and possibly at the fusion step of HCV infection. The fusion process appears to be structurally conserved among many enveloped viruses and can be classified into types I and II.³⁴ The type I membrane fusion is exemplified by the influenza and HIV-1 via hemagglutinin and gp41, respectively. The type II fusion includes the alpha-viruses and flaviviruses.³⁴⁻³⁶ It has been proposed that HCV uses a type II fusion process because of its similarity to flaviviruses.³⁷ Our recent study suggests that HCV and flaviviruses are indeed structurally similar.³⁸ It is conceivable that the fusion process of HCV may be susceptible to inhibition by the amphipathic structure of PS-ON, but further confirmation is necessary. HCV entry has been shown to occur via receptor-mediated endocytosis and is sensitive to lysosomotropic agents and inhibitors of vacuolar ATPases.³⁹ The finding that PS-ON acts at the postbinding step like concanamycin A and bafilomycin A1, which are potent inhibitors of the vacuolar ATPases, supports this hypothesis. Furthermore, all HCV genotypes appeared to be susceptible to the APs equally, suggesting that the process involved is highly conserved.

HCV entry involves multiple cellular factors, such as CD81, SR-B1, Claudin-1, heparin sulfate, DC-SIGN, and L-SIGN, and possibly LDL receptor.^{5-10,31} CD81 and Claudin-1 have been postulated to act on the postbinding step.^{6,40} SR-B1 is likely involved in an early viral entry step to the cells. Its interaction with apolipoproteins and cholesterol transfer property appear to be important for viral entry,⁴¹ possibly at the level of membrane fusion.⁴² The overall mechanism of HCV entry is complex and involves multiple factors and steps. The APs likely interact with 1 of these essential steps to abort HCV entry. The unique inhibitory effect of the APs on HCV infection makes it a valuable reagent to study the molecular pathway of HCV entry. The APs can also be developed as a molecular probe to image and dissect biochemically this complex process.

Our study demonstrates that APs are potent inhibitors of HCV infection. APs are equally effective against all HCV genotypes and can inhibit de novo HCV infection in the human hepatocyte-transplanted uPA/SCID mouse model. This approach has the advantage of a novel and highly conserved target mechanism that is distinct from the small molecule inhibitors being developed clinically as well as the well-established pharmacology of antisense-based nucleic acid molecules in clinical trials. The effectiveness of this class of compounds in blocking de novo HCV infection supports its value in liver transplantation to prevent reinfection, which occurs invariably and presents a major problem for the management of these patients.⁴³ So far, prophylactic reagents based on neutralizing antibodies have been disappointing in clinical trials

of liver transplantation.⁴⁴ Our studies illustrate the promise of this class of compounds as a potent antiviral against HCV and support its further development in the therapy of hepatitis C.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2009.04.048.

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Reprint requests

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Conflicts of Interest

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A genome-wide association study identifies variants in the *HLA-DP* locus associated with chronic hepatitis B in Asians

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Chronic hepatitis B is a serious infectious liver disease that often progresses to liver cirrhosis and hepatocellular carcinoma; however, clinical outcomes after viral exposure vary enormously among individuals¹. Through a two-stage genome-wide association study using 786 Japanese chronic hepatitis B cases and 2,201 controls, we identified a significant association of chronic hepatitis B with 11 SNPs in a region including *HLA-DPA1* and *HLA-DPB1*. We validated these associations by genotyping two SNPs from the region in three additional Japanese and Thai cohorts consisting of 1,300 cases and 2,100 controls (combined $P = 6.34 \times 10^{-39}$ and 2.31×10^{-38} , OR = 0.57 and 0.56, respectively). Subsequent analyses revealed risk haplotypes (*HLA-DPA1*0202-DPB1*0501* and *HLA-DPA1*0202-DPB1*0301*, OR = 1.45 and 2.31, respectively) and protective haplotypes (*HLA-DPA1*0103-DPB1*0402* and *HLA-DPA1*0103-DPB1*0401*, OR = 0.52 and 0.57, respectively). Our findings show that genetic variants in the *HLA-DP* locus are strongly associated with risk of persistent infection with hepatitis B virus.

Chronic hepatitis B is one of the most common infectious liver diseases caused by hepatitis B virus (HBV). HBV infection shows a marked regional diversity and is very prevalent in the Asia-Pacific region; HBsAg seropositivity rates are as high as 5–12% in Thai and China, but as low as 0.2–0.5% in North America and Europe². It is estimated that, at present, more than 400 million people worldwide are chronically infected with HBV, and nearly 60% of liver cancers are considered to be related to chronic hepatitis B and subsequent liver cirrhosis³. Most HBV carriers are considered to have been infected

through maternal transmission in the neonatal period or infancy, particularly in Japan⁴. Although some HBV carriers spontaneously eliminate the virus, 2–10% of individuals with chronic hepatitis B are estimated to develop liver cirrhosis every year, and a subset of these individuals suffer from liver failure or hepatocellular carcinoma¹. Because clinical outcomes after exposure to HBV are highly variable, identification of genetic and environmental factors that are related to progression of HBV-induced liver diseases is critical.

Several epidemiological factors such as age at infection, sex, chronic alcohol abuse⁵ and co-infection with other hepatitis viruses⁶ were suspected to affect viral persistence. In addition, a twin study in Taiwan indicated that host genetic background influences infection outcome⁷. Although genetic variants in *IFNG*, *TNF*, *VDR*, *ESR1* and several *HLA* loci were shown to associate with chronic hepatitis B^{8–12}, none of the associations has been proven to be conclusive. To identify disease-predisposing variants, we carried out a two-stage association study for chronic hepatitis B using genome-wide SNPs as genetic markers.

Characteristics of each cohort group are shown in **Supplementary Table 1** online. We carried out a two-stage genome-wide association approach as described in the Methods. In the first stage, we genotyped 179 Japanese individuals with chronic hepatitis B and 934 control individuals using Illumina HumanHap550 BeadChip (**Fig. 1a**). For the second stage, we selected the top 12,000 SNPs that had the smallest *P* values on the basis of minimum *P* value considering three genetic models: allelic, dominant or recessive. Analysis of an independent set of 607 cases and 1,267 controls using these sub-selected SNPs showed 11 SNPs to be significantly associated ($P = 3.62 \times 10^{-8} \sim 1.16 \times 10^{-13}$) with chronic hepatitis B after Bonferroni correction (**Fig. 1b** and **Supplementary Table 2** online). Application of the Cochrane-Armitage

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test to all the tested SNPs indicated that the genetic inflation factor lambda was 1.02 for the second stage (Supplementary Fig. 1a online), implying a low possibility of false positive associations due to population stratification. All 11 SNPs are located within or around the *HLA-DPA1* and *HLA-DPB1* locus (Fig. 2). We also conducted age- and sex-adjusted analysis using a logistic regression model, and confirmed similar association after adjustment (data not shown).

To validate the result of the discovery-phase analysis, we carried out replication analyses using three independent cohorts. We selected the most or second-most strongly associated SNPs from each *HLA-DP* locus (rs9277535 on *HLA-DPB1* and rs3077 on *HLA-DPA1*, respectively), as we failed to design a Taqman or Invader probe for rs2395309 on *HLA-DPA1*. We first examined two independent sets of Japanese case-control samples comprising 274 cases and 274 controls (age-, sex- and alcohol consumption-matched cohort from BioBank Japan) as well as 718 cases and 1,280 controls. We found significant associations at two SNP loci in both studies ($P = 1.06 \times 10^{-16} \sim 1.96 \times 10^{-6}$; Table 1). We also genotyped 308 individuals with chronic hepatitis B and 546 healthy controls in Thailand, and further confirmed the association at the two loci, rs3077 ($P = 6.53 \times 10^{-6}$) and rs9277535 ($P = 6.52 \times 10^{-8}$).

To combine these studies, we conducted a meta-analysis with a fixed-effects model using the Mantel-Haenszel method. As shown in Table 1 and Supplementary Figure 1b, the odds ratios (OR) were quite similar across the four studies (the second stage of GWAS and three replication studies) and no heterogeneity was observed. Mantel-Haenszel P values for independence were 2.31×10^{-38} for

rs3077 (OR = 0.56, 95% confidence interval (CI) = 0.51–0.61), and 6.34×10^{-39} for rs9277535 (OR = 0.57, 95% CI = 0.52–0.62).

The 11 SNPs showing significant associations are located within a 50-kb region including *HLA-DPA1* and *HLA-DPB1* (Fig. 2). Although the *HLA* region is known to show extensive linkage disequilibrium (LD) spanning over 7 Mb, the LD block including these 11 SNPs (surrounded by a bold line in Fig. 2a) was not in strong LD with the other *HLA* loci. In accordance with the extent of LD, only SNPs around the *HLA-DPA1* and *HLA-DPB1* genes showed very strong associations with chronic HBV (surrounded by a bold line in Fig. 2b), and SNPs outside of this particular LD block did not have significant association.

HLA-DPA1 and *HLA-DPB1* encode the HLA-DP α and β chains, respectively. HLA-DPs belong to the HLA class II molecules that form heterodimers on the cell surface and present antigens to CD4-positive T lymphocytes. HLA-DPs are highly polymorphic, especially in exon 2, which encodes antigen-binding sites. We thus considered that the association of these SNPs with chronic HBV might reflect variations in antigen-binding sites that might affect the immune response to HBV. We genotyped *HLA-DPA1* and *HLA-DPB1* alleles by direct sequencing of exon 2 (cases at second stage and controls at first stage) and found significant association of chronic hepatitis B with *HLA-DPA1**0103, *DPA1**0202, *DPB1**0402 and *DPB1**0501 ($P = 2.93 \times 10^{-11}$, 4.45×10^{-8} , 2.27×10^{-7} and 6.98×10^{-7} , respectively; Supplementary Table 3 online). Because sequence variants in exon 2 of *HLA-DPA1* and *HLA-DPB1* could be linked to individual nucleotide variants, we inferred haplotypes using the 11 SNPs and variants in exon 2, and found very strong LD among them (Supplementary Fig. 2

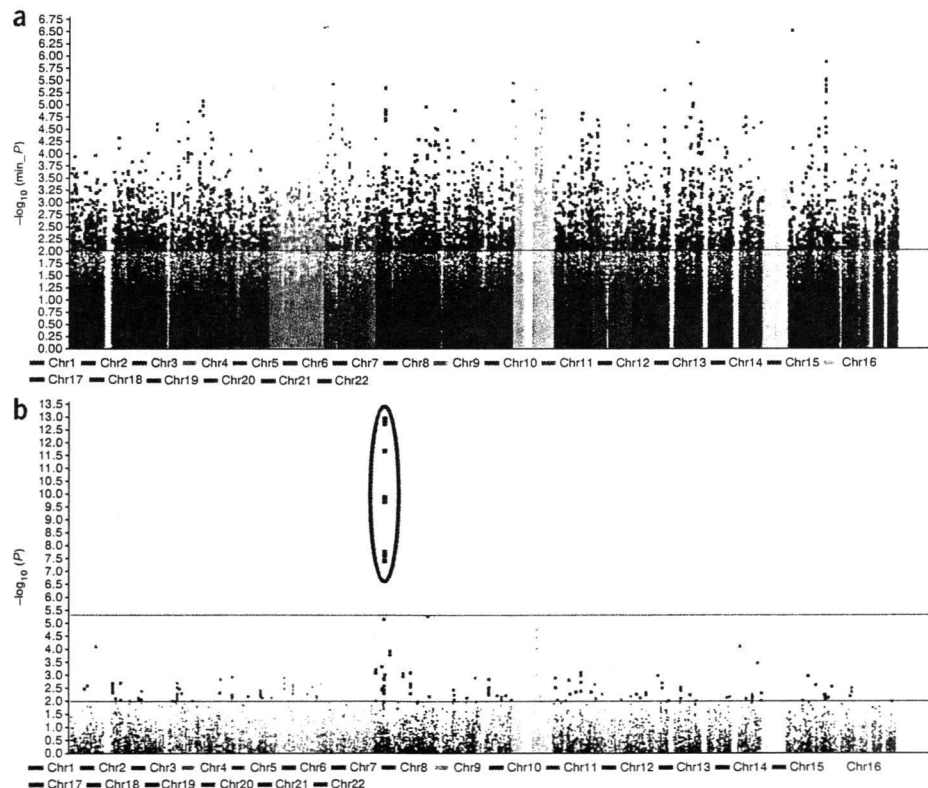


Figure 1 Results from a two-stage genome-wide association study. (a) $-\log_{10} P$ value plot at the first stage. Each P value is the minimum of Fisher's exact tests for three models: dominant, recessive and allele frequency model. (b) $-\log_{10} P$ value plot at the second stage. P values were calculated by 1-d.f. Cochran-Armitage trend test. The large dots circled by red on the chromosome 6 showed significant associations ($P < 5.06 \times 10^{-6}$) with chronic hepatitis B.

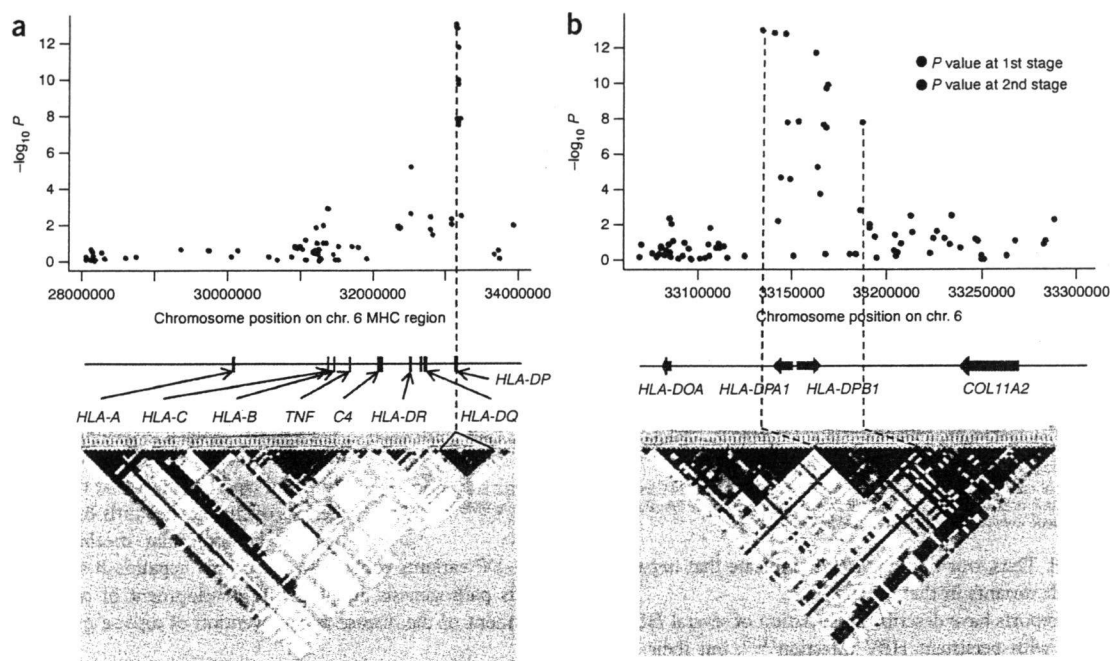


Figure 2 Case-control association results and linkage disequilibrium map of the MHC region. (a) P -value plot, genomic structure and LD map of the second stage within the extended MHC region of chromosome 6. The LD map based on D' was drawn using the genotype data of the cases and the controls in the second stage. (b) P -value plot, genomic structure and LD map around the *HLA-DPA1* and *HLA-DPB1* region. Black dots and red dots represent P values in the first and the second stage, respectively. The LD map based on D' was drawn using the genotype data of the cases and the controls in the first stage.

online). Case-control analyses revealed four associated haplotypes: *DPA1**0103-*DPB1**0402 and *DPA1**0103-*DPB1**0401 showed protective effects ($P = 6.00 \times 10^{-8}$, OR = 0.52, 95% CI = 0.35–0.75 and $P = 0.002$, OR = 0.57, 95% CI = 0.33–0.96, respectively), whereas *DPA1**0202-*DPB1**0501 and *DPA1**0202-*DPB1**0301 were associated with susceptibility to chronic hepatitis B ($P = 5.79 \times 10^{-6}$, OR = 1.45, 95% CI = 1.16–1.81 and $P = 0.002$, OR = 2.31, 95% CI = 1.39–3.84, respectively; Table 2). We also found various sets of SNPs (tagging SNPs) that could predict *HLA-DP* alleles (Supplementary Table 4 online). Taken together, our findings strongly implicate an association of genetic variants in the *HLA-DPA1* and *HLA-DPB1* genes with chronic hepatitis B.

HLA-DR13 was reported to have a protective effect against persistent HBV infection in different populations^{9,13,14}. Comparison of genotypes of *HLA-DRB1**1301 and *1302 alleles (both corresponding to *HLA-DR13*) and Illumina HumanHap550 SNPs in 333 of the first-stage control samples revealed that the A allele of rs11752643 was in strong LD with *HLA-DR13* ($r^2 = 0.83$, $D' = 1$). However, the association between rs11752643 and chronic hepatitis B was not significant in our second stage GWAS, with an uncorrected P value of 1.04×10^{-4} (Supplementary Table 5 online). In addition, the association of chronic hepatitis B with rs3077 and rs9277535 remained highly significant ($P = 2.11 \times 10^{-10}$ and 1.73×10^{-9} , respectively) after adjustment for rs11752643 using a logistic

Table 1 Results of replication studies and meta-analysis

SNP	Nearest gene	Allele (1/2)	Stage	Cases			Controls			OR (95%CI) ^a	P^b	P_{het}^c
				11	12	22	11	12	22			
rs3077	<i>HLA-DPA1</i>	A/G	GWAS second stage	42	240	324	197	598	472	0.57 (0.49–0.66)	1.26E–13	0.84
			First replication	25	95	152	50	122	102	0.53 (0.41–0.69)	1.73E–06	
			Second replication	64	237	410	197	596	485	0.55 (0.47–0.63)	1.06E–16	
			Third replication	28	109	163	85	250	210	0.61 (0.49–0.75)	6.53E–06	
			Meta-analysis ^d							0.56 (0.51–0.61)	2.31E–38	
rs9277535	<i>HLA-DPB1</i>	A/G	GWAS second stage	58	254	294	230	619	418	0.59 (0.51–0.69)	1.78E–12	0.85
			First replication	26	102	144	49	132	91	0.54 (0.42–0.69)	1.96E–06	
			Second replication	68	264	376	227	604	445	0.56 (0.48–0.64)	1.81E–16	
			Third replication	29	136	139	107	273	155	0.56 (0.46–0.69)	6.52E–08	
			Meta-analysis ^d							0.57 (0.52–0.62)	6.34E–39	

Odds ratio and P values for independence test were calculated by the Mantel-Haenszel method.

^aOdds ratio of minor allele from two-by-two allele frequency table. ^b P values of Pearson's χ^2 test for allele model. ^cResult of Breslow-Day test. ^dMeta-analysis of all four studies.

Table 2 Haplotype analysis

No.	Haplotype ^a	Frequency (cases)	Frequency (controls)	<i>P</i> ^b	OR ^b (95% CI)
1	GG-DPA1*0202-TCG-DPB1*0501-GAGATT	0.428	0.347	5.79E-06	1.45 (1.16–1.81)
2	AA-DPA1*0103-CCA-DPB1*0201-AGTGCC	0.165	0.192	0.052	Reference
3	GG-DPA1*0201-TCG-DPB1*0901-GGGGTC	0.129	0.124	0.642	1.21 (0.91–1.61)
4	AA-DPA1*0103-CTA-DPB1*0402-AGTGCC	0.042	0.096	6.00E-08	0.52 (0.35–0.75)
5	AA-DPA1*0103-CCA-DPB1*0401-AGTGCC	0.018	0.038	0.002	0.57 (0.33–0.96)
6	GG-DPA1*0202-TCG-DPB1*0301-GGGGTC	0.036	0.018	0.002	2.31 (1.39–3.84)
7	GG-DPA1*0202-TCG-DPB1*0202-AGTGCC	0.020	0.027	0.257	0.88 (0.51–1.52)
8	GG-DPA1*0202-TCG-DPB1*0201-AGTGCC	0.022	0.024	0.662	0.97 (0.57–1.65)
9	GG-DPA1*0201-TCG-DPB1*0501-GAGATT	0.029	0.018	0.057	1.81 (1.06–3.08)
10	GG-DPA1*0201-TCA-DPB1*1301-GGTGCC	0.022	0.016	0.172	1.69 (0.95–3.03)
11	AA-DPA1*0103-CTG-DPB1*0301-GGGGTC	0.011	0.016	0.246	0.74 (0.36–1.53)
12	GG-DPA1*0201-TCG-DPB1*1401-GGGGTC	0.012	0.012	0.877	1.25 (0.61–2.53)

Controls of the first stage and cases of the second stage were analyzed.

^aHaplotypes consisting of rs2595309, rs3077, HLA-DPA1, rs2301220, rs9277341, rs3135021, HLA-DPB1, rs9277535, rs10484569, rs3128917, rs2281388, rs3117222 and rs9380343 are shown. ^b*P* values, odds ratios and its 95% confidence intervals of each haplotype were calculated as described in the Methods.

regression model. Thus, our findings clearly indicate that hepatitis B is associated with variants in the HLA-DP loci.

A number of reports have described association of several HLA and non-HLA genes with persistent HBV infection^{12,15}, but their results were not consistent among the studies, and none of them indicated a possible involvement of the HLA-DP locus. This study is the first GWAS to investigate host genetic factors associated with chronic hepatitis B. One genome-wide linkage analysis using 318 microsatellite markers in the Gambian population suggested that the chromosome 21q22 region contains a susceptibility locus for persistent HBV infection¹⁶. However, our GWAS analysis failed to support this result, possibly owing to ancestry differences or different modes of viral transmission (the vertical transmission in Japan versus the horizontal transmission in Gambia).

To investigate the correlation between the incidence of hepatitis B infection and these polymorphisms, we evaluated the frequencies of rs3077 and rs9277535 in 11 different HapMap3 populations (Supplementary Table 6 online). Our association analysis indicated that A alleles at both rs3077 and rs9277535 were associated with protective effects for chronic hepatitis B. Notably, the frequencies of these two alleles were lower in Asian and African populations, especially in the Chinese population, compared with European and Central American populations. Although disease prevalence is not determined solely by genetic factors, the findings presented in our manuscript suggest that genetic factors might exert substantial influence on the prevalence of infectious disease.

Antigen presentations on HLA class II molecules to CD4-positive helper T cells and on class-I molecules to CD8-positive cytotoxic T cells are considered to be critical for the immune response against exposure to HBV. Although cytotoxic T cells are suspected to have major roles in viral clearance, helper T cells are also essential in the immune response to acute infections¹⁷. HLA-DPs have a structure similar to other classical HLA class II molecules, but their roles in the immune response have not been well characterized, except the association with berylliosis¹⁸. The 11 SNPs we found showing strong association with chronic HBV infection were in very strong LD with HLA-DP alleles. Because the subsequent haplotype analyses identified significant association of chronic hepatitis B with haplotypes containing the HLA-DPA1 and HLA-DPB1 genes, we suspected that variations in HLA-DP molecules would affect the ability for antigen presentation of HLA class II molecules on immune cells and result in weak

(or no) immune response and persistent HBV infection. A previous report that implicated HLA-DPA1*0103 and DPB1*0402 to be candidate predictive factors for antibody production after HBV vaccination¹⁹ supports this hypothesis. It should be noted that the lack of information regarding exposure to HBV for each control might underestimate the effect size obtained in this study but does not inflate the type 1 error rate.

In summary, we have demonstrated that genetic variants in the HLA-DP genes are strongly associated with chronic hepatitis B in the Asian population. Considering the function of HLA-DP molecules, our findings suggest that antigen presentation on HLA-DP molecules might be critical for virus elimination and have an important role in the pathogenesis of chronic hepatitis B. An understanding of the molecular mechanism by which

HLA-DP variants confer risk of chronic hepatitis B should shed light on its pathogenesis and facilitate development of new therapies for treatment of the disease and prevention of disease progression.

METHODS

Samples. Characteristics of each cohort group are shown in Supplementary Table 1. Case and control samples used in this study for the Japanese population were obtained from the BioBank Japan at the Institute of Medical Science, the University of Tokyo²⁰, except case samples of the second replication and control samples of the first stage of the GWAS. From the registered samples in BioBank Japan, we selected individuals that were clinically diagnosed as having chronic hepatitis B. The diagnosis of chronic hepatitis B was conducted based on HBsAg-seropositivity and elevated serum aminotransferase levels for more than six months according to the guideline for diagnosis and treatment of chronic hepatitis (see URLs section below). The control groups consisted of 2,821 individuals that were registered in BioBank Japan as subjects with diseases other than chronic hepatitis B. Subjects who were positive for HBsAg were excluded from the controls. We obtained 934 Japanese control DNAs in the first stage from volunteers in the Osaka-Midosuji Rotary Club, Osaka, Japan. Case samples for the second replication cohort (*n* = 718, RIKEN) were collected at Toranomon Hospital as well as at hospitals participating in the Hiroshima Liver Study Group (for a list of doctors participating in this study group, see URLs section below). Cases and controls for the Thai replication study (*n* = 308 and 546, respectively) were collected at Ramathibodi Hospital, Mahidol University, Thailand. The diagnosis of chronic hepatitis B was based on HBsAg-seropositivity and elevated serum aminotransferase levels. All participants provided written informed consent. This research project was approved by the ethical committees at the Institute of Medical Science, the University of Tokyo, the Center for Genomic Medicine (formerly SNP Research Center), RIKEN and Ramathibodi Hospital, Mahidol University.

SNP genotyping. We applied the two-stage approach as described previously²¹. For the first stage, we genotyped 188 individuals with chronic hepatitis B and 934 controls using the Illumina HumanHap550v3 Genotyping BeadChip. After excluding nine cases with call rate of <0.98, we applied SNP quality control (call rate of ≥0.99 in both cases and controls and *P* value of Hardy-Weinberg equilibrium test of ≥1.0 × 10⁻⁶ in controls): 499,544 SNPs on autosomal chromosomes passed the quality control filters and were further analyzed. Among the SNPs analyzed in the first stage, we selected the top 12,000 SNPs showing the smallest *P* values for the second stage. SNPs with minor allele frequency (MAF) of ≤0.1 in both case and control samples were excluded from the further analysis. In the second stage, we genotyped an additional panel of 616 cases using an

Affymetrix GeneChip Custom 10K array. After excluding nine cases with call rate of <0.95, all cluster plots were checked by visual inspection by trained staff, and SNPs with ambiguous calls were excluded. Ninety-four randomly selected case samples in the first stage were re-genotyped in the second stage, and SNPs with concordance rates of <98% between two assays (Illumina and Affymetrix) were excluded from the further analysis. We used genome-wide screening data of other diseases (uterine cervical cancer, esophageal cancer, hematological cancer, pulmonary tuberculosis, ovarian cancer, uterine body cancer and keroid) as controls for the second stage. All the samples were genotyped using the Illumina HumanHap550v3 Genotyping BeadChip, and the same quality-control filters as the first screening were applied. As a result, we analyzed 9,875 SNPs in 607 cases and 1,267 controls in the second stage and found 11 SNPs ($P < 5.06 \times 10^{-6}$) to be significantly associated with chronic hepatitis B after Bonferroni correction. These first and second stages are defined as the discovery phase of the research, and the following replication studies are defined as the replication phase. In the replication analyses, we used TaqMan genotyping system (Applied Biosystems) or the multiplex PCR-based Invader assay (Third Wave Technologies).

HLA-DPA1 and HLA-DPB1 genotyping. We analyzed HLA-DP genotypes using 607 cases (in the second stage of GWAS) and 934 controls (in the first stage of GWAS). Exon 2 of the HLA-DPA1 and HLA-DPB1 genes were amplified and directly sequenced according to the protocol of International Histocompatibility Workshop Group²². HLA-DPA1 and DPB1 alleles were determined based on the alignment database of dbMHC.

Statistical analysis. In the first stage of the GWAS, Fisher's exact test was applied to a two-by-two contingency table in three genetic models: an allele frequency model, a dominant-effect model and a recessive-effect model. At the second stage of GWAS and replication analyses, statistical significance of the association with each SNP was assessed using a 1-degree-of-freedom Cochran-Armitage trend test. Significance levels after Bonferroni correction for multiple testing were $P = 5.06 \times 10^{-6}$ (0.05/9,875) in the second stage and $P = 0.025$ (0.05/2) in replication analyses. Age- and sex-adjusted odds ratios were obtained by logistic regression analysis. Odds ratios and confidence intervals were calculated using the major allele as a reference. The meta-analysis was conducted using the Mantel-Haenszel method. Heterogeneity among studies was examined by using the Breslow-Day test. To assess the association of each HLA allele, we used Fisher's exact tests on two-by-two contingency tables with or without each HLA allele. To analyze the association of haplotypes, we used R package haplo.stats. *P* values for each haplotype were given by the results of a score test, and odds ratios and 95% confidence intervals were calculated from coefficients of GLM model. Odds ratios of each haplotype were calculated relative to the second major haplotype in Table 2, because the most common haplotype was the disease-associated haplotype. All of these statistical values were calculated by function haplo.cc. We used Haploview software to analyze linkage disequilibrium values between HLA-DR13 and SNPs.

Software. For general statistical analysis, we used R statistical environment version 2.6.1 or PLINK1.03 (ref. 23). To draw the LD map, we used Haploview software²⁴. Estimation of haplotype frequencies and analysis of haplotype association were performed by R package haplo.stats²⁵. Sequence variants in exon2 of HLA-DPA1 and HLA-DPB1 were analyzed by Polyphred.

URLs. The Japan Society of Hepatology, <http://www.jsh.or.jp/medical/guidelines/index.html>; Hiroshima Liver Study Group, <http://home.hiroshima-u.ac.jp/naika1/hepatology/english/study.html>; PLINK1.03, <http://pngu.mgh.harvard.edu/~purcell/plink/>; R package haplo.stats, http://mayoresearch.mayo.edu/mayo/research/schaid_lab/software.cfm; Polyphred, <http://droog.gs.washington.edu/polyphred/>.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

Y.N. conceived the study; Y.N., Y.K., Y.D., M.K. and K.M. designed the study; Y.K., S.W., H.O. and N.H. performed genotyping; Y.K., T.T., M.K., N.K., Y.N. and K.M. wrote the manuscript; T.K., A.T., T.T. and N.K. performed data analysis at the genome-wide phase; Y.N., K.M. and M.K. managed DNA samples belong to BioBankJapan; K.C. and H.K. managed second replication samples; W.C., A.P. and T.S. managed third replication samples in Thailand; Y.K. summarized the whole results; Y.N. obtained funding for the study.

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Intra-arterial 5-fluorouracil/interferon combination therapy for advanced hepatocellular carcinoma with or without three-dimensional conformal radiotherapy for portal vein tumor thrombosis

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Abstract

Background The aim of this study was to elucidate the efficacy of intra-arterial 5-fluorouracil (5-FU) and interferon (IFN) α combined with three-dimensional conformal radiotherapy (3D-CRT) for portal vein tumor thrombosis (PVTT).

Methods The study groups were 16 HCC patients with PVTT treated with 5-FU/IFN combined with 3D-CRT (RT group) and 16 matched controls treated with 5-FU/IFN alone (non-RT group). We compared the survival rate, response, time to progression (TTP), portal hypertension-related events (PREs) and safety.

Result Complete response (CR) of PVTT, partial response (PR), stable disease (SD) and progressive disease (PR) were noted in three (19%), nine (56%), four (25%) and zero patients of the RT group, one (6%), three (19%), seven (44%) and five (31%) patients of the non-RT group, respectively. The objective response rate of

PVTT was higher in the RT group ($P = 0.012$). The rate of PREs (variceal rupture, worsening of esophagogastric varices and emerging of uncontrollable ascites) was lower in the RT group than in the non-RT group ($P = 0.0195$). The median survival time of the RT group (7.5 months) was not significantly different from that of the non-RT group (7.9 months). RT-induced liver disease was not observed.

Conclusion 5-FU/IFN combination with 3D-CRT for PVTT improved the response rate of PVTT and reduced the incidence of portal hypertension-related events.

Keywords Hepatocellular carcinoma · Portal vein tumor thrombosis · Radiotherapy · 5-FU · IFN

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers and causes of cancer death worldwide [1–3]. Development of new diagnostic techniques, such as ultrasonography, computed tomography (CT), magnetic resonance imaging and angiography, and advancements in therapeutic modalities, such as surgical resection, radiofrequency ablation (RFA), percutaneous ethanol injection (PEI), transcatheter arterial chemoembolization (TACE), radiotherapy (RT) and intra-arterial infusion via implantable drug delivery systems have gradually improved the prognosis of HCC patients [4–8]. Nevertheless, the prognosis of patients with advanced HCC and portal vein tumor thrombosis (PVTT) is still poor [9–13]. PVTT causes widespread intrahepatic and extrahepatic dissemination by spreading out of tumor cells through the portal tract. Furthermore, PVTT, especially PVTT in the first branch (Vp3)

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or in the main trunk (Vp4), causes portal hypertension resulting in variceal rupture, uncontrollable ascites, ischemic liver failure and worsening of performance status (PS) [14]. These pathological states lead to simultaneous worsening of quality of life (QOL); hence, any treatment for HCC is contraindicated under such conditions, especially for those patients with HCC and PVTT in Vp3 or Vp4.

Recent advances in implantable drug delivery systems have facilitated repeated arterial infusion of chemotherapy agents. Because hepatic arterial infusion chemotherapy (HAIC) increases local tissue drug concentrations and consequently reduces the side effects of anticancer agents, this therapeutic modality is suitable for HCC patients with PVTT and poor hepatic reserve. Among several anticancer agents, intra-arterial 5-fluorouracil (5-FU) and systemic interferon (IFN) α were reported as one of the most effective combination chemotherapies for HCC with PVTT [15–18]. On the other hand, advances in three-dimensional conformal radiotherapy (3D-CRT) have allowed the delivery of higher radiation doses to the tumor and at the same time minimized the radiation dose to normal tissue, resulting in improvement of the antitumor effect and minimization of damage to normal tissue. This modality makes possible local irradiation for PVTT in patients with poor hepatic reserves [19, 20]. Despite the development of new chemotherapies and radiotherapies, the prognosis of HCC patients with Vp3/4 is still less than 1 year, and the response rate is less than about 50%.

In various malignancies such as lung cancer and esophageal cancer, the synergistic effects of the combination of chemotherapy and radiotherapy have been reported [21–24]. Recently, Han et al. [25] reported that the use of combination therapy of HAIC containing 5-FU/cisplatin (CDDP) and 3D-CRT in HCC patients with Vp3/4 showed a response rate of 45%. To our knowledge, however, there is no report about the combination therapy of 5-FU/IFN and 3D-CRT. In addition, it is still unclear whether RT has any additional effects on 5-FU/IFN. Accordingly, we performed a retrospective case-control study of intra-arterial 5-FU/IFN combination therapy with or without 3D-CRT for advanced HCC with Vp3/4. The aim of the present study was to elucidate the efficacy of 5-FU/IFN combination therapy with 3D-CRT by comparing survival, response, time to progression (TTP), portal hypertension related-events (PREs) and safety in the two groups.

Patients and methods

Study design and eligibility

The enrollment criteria were as follows: HCC with Vp 3 or Vp 4, PS 0 or 1, Child-Pugh A or B, serum total bilirubin

<3.0 mg/dl, leukocyte count >2,000/ml, platelet count >50,000/ml, serum creatinine <1.5 mg/dl, at least a 4-week rest period of no treatment since any previous treatment for HCC, no recent history of upper gastrointestinal bleeding, no history of heavy alcohol abuse, and no other serious medical condition that would interfere with participation in this study. Extrahepatic metastases were not exclusion criteria when we considered these were not prognostic factors of the patients. The exclusion criteria included HCC with inferior vena cava or hepatic vein tumor thrombosis. All patients were asked to provide a written informed consent for this study, which was approved by the Institutional Review Board of Hiroshima University.

From June 2003 to January 2008, 18 patients met the above criteria for 5-FU/IFN combined with 3D-CRT for Vp3/4. Two patients refused the therapy; thus, 16 patients were enrolled for the therapy. The 16 patients were defined as the RT group. To compare the clinical efficacy of the therapy, we retrospectively selected 16 patients treated with 5FU/IFN alone, matched 1:1 with patients of the RT group for sex, age, grade of portal vein invasion, grade of ascites and status of esophagogastric varices (form and red color sign) [26]. These patients were defined as the non-RT group. Patients of the non-RT group were selected from among 29 HCC patients with PVTT treated by 5-FU/IFN. The decision to use or not to use 3D-CRT was left to the attending physician. The baseline characteristics of patients of the two groups are shown in Table 1. There were no differences between the two groups with regard to the PS, etiology, HCC stage, main tumor size, tumor volume, extrahepatic metastases, α -fetoprotein (AFP), AFP-L3, des- γ -carboxy prothrombin (DCP), Child-Pugh grade, leukocyte count, hemoglobin, platelet count, total bilirubin, albumin and indocyanine green retention rate at 15 min (ICG-R (15)).

Treatment protocol

For the RT group, patients received 3D-CRT concomitantly with the first course of intra-arterial 5-FU/IFN. Patients received 3D-CRT in the Division of Radiation Oncology at our hospital. Patients of the RT group received high-energy photon beam irradiation using 18 or 6 MV, delivered by a three-dimensional conformal technique (CLINAC 2300 C/D linear accelerators, Varian Medical Systems Inc., Palo Alto, CA). The planning CT determined the gross tumor volume (GTV) as only the PVTT. The clinical target volume (CTV) was determined including GTV and intrahepatic tumor forming the basal part of PVTT. The planning target volume (PTV) represented the CTV plus a 10–20-mm margin in all directions for internal motion and set-up error. Four to five portal fields were

Table 1 Clinical profile of 32 patients with hepatocellular carcinoma and portal vein tumor thrombosis

	5FU/IFN combination with 3D-CRT	5FU/IFN alone	P value
Number	16	16	
Age (years) ^a	65.5 (35–79)	64 (53–76)	Matched
Sex (male/female)	15/1	15/1	Matched
PS (0/1)	14/2	12/4	NS
Etiology: HBV/HCV/other	4/9/3	5/8/3	NS
HCC stage (IVA/IVB)	11/5	13/3	NS
Grade of portal vein invasion (Vp 3/4) ^b	8/8	8/8	Matched
Main tumor size ^a	70 (36–130)	64 (22–115)	NS
Tumor volume ($\leq 50/\gt 50\%$)	11/5	13/3	NS
Extrahepatic metastases (yes/no)	6/10	4/12	NS
AFP (ng/ml) ^a	184.8 (<5–153,200)	586.7 (6–165,500)	NS
AFP-L3 (%) ^a	48.1 (<0.5–88.8)	27.1 (<0.5–87.6)	NS
DCP (mAU/ml) ^a	8,992 (36–392,790)	9,513 (61–722,140)	NS
Child-Pugh grade (A/B)	12/4	13/3	NS
Platelet count ($\times 10^3/\mu\text{l}$) ^a	11.3 (5.7–32.8)	14.4 (6.7–54.4)	NS
Total bilirubin (mg/dl) ^a	0.9 (0.4–1.9)	0.9 (0.6–1.9)	NS
Albumin (g/dl) ^a	3.8 (2.6–5)	3.7 (3.1–4.3)	NS
Ascites (absent/present)	12/4	12/4	Matched
ICG-R(15) (%) ^a	17.6 (3.2–32)	25.4 (6.8–47)	NS
Previous treatment (performed/not performed)	5/11	5/11	NS
Radiation dose (Gy) ^a	39 (30–45)	–	
Form of EV (F0/F1/F2) ^c	4/10/2	9/5/2	Matched
Red color sign of EV (RC0/RC1/RC2) ^c	12/3/1	13/2/1	Matched
Form of GV (F0/F1/F2) ^c	10/6/0	11/3/2	Matched
Red color sign of GV (RC0/RC1/RC2) ^c	16/0/0	15/1/0	Matched

PS Eastern Cooperative Oncology Group performance status, HBV hepatitis B virus, HCV hepatitis C virus, HCC hepatocellular carcinoma, AFP α -fetoprotein, AFP-L3 lens culinaris agglutininreactive fraction of α -fetoprotein, DCP des- γ -carboxy prothrombin, ICG-R (15) indocyanine green retention rate at 15 min, EV esophageal varices, GV gastric varices

^a Data are median values (range)

^b PVTT grade: Vp3 tumor thrombus in the first branch of the portal vein, Vp4 tumor thrombus in the trunk of the portal vein

^c Endoscopic findings of esophagogastric varices: F form, F0 absence of varices, F1 small straight, F2 enlarged tortuous, RC red color sign, RC0 no RC, RC1 localized RC, RC2 between localized and entire circumference RC

used. Outlined target volumes, total liver tissue and organ at risk structures, including the spinal cord, bilateral kidneys and intestinal tract nearby targets, were transferred to the treatment planning system (Pinnacle3, Philips Medical Systems, Eindhoven, The Netherlands) with reference to the diagnostic enhanced CT images. The prescribed dose was 30, 39 or 45 Gy in accordance with the dose-volume constraint of normal tissue and liver function. Ninety-five percent of the PTV should receive at least 95% of the prescribed dose. 50% of the liver tissue should not receive more than 25 Gy, 50% of each kidney not more than 20 Gy and maximum dose to the spinal cord, intestinal tract and esophagus not more than 40 Gy. Five, eight and three patients received a dose of 30, 39 and 45 Gy, in daily doses of 3 Gy per fraction, respectively.

In both groups, patients received repeated arterial infusions of anticancer agents via an injection port. One course of chemotherapy represented 2 weeks. 5-FU (500 mg/body weight/day; Kyowa Hakko, Tokyo) was administered within 5 h using a mechanical infusion pump on days 1–5 of the first and second weeks (5 g in one course). Recombinant IFN α -2b (Intron A, Schering-Plough Pharmaceuticals, Osaka, Japan), 3×10^6 U (3 MU), or natural IFN α (OIF, Otsuka Pharmaceuticals, Tokyo), 5×10^6 U (5 MU), was administered intramuscularly on days 1, 3 and 5 of each week (total dose, 18 and 30 MU, respectively). In principle, treatment was repeated several times unless PS changed to 3 or 4 during the treatment. A 2- to 4-week rest period of no treatment was allowed after each treatment course. As for the two types of IFN, we reported previously

similar effects of recombinant IFN α -2b and natural IFN α when combined with intraarterial 5-FU for the treatment of advanced HCC [15]. The arterial catheter was implanted as described previously [27].

Evaluation

The maximum response to therapy was assessed with contrast-enhanced CT at 1–2 months after completion of the first course of the treatment, and then every 2–3 months. The response was defined according to the response evaluation criteria in solid tumors (RECIST) [28]. A complete response (CR) was defined as disappearance of all target/non-target lesions, no appearance of any other lesion, and normalization of AFP and DCP. CR was confirmed at 4 weeks after the first evaluation of CR. A partial response (PR) was defined as a decrease of at least 30% in the sum of the longest diameter of target lesions with the baseline sum of the longest diameter of target lesions as the reference. Progressive disease (PD) was defined as an increase of at least 20% in the sum of the longest diameter of target lesions. Stable disease (SD) was defined as meeting neither the PR nor PD criteria. We evaluated the response to the therapies of PVTT and intrahepatic tumor as well as overall response. We also evaluated changes in ascites, esophagogastric varices and PS during the clinical course. We defined variceal rupture, worsening of esophagogastric varices and emerging of uncontrollable ascites as portal hypertension-related events (PREs).

Esophagogastric varices were assessed by endoscopic examination at 2–3 months after completion of the first course of treatment and then every 3–6 months. The varices-related endoscopic findings were evaluated according to the general rules proposed by the Japanese Research Society for Portal Hypertension [26]. In brief, the form of the varices (F factor) was classified as small straight (F1), enlarged tortuous (F2) or large coil-shaped (F3). The red color (RC) sign of the mucosal area covering the varices (red colored blood visualized underneath a very thin vascular wall) was classified according to the criteria of the Japanese Research Society for Portal Hypertension as negative (RC0), localized (RC1), between localized and entire circumference (RC2), and entire circumference (RC3). Worsening of esophagogastric varices was defined as deterioration in the F or RC factor of esophagogastric varices.

Adverse reactions were assessed every week during the treatment using the National Cancer Institute Common Toxicity Criteria (NCI-CTC) (version 3.0) [29].

Radiotherapy-induced liver disease (RILD) manifested by the development of anicteric elevation of the alkaline phosphatase level of at least twofold and nonmalignant ascites in the absence of documented progressive disease,

or elevated transaminases of at least fivefold the upper limit of normal or of pretreatment level, as proposed by Lawrence et al. [30].

Statistical analysis

Data were analyzed statistically on 1 July 2008. Differences between groups were examined for statistical significance using the Mann–Whitney test (*U* test), logistic regression test and χ^2 test where appropriate. The cumulative survival rate, TTP and PREs were calculated from the initial date of the therapy and assessed by the Kaplan–Meier life-table method, and differences were evaluated by the log rank test. Univariate analyses of predictors of survival and TTP were assessed by the Kaplan–Meier life-table method, and differences were evaluated by the log rank test. Variables that achieved statistical significance ($P < 0.05$) or those with P value of less than 0.10 on univariate analysis were entered into the multivariate analysis. Multivariate analyses of predictors of survival and TTP were assessed by Cox proportional hazard model. All aforementioned analyses were performed using the SPSS program (version 11, SPSS Inc., Chicago, IL). In this study, we assessed the survival benefits, PREs and safety of 5FU/IFN combined with 3D-CRT for HCC patients with PVTT.

Results

Response

Response of intrahepatic HCC

All patients received at least one course of the therapy. In the RT group, three patients were treated with 1 course, six with 2 courses, 5 with three courses and two patients with >4 courses. In the non-RT group, four patients were treated with 1 course, six with 2 courses, two with 3 courses and four with >4 courses. With regard to the maximum response of the intrahepatic tumor, of the RT group, one (6%), two (13%), eight (50%) and five (31%) patients and of the non-RT group, one (6%), three (19%), eight (50%) and four (25%) patients showed CR, PR, SD and PD, respectively. There were no statistically significant differences with regard to objective response (CR and PR) rates of intrahepatic HCC between the two groups ($P = 1.0$, Table 2).

Response of portal vein tumor thrombosis

With regard to the maximum response of PVTT, of the RT group, three (19%), nine (56%), four (25%) and zero (0%) patients and of the non-RT group, one (6%), three

Table 2 Response of intrahepatic HCC to treatment

	CR	PR	SD	PD	Response rate ^a (%)	P value
5-FU/IFN combination with 3D-CRT	1	2	8	5	19	1.0
5-FU/IFN alone	1	3	8	4	25	

HCC hepatocellular carcinoma, CR complete response, PR partial response, SD stable disease, PD progressive disease, 5-FU 5-fluorouracil, IFN interferon, 3D-CRT three-dimensional conformal radiotherapy

^a Response rate = CR + PR/CR + PR + SD + PD

Table 3 Response of portal vein tumor thrombosis to treatment

	CR	PR	SD	PD	Response rate ^a (%)	P value
5-FU/IFN combination with 3D-CRT	3	9	4	0	75	0.012
5-FU/IFN alone	1	3	7	5	25	

CR complete response, PR partial response, SD stable disease, PD progressive disease, 5-FU 5-fluorouracil, IFN interferon, 3D-CRT three-dimensional conformal radiotherapy

^a Response rate = CR + PR/CR + PR + SD + PD

(19%), seven (44%) and five (31%) patients showed CR, PR, SD and PD, respectively. The objective response rates were 75 and 25% in the RT group and the non-RT group, respectively. The objective response rate of PVTT was significantly higher in the RT group than the non-RT group ($P = 0.012$, Table 3).

Overall response

With regard to the maximum overall response, one (6%), two (13%), eight (50%) and five (31%) patients of the RT group and one (6%), three (19%), eight (50%) and four (25%) patients of the non-RT group showed CR, PR, SD and PD, respectively. There were no statistically significant differences with regard to overall response rates between the two groups.

Three (19%) patients of the RT group and four (25%) patients of the non-RT group showed objective response of both intrahepatic HCC and PVTT. Nine (56%) patients of the RT group and none of the non-RT group showed objective response of PVTT alone. None of each group showed objective response of intrahepatic HCC only (Table 4).

Time to progression

Overall time to progression

In the RT group, the cumulative overall TTP rates at 3, 6 and 12 months were 37.5, 62.5 and 85.0%, respectively. In the non-RT group, the cumulative overall TTP rates at 3, 6

Table 4 Objective response of intrahepatic HCC and portal vein tumor thrombosis

	5-FU/IFN combination with 3D-CRT (%)	5-FU/IFN alone (%)
Both intrahepatic HCC and PVTT	3/16 (19)	4/16 (25)
PVTT only	9/16 (56)	0/16 (0)
Intrahepatic HCC only	0/16 (0)	0/16 (0)

HCC hepatocellular carcinoma, 5-FU 5-fluorouracil, IFN interferon, 3D-CRT three-dimensional conformal radiotherapy, PVTT portal vein tumor thrombosis

and 12 months were 38.1, 58.7 and 72.5%, respectively. There were no significant differences with regard to overall TTP between the two groups ($P = 0.792$). The median TTP was 3.6 months (95% CI, 3.1–4.1 months) for the RT group and 3.8 months (95% CI, 1.2–6.4 months) for the non-RT group.

Time to progression of portal vein tumor thrombosis

In the RT group, the cumulative TTP of PVTT rates at 3, 6 and 12 months were 6.7, 27.4 and 27.4%, respectively. In the non-RT group, the cumulative TTP of PVTT rates at 3, 6 and 12 months were 38.1, 51.9 and 51.9%, respectively (Fig. 1). Univariate analysis identified positivity of HCV antibody ($P = 0.0134$), PS = 0 ($P = 0.0001$) and absence of extrahepatic metastases ($P = 0.0044$) as significant factors of TTP of PVTT. Multivariate analysis identified PS = 0 ($P = 0.012$), absence of extrahepatic metastases ($P = 0.005$) and 5-FU/IFN combined with RT ($P = 0.020$)

as significant and independent factors of TTP of PVTT (Table 5).

Incidence of portal hypertension-related events

During the observation period, rupture of esophageal varices occurred in zero patients and five (31.3%) patients of the RT and non-RT groups, respectively. Rupture of gastric varices did not occur in this cohort. The cumulative rupture rate of the esophageal varices at 6 and 12 months in the non-RT group were 21 and 37%, respectively. Worsening of esophagogastric varices was observed in two and ten patients of the RT group and non-RT group, respectively. The cumulative worsening of the esophagogastric varices rate at 6, 12 and 24 months were 14.3, 14.3 and 57.1% in

the RT group, respectively, and 57.6, 68.2 and 84.0% in the non-RT group, respectively. The cumulative rupture rate of esophageal varices was significantly higher in the non-RT group than in the RT group ($P = 0.040$). The worsening of the esophagogastric varices rate was significantly higher in the non-RT group than in the RT group ($P = 0.0244$, Fig. 2). In addition, none of the RT group and three patients of the non-RT group required preventive therapy for varices, such as Hassab’s operation, endoscopic injection sclerotherapy (EIS) and endoscopic variceal ligation (EVL) due to pending variceal rupture during the follow-up period.

At the commencement of this study, four (25%) patients of the RT group and four (25%) of the non-RT group developed controllable ascites. None showed uncontrollable ascites. During the follow-up period, uncontrollable ascites was noted in four (25%) patients of the RT group and ten (62.5%) of the non-RT group. In the RT group, the cumulative uncontrollable ascites rates at 6, 12, 24 months were 13, 13 and 57%, respectively, and in the non-RT group were 52, 60 and 74%, respectively. Although there was no statistical difference between the RT and non-RT group, the cumulative uncontrollable ascites rate of the RT group tended to be lower than that of the non-RT group ($P = 0.064$).

We defined variceal rupture, worsening of esophagogastric varices and development of uncontrollable ascites as portal hypertension-related events (PREs). In the RT group, the cumulative PREs-free rates at 3, 6, 12 and 24 months were 93.8, 79.3, 79.3 and 19.8%, respectively. In the non-RT group, the cumulative PREs-free rate at 3, 6, 12 and 24 months were 37.5, 30, 30 and 15%, respectively. The

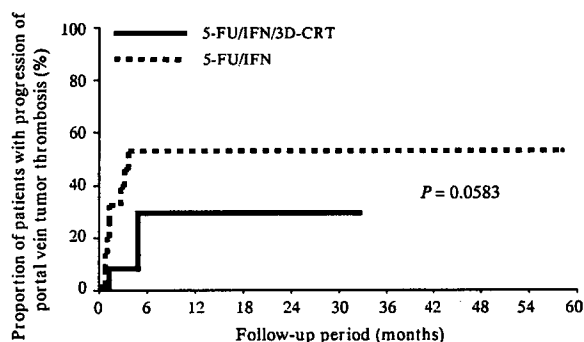


Fig. 1 Proportion of patients who showed progression of portal vein tumor thrombosis. Comparison between patients treated with 5-FU/IFN/three-dimensional conformal radiotherapy and 5-FU/IFN alone (log-rank test)

Table 5 Univariate and multivariate analyses of predictors of time to progression of portal vein tumor thrombosis

Variable	Univariate analysis P value	Multivariate analysis		
		Hazard ratio	95% CI	P value
Age (≤ 65 vs. >65)	0.1738			
Sex (M vs. F)	0.4026			
HCV antibody (positive vs. negative)	0.0134	–	–	0.187
Child Pugh stage (A vs. B, C)	0.0716	–	–	0.140
PS (0 vs. 1)	0.0001	6.726	1.532–29.527	0.012
Intrahepatic tumor volume (≤ 50 vs. $>50\%$)	0.5577			
Extrahepatic metastases (absence vs. presence)	0.0044	9.988	1.992–50.077	0.005
Vp (3 vs. 4)	0.8389			
AFP ($\leq 1,000$ vs. $>1,000$)	0.7778			
AFP-L3 (≤ 40 vs. >40)	0.5893			
DCP ($\leq 10,000$ vs. $>10,000$)	0.2378			
3D-CRT (combination with vs. without)	0.0583	6.287	1.340–29.505	0.020

HCV hepatitis C virus, PS Eastern Cooperative Oncology Group performance status, Vp3 tumor thrombus in the first branch of the portal vein, Vp4 tumor thrombus in the trunk of the portal vein, AFP α -fetoprotein, AFP-L3 lens culinaris agglutininreactive fraction of α -fetoprotein, DCP des- γ -carboxy prothrombin, 3D-CRT three-dimensional conformal radiotherapy

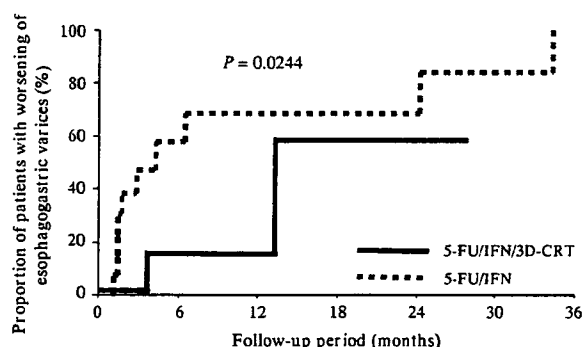


Fig. 2 Proportion of patients with worsening of esophagogastric varices. Comparison between patients treated with 5-FU/IFN/three-dimensional conformal radiotherapy and 5-FU/IFN alone (log-rank test)

difference in the cumulative PREs-free rate between the RT group and the non-RT group was significant ($P = 0.0195$, Fig. 3).

The median PS worsening-free periods were 6.6 (range 1.2–35.2) and 2.8 months (range 1.0–59.0) for the RT group and non-RT group, respectively. The median PS worsening-free period was longer in the RT group than in the non-RT group.

Survival

Data of the 32 patients showed that the median survival time (MST) was 7.9 months (95% CI, 4.6–11.2 months), and the cumulative survival rates at 6, 12 and 24 months were 61.3, 30.1 and 21.5%, respectively. The MST of the RT group [7.5 months (95% CI, 0.0–15.0 months)] was not significantly different from that of the non-RT group [7.9 months (95% CI, 6.1–9.7 months)] ($P = 0.871$, Fig. 4).

Univariate analysis identified positivity of HCV antibody ($P = 0.0009$), PS = 0 ($P = 0.0003$), absence of extrahepatic metastases ($P = 0.0002$) and objective response of both intrahepatic HCC and PVTT ($P = 0.0020$) as significant factors of overall survival. Multivariate analysis identified PS = 0 ($P = 0.020$), absence of extrahepatic metastases ($P = 0.001$) and objective response of intrahepatic HCC and PVTT ($P = 0.005$) as significant and independent factors of overall survival (Table 6).

Adverse reactions and complications

Table 7 lists the toxicity data for all patients during and after the treatment. Fever, fatigue, nausea and anorexia were the most common adverse events, but these were mostly NCI-CTC grade 1 or 2. NCI-CTC grade 3 or 4 adverse reactions relative to the RT group and the non-RT

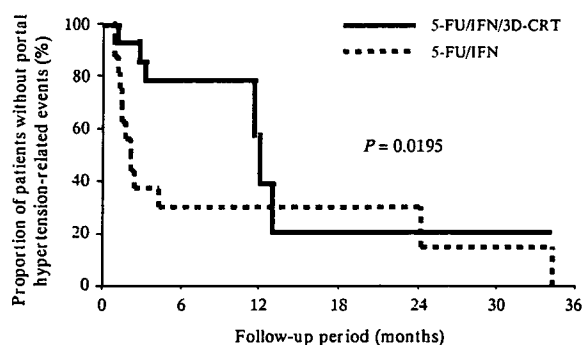


Fig. 3 Proportion of patients without portal hypertension-related events (variceal rupture, worsening of esophagogastric and emerging of uncontrollable ascites). Comparison between patients treated with 5-FU/IFN/three-dimensional conformal radiotherapy and 5-FU/IFN alone (log-rank test)

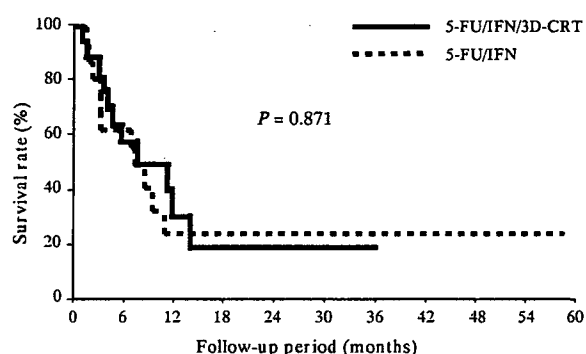


Fig. 4 Overall survival rate of patients treated with 5-FU/IFN/three-dimensional conformal radiotherapy and 5-FU/IFN alone (log-rank test)

group were as follows: leukopenia was observed in nine (56.3%) and three (18.8%) patients, thrombocytopenia in six (37.5%) and five (31.3%) patients and anorexia in one (6.3%) and one (6.3%) patient, respectively. The proportion of patients with NCI-CTC grade 3 or 4 leukopenia tended to be higher in the RT group than in the non-RT group ($P = 0.066$). Two patients of the RT group developed NCI-CTC grade 4 leukopenia and required administration of granulocyte colony-stimulating factor. None required platelet transfusion. None developed upper gastrointestinal ulcers associated with 3D-CRT. Furthermore, none developed RILD, and hepatic failure related to 5-FU/IFN with 3D-CRT was not observed during the follow-up period.

After 3D-CRT and one course of 5-FU/IFN, Child-Pugh classification did not change in the RT group: 13 patients, from A to B: 2 patients and from A to C: 1 patient. After one course of 5-FU/IFN, Child-Pugh classification did not change in the non-RT group: 13 patients, from A to B: 2 patients and from B to A: 1 patient. In the RT group, one