GASTROENTEROLOGY 2009;137:673–681

# Amphipathic DNA Polymers Inhibit Hepatitis C Virus Infection by Blocking Viral Entry

TAKUYA MATSUMURA,* ZONGYI HU,* TAKANOBU KATO,* MARLENE DREUX,‡ YONG-YUAN ZHANG,* MICHIO IMAMURA,§ NOBUHIKO HIRAGA,§ JEAN-MARC JUTEAU,‖ FRANCOIS-LOIC COSSET,‡ KAZUAKI CHAYAMA,§ ANDREW VAILLANT,‖ and T. JAKE LIANG*

*Liver Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland; ‡Universite de Lyon, INSERM U758, and Ecole Normale Superieure de Lyon, Lyon, France; §Department of Medicine and Molecular Science, Division of Frontier Medical Science, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan; and ‖REPLICor Inc, Laval, Quebec, Canada

**See editorial on page 427.**

**BACKGROUND & AIMS:** Hepatitis C virus (HCV) gains entry into susceptible cells by interacting with cell surface receptor(s). Viral entry is an attractive target for antiviral development because of the highly conserved mechanism. **METHODS:** HCV culture systems were used to study the effects of phosphorothioate oligonucleotides (PS-ONs), as amphipathic DNA polymers (APs), on HCV infection. The in vivo effects of APs were tested in urokinase plasminogen activator (uPA)/severe combined immunodeficient (SCID) mice engrafted with human hepatocytes. **RESULTS:** We show the sequence-independent inhibitory effects of APs on HCV infection. APs were shown to potently inhibit HCV infection at submicromolar concentrations. APs exhibited a size-dependent antiviral activity and were equally active against HCV pseudoparticles of various genotypes. Control phosphodiester oligonucleotide (PO-ON) polymer without the amphipathic structure was inactive. APs had no effect on viral replication in the HCV replicon system or binding of HCV to cells but inhibited viral internalization, indicating that the target of inhibition is at the postbinding, cell entry step. In uPA/SCID mice engrafted with human hepatocytes, APs efficiently blocked de novo HCV infection. **CONCLUSIONS: Our results demonstrate that APs are a novel class of antiviral compounds that hold promise as a drug to inhibit HCV entry.**

Hepatitis C virus (HCV) infects approximately 200 million people worldwide.[1] The majority of HCV-infected patients fails to clear the virus, and many develop chronic liver disease including cirrhosis with a risk of hepatocellular carcinoma. Treatment of chronic hepatitis C is currently based on peginterferon-alfa and ribavirin, which is accompanied by substantial adverse effects and is only effective in approximately half of the patients.[2,3] In addition to other viral targets, viral entry is an attractive target for antiviral development because of the potentially conserved mechanism of viral entry.[4] Although several candidate receptors for HCV have been identified,[5–10] the mechanism of HCV entry still remains largely unknown. Previous reports have indicated a pH dependency for entry of HCV pseudoparticles (HCVpp) as well as cell culture-generated HCV (HCVcc), suggesting that HCV enters cells by receptor-mediated endocytosis.[7,11,12] Antiviral compounds targeting the entry step of viral infection have been successfully developed in other viral infections.[13] Recent studies have shown that phosphorothioate oligonucleotides (PS-ONs), as amphipathic DNA polymers (APs), have a sequence-independent antiviral activity against human immunodeficiency virus type 1 (HIV-1) as entry inhibitors.[14] The antiviral effect of APs appears to be specific to the phosphorothioate backbone, which confers an amphipathic structure, because the phosphodiester oligonucleotides (PO-ONs) as nonamphipathic polymers are ineffective.[14]

## Materials and Methods

### *Cell Culture and Oligonucleotide Synthesis*

Huh7.5 (provided by Charles Rice), Huh7.5.1 (provided by Francis Chisari), Huh7, and Hep3B cells were maintained at 37°C, 5% $CO_2$ in Dulbecco's modified Eagle medium, containing 10% fetal bovine serum. All PS-ONs and PO-ONs were synthesized as described previously.[14] Oligonucleotides lacking the phosphorothioate modification (PO-ONs) were synthesized with the addition of 2′-O-methyl ribose modification, which stabilizes oligonucleotides from nuclease degradation.[14] Compounds used in the in vivo experiment were synthesized under good manufacturing practice (GMP) conditions to yield high-purity sodium salts.

*Abbreviations used in this paper:* **APs, amphipathic DNA polymers; HCV, hepatitis C virus; HCVcc, cell culture-generated HCV; HCVpp, HCV pseudoparticles; VSVGpp, vesicular stomatitis virus G protein pseudoparticle; PO-ON, phosphodiester oligonucleotide; PS-ONs, phosphorothioate oligonucleotides.**


0016-5085/09/$36.00
doi:10.1053/j.gastro.2009.04.048

### *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 antibody[17] (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 $CaCl_2$) buffer containing 1% bovine serum albumin as reported previously[20] 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 reported[21] 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 AC PS-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 $3.9 \times 10^3$ 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 ± 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 1*A*) and intracellular HCV RNA quantification (Figure 1*B*). 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 1*C* 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 1*E*). 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 1*F*). 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 2*A*). Similar effects were also observed on HCV core antigen and HCV RNA levels in the culture supernatant (Figure 2*B* and *C*). In the HCVpp system, these PS-ONs also had similar inhibitory effects (Figure 2*D*).

### *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

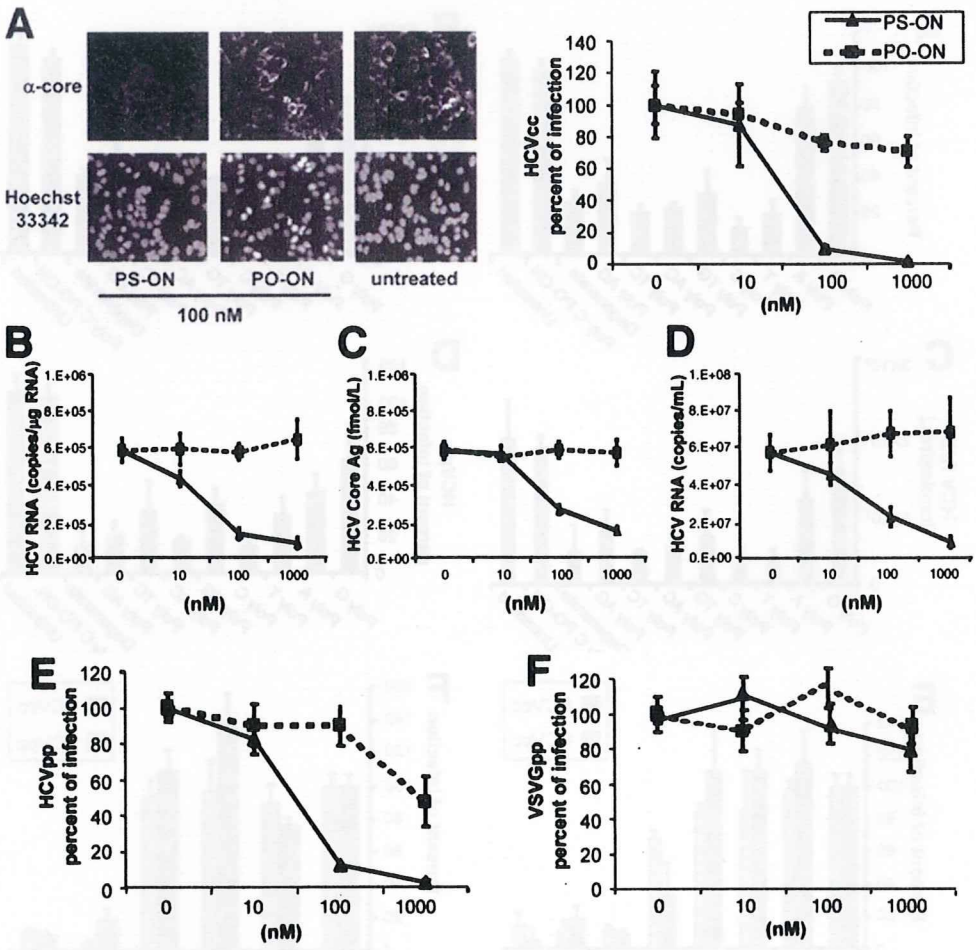


**Figure 1.** Effect of PS-ON on HCV infection. (*A*) Huh7.5 cells were infected with HCVcc in the presence of various concentrations of 40mer PS-ON or PO-ON (degenerate sequence). Two days after infection, infected cells were detected by immunofluorescence assay using anticore antibodies (*left panel*). 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 2*E*). 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 2*F*). 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 3*A*). Similar observation was obtained with the poly C compounds (Supplementary Figure 2*D*).

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 3*B*). 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

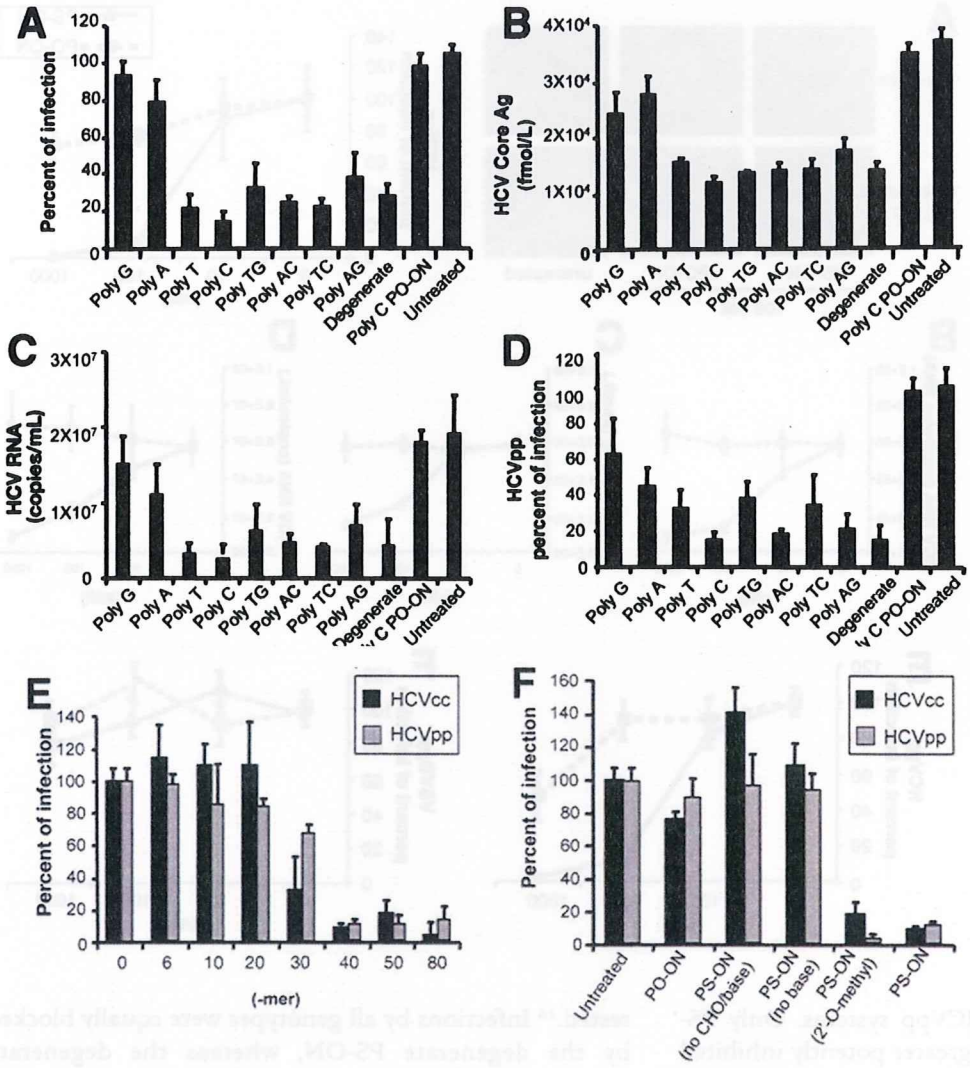


**Figure 2.** Sequence-independent and size- and phosphorothioation-dependent effects of PS-ON on HCV infection. A series of 40mer PS-ONs with specific sequences including poly poly G, A, T, C, TG AC, TC, and AG were tested for their inhibitory effect on HCV infection. (*A*) HCVcc was inoculated with 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 3*C*). 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 3*D*, 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 3*E*), 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

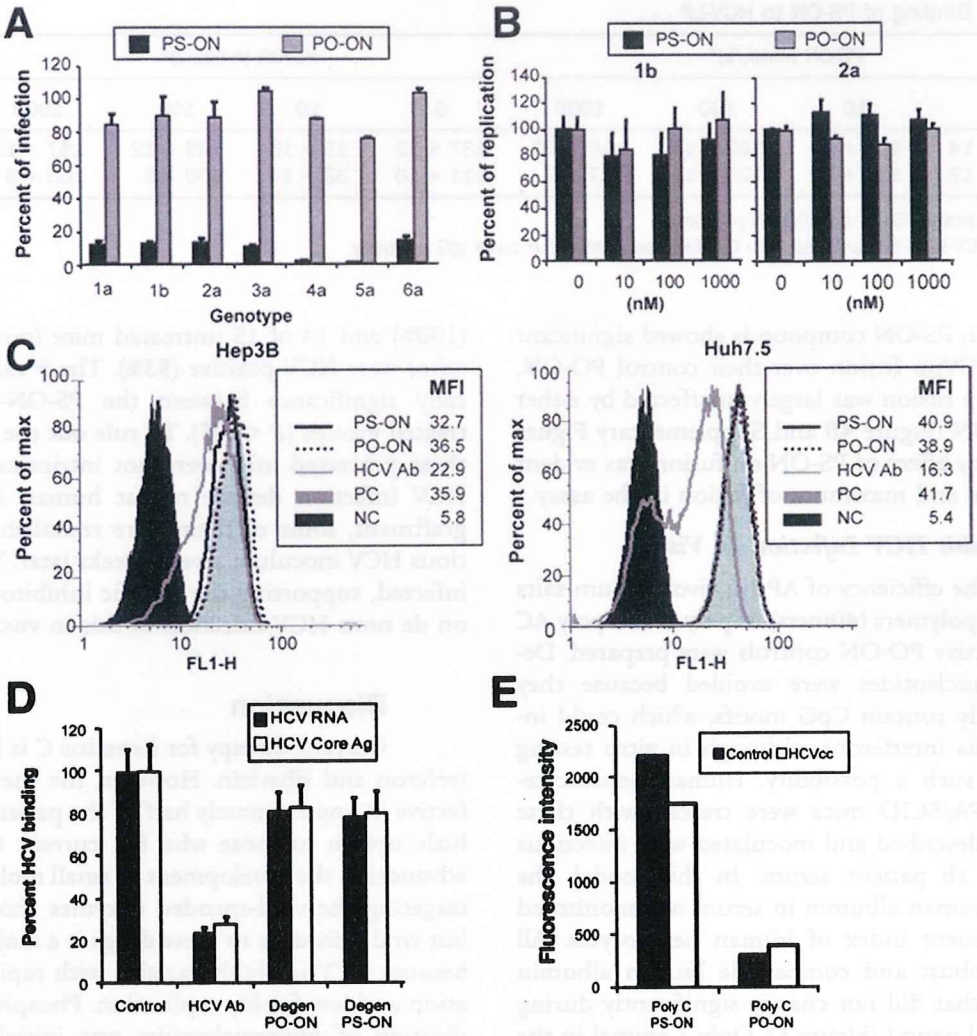


**Figure 3.** Effects of PS-ON on infection of various HCV genotypes, HCV replication, and cell binding. (*A*) HCVpp harboring E1/E2 glycoproteins from genotypes 1a, 1b, 2a, 3a, 4a, 5a, and 6a were inoculated into Hep3B cells and simultaneously treated with 100 nmol/L of degenerate PS-ON and PO-ON (40mer). 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 μg/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 iodixinol 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 μg/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 4*A*, 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

**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 μg/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 |

[a]Cy3 labeled degenerate PS-ON and PO-ON (40mer).
[b]AP33 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 4*B* 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



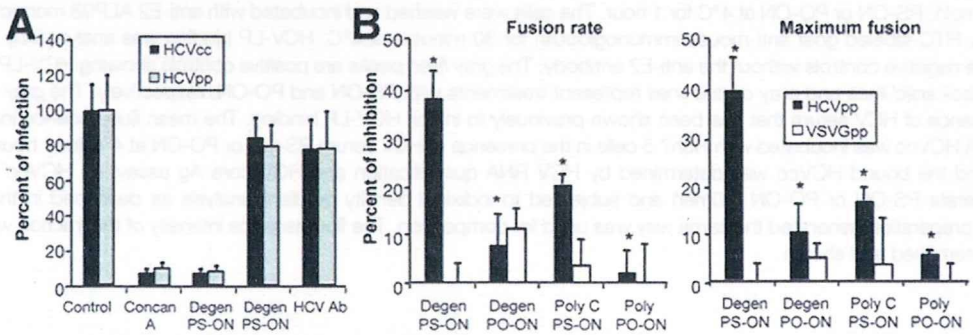


**Figure 4.** Effects of PS-ON on HCV viral entry. (*A*) Hep3B cells were incubated with HCVpp at 4°C for 1 hour to bind the virus and washed to remove the unbound virus. Cells were then incubated with fresh culture medium containing 25 nmol/L concanamycin A, 100 nmol/L PS-ON, 100 nmol/L PO-ON, or 25 μg/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.

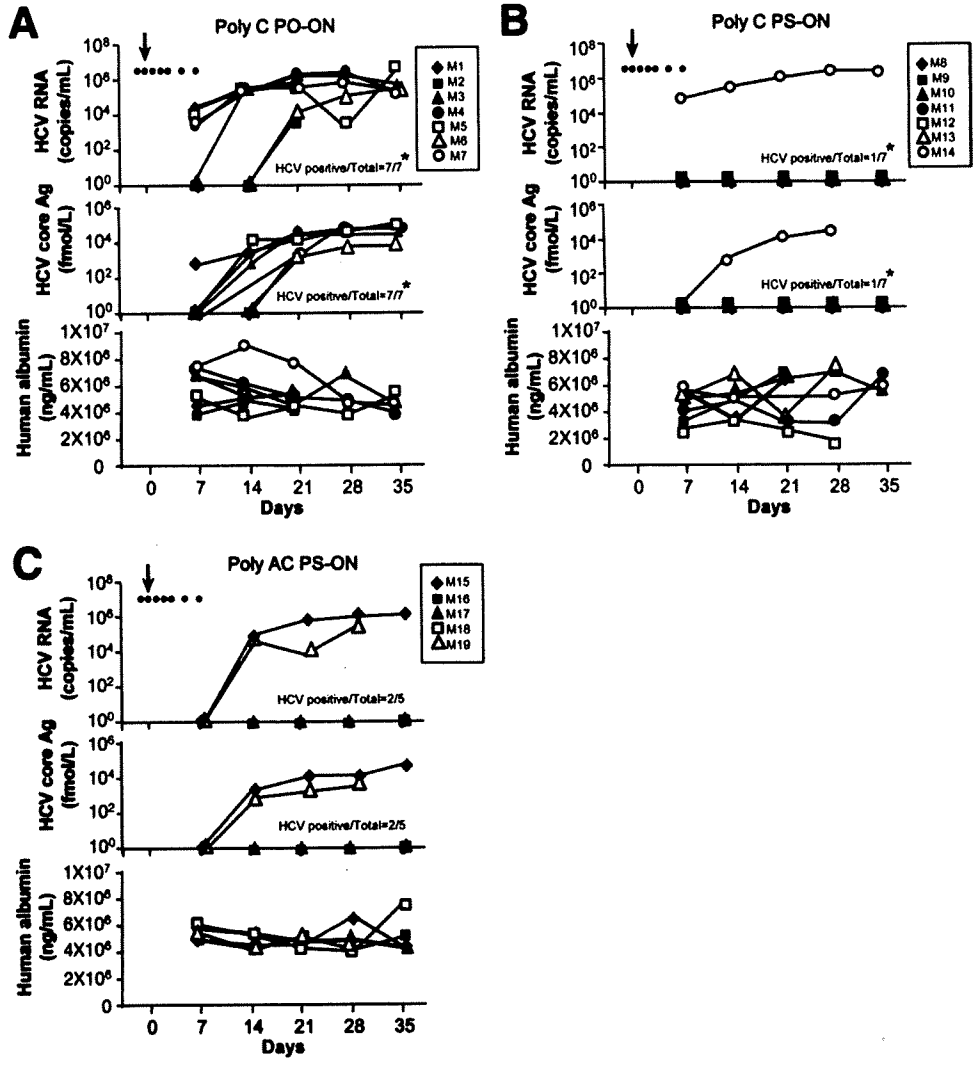


**Figure 5.** Effects of PS-ON on HCV infection in vivo. Human hepatocytes-transplanted uPA/SCID mice were treated intraperitoneally with 10 mg/kg of PS-ON (poly C) (n = 7) or (poly AC) (n = 5) on days −1, 0, 1, 3, 5, and 7 (indicated by *dots*). The corresponding control PO-ON (poly C) was also tested (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

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.

### References


1. Liang TJ, Rehermann B, Seeff LB, et al. Pathogenesis, natural history, treatment, and prevention of hepatitis C. Ann Intern Med 2000;132:296–305.
2. Feld JJ, Hoofnagle JH. Mechanism of action of interferon and ribavirin in treatment of hepatitis C. Nature 2005;436:967–972.
3. Pawlotsky JM. Therapy of hepatitis C: from empiricism to eradication. Hepatology 2006;43:S207–S220.
4. Smith AE, Helenius A. How viruses enter animal cells. Science 2004;304:237–242.
5. Bartosch B, Dubuisson J, Cosset FL. Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. J Exp Med 2003;197:633–642.
6. Evans MJ, von Hahn T, Tscherne DM, et al. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. Nature 2007;446:801–805.
7. Hsu M, Zhang J, Flint M, et al. Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. Proc Natl Acad Sci U S A 2003;100:7271–7276.
8. Lindenbach BD, Evans MJ, Syder AJ, et al. Complete replication of hepatitis C virus in cell culture. Science 2005;309:623–626.
9. Lozach PY, Lortat-Jacob H, de Lacroix de Lavalette A, et al. DC-SIGN and L-SIGN are high affinity binding receptors for hepatitis C virus glycoprotein E2. J Biol Chem 2003;278:20358–20366.
10. Scarselli E, Ansuini H, Cerino R, et al. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. EMBO J 2002;21:5017–5025.
11. Bartosch B, Vitelli A, Granier C, et al. Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. J Biol Chem 2003; 278:41624–41630.
12. Koutsoudakis G, Kaul A, Steinmann E, et al. Characterization of the early steps of hepatitis C virus infection by using luciferase reporter viruses. J Virol 2006;80:5308–5320.
13. Rusconi S, Scozzafava A, Mastrolorenzo A, et al. An update in the development of HIV entry inhibitors. Curr Top Med Chem 2007; 7:1273–1289.
14. Vaillant A, Juteau JM, Lu H, et al. Phosphorothioate oligonucleotides inhibit human immunodeficiency virus type 1 fusion by blocking gp41 core formation. Antimicrob Agents Chemother 2006;50:1393–1401.
15. Wakita T, Pietschmann T, Kato T, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005;11:791–796.
16. Lavillette D, Tarr AW, Voisset C, et al. Characterization of host-range and cell entry properties of the major genotypes and subtypes of hepatitis C virus. Hepatology 2005;41:265–274.
17. Owsianka A, Tarr AW, Juttla VS, et al. Monoclonal antibody AP33 defines a broadly neutralizing epitope on the hepatitis C virus E2 envelope glycoprotein. J Virol 2005;79:11095–11104.
18. Kato T, Date T, Miyamoto M, et al. Detection of anti-hepatitis C virus effects of interferon and ribavirin by a sensitive replicon system. J Clin Microbiol 2005;43:5679–5684.

19. Nanda SK, Herion D, Liang TJ. The SH3 binding motif of HCV (corrected) NS5A protein interacts with Bin1 and is important for apoptosis and infectivity. Gastroenterology 2006;130:794–809.
20. Triyatni M, Saunier B, Maruvada P, et al. Interaction of hepatitis C virus-like particles and cells: a model system for studying viral binding and entry. J Virol 2002;76:9335–9344.
21. Lavillette D, Bartosch B, Nourrisson D, et al. Hepatitis C virus glycoproteins mediate low pH-dependent membrane fusion with liposomes. J Biol Chem 2006;281:3909–3917.
22. Tateno C, Yoshizane Y, Saito N, et al. Near completely humanized liver in mice shows human-type metabolic responses to drugs. Am J Pathol 2004;165:901–912.
23. Lamond AI, Sproat BS. Antisense oligonucleotides made of 2′-O-alkylRNA: their properties and applications in RNA biochemistry. FEBS Lett 1993;325:123–127.
24. Steinmann D, Barth H, Gissler B, et al. Inhibition of hepatitis C virus-like particle binding to target cells by antiviral antibodies in acute and chronic hepatitis C. J Virol 2004;78:9030–9040.
25. Baumert TF, Vergalla J, Satoi J, et al. Hepatitis C virus-like particles synthesized in insect cells as a potential vaccine candidate. Gastroenterology 1999;117:1397–1407.
26. Wellnitz S, Klumpp B, Barth H, et al. Binding of hepatitis C virus-like particles derived from infectious clone H77C to defined human cell lines. J Virol 2002;76:1181–1193.
27. Pawlotsky JM, Chevaliez S, McHutchison JG. The hepatitis C virus life cycle as a target for new antiviral therapies. Gastroenterology 2007;132:1979–1998.
28. Agrawal S, Tang JY, Brown DM. Analytical study of phosphorothioate analogues of oligodeoxynucleotides using high-performance liquid chromatography. J Chromatogr 1990;509:396–399.
29. Lee AM, Rojek JM, Gundersen A, et al. Inhibition of cellular entry of lymphocytic choriomeningitis virus by amphipathic DNA polymers. Virology 2008;372:107–117.
30. Moulard M, Lortat-Jacob H, Mondor I, et al. Selective interactions of polyanions with basic surfaces on human immunodeficiency virus type 1 gp120. J Virol 2000;74:1948–1960.
31. Barth H, Schafer C, Adah MI, et al. Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. J Biol Chem 2003;278:41003–41012.
32. Kim J, Cheong C, Moore PB. Tetramerization of an RNA oligonucleotide containing a GGGG sequence. Nature 1991;351:331–332.
33. Preiss S, Thompson A, Chen X, et al. Characterization of the innate immune signalling pathways in hepatocyte cell lines. J Viral Hepat 2008;15:888–900.
34. Kielian M, Rey FA. Virus membrane-fusion proteins: more than one way to make a hairpin. Nat Rev Microbiol 2006;4:67–76.
35. Kielian M. Class II virus membrane fusion proteins. Virology 2006;344:38–47.
36. Lescar J, Roussel A, Wien MW, et al. The fusion glycoprotein shell of Semliki Forest virus: an icosahedral assembly primed for fusogenic activation at endosomal pH. Cell 2001;105:137–148.
37. Yagnik AT, Lahm A, Meola A, et al. A model for the hepatitis C virus envelope glycoprotein E2. Proteins 2000;40:355–366.
38. Yu X, Qiao M, Atanasov I, et al. Cryo-electron microscopy and three-dimensional reconstructions of hepatitis C virus particles. Virology 2007;367:126–134.
39. Tscherne DM, Jones CT, Evans MJ, et al. Time- and temperature-dependent activation of hepatitis C virus for low-pH-triggered entry. J Virol 2006;80:1734–1741.
40. Cormier EG, Tsamis F, Kajumo F, et al. CD81 is an entry coreceptor for hepatitis C virus. Proc Natl Acad Sci U S A 2004;101:7270–7274.
41. Bartosch B, Verney G, Dreux M, et al. An interplay between hypervariable region 1 of the hepatitis C virus E2 glycoprotein, the scavenger receptor BI, and high-density lipoprotein promotes both enhancement of infection and protection against neutralizing antibodies. J Virol 2005;79:8217–8229.
42. Dreux M, Boson B, Ricard-Blum S, et al. The exchangeable apolipoprotein ApoC-I promotes membrane fusion of hepatitis C virus. J Biol Chem 2007;282:32357–32369.
43. Samuel D, Bizollon T, Feray C, et al. Interferon-$\alpha$ 2b plus ribavirin in patients with chronic hepatitis C after liver transplantation: a randomized study. Gastroenterology 2003;124:642–650.
44. Schiano TD, Charlton M, Younossi Z, et al. Monoclonal antibody HCV-AbXTL68 in patients undergoing liver transplantation for HCV: results of a phase 2 randomized study. Liver Transpl 2006;12:1381–1389.

**Received December 28, 2008. Accepted April 16, 2009.**

*Reprint requests*

**Address requests for reprints to: T. Jake Liang, MD, LDB/NIDDK/NIH, Bldg 10-9B16, 10 Center Dr, Bethesda, Maryland. e-mail: JakeL@bdg10.niddk.nih.gov; fax: (301) 402-0491.**

*Acknowledgments*

**The authors thank Charles Rice, Robert Purcell, Jens Bukh, and Thomas Baumert for providing various valuable reagents.**

*Conflicts of Interest*

**The authors diclose the following: J.-M.J. and A.V. are employees of REPLICor, Inc. The remaining authors disclose no conflicts.**

*Funding*

**Supported in part by the Intramural Research Program of the National Institute of Diabetes and Digestive and Kidney Diseases, NIH, and a NIH Cooperative Research and Development Agreement (DK-06-0367) between NIDDK and REPLICor, Inc.**

nature genetics

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

Yoichiro Kamatani[1,2], Sukanya Wattanapokayakit[3], Hidenori Ochi[4,5], Takahisa Kawaguchi[4], Atsushi Takahashi[4], Naoya Hosono[4], Michiaki Kubo[4], Tatsuhiko Tsunoda[4], Naoyuki Kamatani[4], Hiromitsu Kumada[6], Aekkachai Puseenam[7], Thanyachai Sura[7], Yataro Daigo[1,2], Kazuaki Chayama[4,5], Wasun Chantratita[8], Yusuke Nakamura[1,4] & Koichi Matsuda[1]



**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 individuals[1]. 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 $2.31 \times 10^{-38}$, 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 Europe[2]. 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 cirrhosis[3]. Most HBV carriers are considered to have been infected through maternal transmission in the neonatal period or infancy, particularly in Japan[4]. 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 carcinoma[1]. 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 abuse[5] and co-infection with other hepatitis viruses[6] were suspected to affect viral persistence. In addition, a twin study in Taiwan indicated that host genetic background influences infection outcome[7]. 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

[1]Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, the University of Tokyo, Tokyo, Japan. [2]Department of Medical Genome Sciences, Graduate School of Frontier Sciences, the University of Tokyo, Tokyo, Japan. [3]Medical Genetic Section, National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand. [4]Center for Genomic Medicine, RIKEN, Kanagawa, Japan. [5]Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan. [6]Department of Hepatology, Toranomon Hospital, Tokyo, Japan. [7]Department of Medicine, Faculty of Medicine and [8]Virology and Molecular Microbiology Unit, Department of Pathology, Faculty of Medicine, Ramathidi Hospital, Mahidol University, Bangkok, Thailand. Correspondence should be addressed to Y.N. (yusuke@ims.u-tokyo.ac.jp).

Received 10 November 2008; accepted 16 January 2009; published online 6 April 2009; doi:10.1038/ng.348

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 $2.31 \times 10^{-38}$ for rs3077 (OR = 0.56, 95% confidence interval (CI) = 0.51–0.61), and $6.34 \times 10^{-39}$ 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 ($P = 2.93 \times 10^{-11}$, $4.45 \times 10^{-8}$, $2.27 \times 10^{-7}$ and $6.98 \times 10^{-7}$, 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**

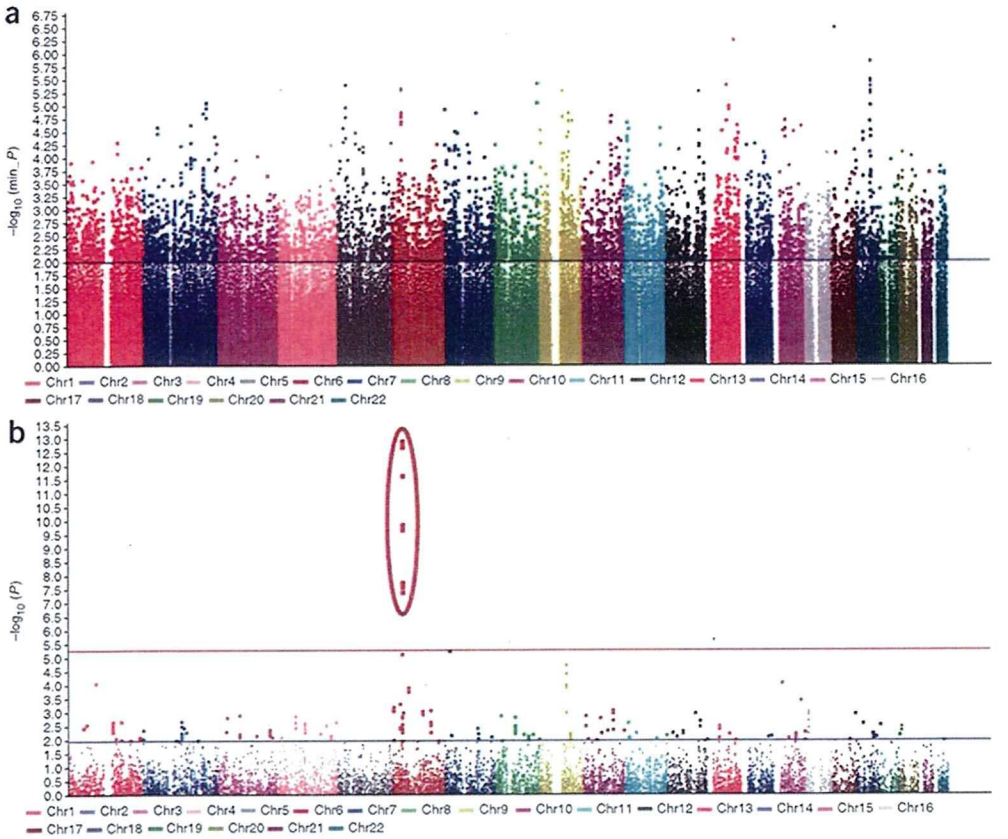


**Figure 1** Results from a two-stage genome-wide association study. **(a)** $-\log_{10}$ $P$ value plot at the first stage. Each $P$ value is the minimum of Fisher's exact tests for three models: dominant, recessive and allele frequency model. **(b)** $-\log_{10}$ $P$ value plot at the second stage. $P$ values were calculated by 1-d.f. Cochrane-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.

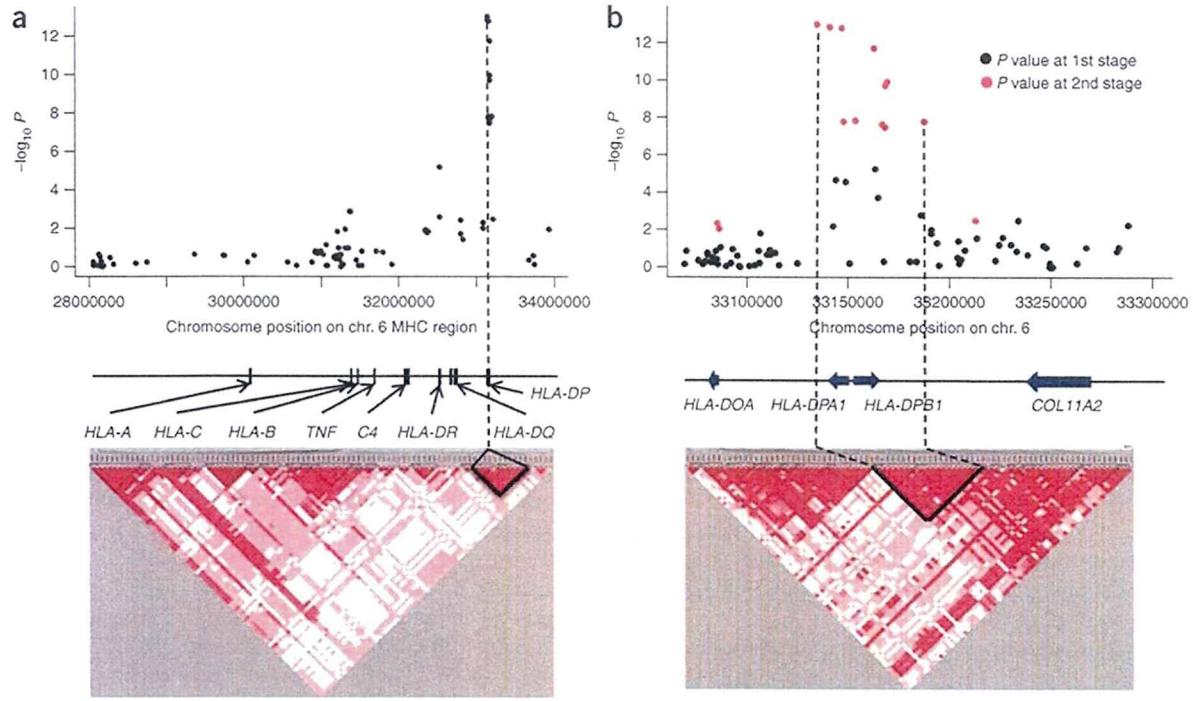


**Figure 2** Case–control association results and linkage disequilibrium map of the MHC region. (**a**) *P*-value plot, genomic structure and LD map of the second stage within the extended MHC region of chromosome 6. The LD map based on *D′* was drawn using the genotype data of the cases and the controls 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 $1.04 \times 10^{-4}$ (**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 $1.73 \times 10^{-9}$, 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 | *HLA-DPA1* | A/G | GWAS second stage | 42 | 240 | 324 | 197 | 598 | 472 | 0.57 (0.49–0.66) | 1.26E-13 | |
| | | | 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 | 0.84 |
| rs9277535 | *HLA-DPB1* | A/G | GWAS second stage | 58 | 254 | 294 | 230 | 619 | 418 | 0.59 (0.51–0.69) | 1.78E-12 | |
| | | | 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 | 0.85 |

Odds ratio and *P* values for independence test were calculated by the Mantel-Haenszel method.
[a]Odds ratio of minor allele from two-by-two allele frequency table. [b]*P* values of Pearson's $\chi^2$ test for allele model. [c]Result of Breslow-Day test. [d]Meta-analysis of all four studies.

**Table 2 Haplotype analysis**

| No. | Haplotype[a] | Frequency (cases) | Frequency (controls) | $P^b$ | 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.
[a]Haplotypes 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 infection[16]. 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 infections[17]. 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 berylliosis[18]. 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 vaccination[19] 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 Tokyo[20], 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 previously[21]. 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 $\geq 0.99$ in both cases and controls and $P$ value of Hardy-Weinberg equilibrium test of $\geq 1.0 \times 10^{-6}$ 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 $\leq 0.1$ 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 Group[22]. *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 Cochrane-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 software[24]. Estimation of haplotype frequencies and analysis of haplotype association were performed by R package haplo.stats[25]. 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/gudelines/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.*

ACKNOWLEDGMENTS

We thank K. Tokunaga for useful advice of on HLA-DP genotyping and interpretation, and technical staff of Laboratory for Genotyping Development at RIKEN for SNP genotyping at the first and second stages of the GWAS. We are also grateful to members of Hiroshima Liver Study Group and The Rotary Club of Osaka-Midosuji District 2660 Rotary International in Japan for supporting our study. This work was conducted as a part of the BioBank Japan Project that was supported by the Ministry of Education, Culture, Sports, Sciences and Technology of the Japanese government.

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.

Published online at http://www.nature.com/naturegenetics/
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions/

1. Pungpapong, S., Kim, W. & Poterucha, J. Natural history of hepatitis B virus infection: an update for clinicians. *Mayo Clin. Proc.* **82**, 967–975 (2007).
2. Custer, B. *et al.* Global epidemiology of hepatitis B virus. *J. Clin. Gastroenterol.* **38**, S158–S168 (2004).
3. Lai, C.L., Ratziu, V., Yuen, M.F. & Poynard, T. Viral hepatitis B. *Lancet* **362**, 2089–2094 (2003).
4. Kobayashi, M. *et al.* Viral genotypes and response to interferon in patients with acute prolonged hepatitis B virus infection of adulthood in Japan. *J. Med. Virol.* **68**, 522–528 (2002).
5. Laskus, T. *et al.* Prevalence of markers of hepatitis viruses in out-patient alcoholics. *J. Hepatol.* **15**, 174–178 (1992).
6. Sheen, I., Liaw, Y., Lin, D. & Chu, C. Role of hepatitis C and delta viruses in the termination of chronic hepatitis B surface antigen carrier state: a multivariate analysis in a longitudinal follow-up study. *J. Infect. Dis.* **170**, 358–361 (1994).
7. Lin, T. *et al.* Hepatitis B virus markers in Chinese twins. *Anticancer Res.* **9**, 737–741 (1989).
8. Ben-Ari, Z. *et al.* Cytokine gene polymorphisms in patients infected with hepatitis B virus. *Am. J. Gastroenterol.* **98**, 144–150 (2003).
9. Thursz, M.R. *et al.* Association between an MHC class II allele and clearance of hepatitis B virus in the Gambia. *N. Engl. J. Med.* **332**, 1065–1069 (1995).
10. Bellamy, R. *et al.* Tuberculosis and chronic hepatitis B virus infection in Africans and variation in the vitamin D receptor gene. *J. Infect. Dis.* **179**, 721–724 (1999).
11. Deng, G. *et al.* Association of estrogen receptor polymorphisms with susceptibility to chronic hepatitis B virus infection. *Hepatology* **40**, 318–326 (2004).
12. Singh, R., Kaul, R., Kaul, A. & Khan, K. A comparative review of HLA associations with hepatitis B and C viral infections across global populations. *World J. Gastroenterol.* **13**, 1770–1787 (2007).
13. Hohler, T. *et al.* HLA-DRB1* 1301 and* 1302 protect against chronic hepatitis B. *J. Hepatol.* **26**, 503–507 (1997).
14. Ahn, S.H. *et al.* Association between hepatitis B virus infection and HLA-DR type in Korea. *Hepatology* **31**, 1371–1373 (2000).
15. Frodsham, A. Host genetics and the outcome of hepatitis B viral infection. *Transpl. Immunol.* **14**, 183–186 (2005).
16. Frodsham, A. *et al.* Class II cytokine receptor gene cluster is a major locus for hepatitis B persistence. *Proc. Natl. Acad. Sci. USA* **103**, 9148–9153 (2006).
17. Jung, M. *et al.* Activation of a heterogeneous hepatitis B (HB) core and e antigen-specific CD4+ T-cell population during seroconversion to anti-HBe and anti-HBs in hepatitis B virus infection. *J. Virol.* **69**, 3358–3368 (1995).
18. Fontenot, A., Torres, M., Marshall, W., Newman, L. & Kotzin, B. Beryllium presentation to CD4+ T cells underlies disease-susceptibility HLA-DP alleles in chronic beryllium disease. *Proc. Natl. Acad. Sci. USA* **97**, 12717–12722 (2000).
19. Mineta, M. *et al.* Contribution of HLA class I and class II alleles to the regulation of antibody production to hepatitis B surface antigen in humans. *Int. Immunol.* **8**, 525–531 (1996).
20. Nakamura, Y. The BioBank Japan project. *Clin. Adv. Hematol. Oncol.* **5**, 696–697 (2007).
21. Saito, A. & Kamatani, N. Strategies for genome-wide association studies: optimization of study designs by the stepwise focusing method. *J. Hum. Genet.* **47**, 360–365 (2002).
22. van der Zwan, A., Griffith, B., Rozemuller, E., Williams, T. & Tilanus, M.G.J. *IHWG Technical Manual Genomic Analysis of the Human MHC: DNA-Based Typing for HLA Alleles and Linked Polymorphisms* (ed. Tilanus, M.G.J.) (Seattle, Washington, International Histocompatibility Working Group, 2002).
23. Purcell, S. *et al.* PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am. J. Hum. Genet.* **81**, 559–575 (2007).
24. Barrett, J., Fry, B., Maller, J. & Daly, M. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* **21**, 263–265 (2005).
25. Schaid, D.J., Rowland, C.M., Tines, D.E., Jacobson, R.M. & Poland, G.A. Score tests for association between traits and haplotypes when linkage phase is ambiguous. *Am. J. Hum. Genet.* **70**, 425–434 (2002).

# A Polymorphism in *MAPKAPK3* Affects Response to Interferon Therapy for Chronic Hepatitis C

HIRONOBU TSUKADA,*,‡ HIDENORI OCHI,* TOSHIRO MAEKAWA,* HIROMI ABE,§ YOSHIFUMI FUJIMOTO,§ MASATAKA TSUGE,§ HIROSHI TAKAHASHI,‖ HIROMITSU KUMADA,¶ NAOYUKI KAMATANI,‖ YUSUKE NAKAMURA,# and KAZUAKI CHAYAMA*,§

*Laboratory for Digestive Diseases, Center for Genomic Medicine, RIKEN (The Institute of Physical and Chemical Research), Kasumi, Minami-ku, Hiroshima, Japan; ‡Pharmacology Research Laboratories, Drug Research Division, Dainippon Sumitomo Pharma Co., Ltd, Kasugade Naka, Konohana-ku, Osaka, Japan; §Department of Medical and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Science, Hiroshima University, Minami-ku, Hiroshima, Japan; ‖Laboratory for Statistical Analysis, Center for Genomic Medicine, RIKEN (The Institute of Physical and Chemical Research), Shirokanedai, Minato-ku, Tokyo, Japan; ¶Department of Hepatology, Toranomon Hospital, Toranomon, Minato-ku, Tokyo, Japan; #Laboratory of Molecular Medicine, Human Genome Center, The Institute of Medical Science, University of Tokyo, Shirokanedai, Minato-ku, Tokyo, Japan

***Background & Aims:*** **This study aimed to identify host single nucleotide polymorphisms (SNPs) that are associated with the efficacy of interferon (IFN) therapy in patients with chronic hepatitis C.** ***Methods:*** **We examined whether 116 tagging-SNPs from 13 genes that are involved in type I IFN signaling associate with the outcome of IFN therapy in Japanese case-control groups; the study included 468 sustained responders and 587 nonresponders.** ***Results:*** **We identified 2 SNPs (rs3792323 [A/T] and rs616589 [G/A]), located in intron 2 of mitogen-activated protein kinase–activated protein kinase 3 (MAPKAPK3) that were associated with the outcome of IFN therapy in patients infected with hepatitis C virus (HCV) genotype 1b ($P = 4.6 \times 10^{-5}$ and $4.8 \times 10^{-5}$, respectively). The 2 SNPs were in strong linkage disequilibrium and multivariate logistic regression analysis showed that rs3792323 is an independent factor associated with the IFN efficacy (genotype 1b; $P = .0011$). *MAPKAPK3* is a kinase involved in the mitogen and stress responses, but the biological significance of MAPKAPK3 in IFN responses is poorly understood. By using an allele-specific transcript quantification assay in liver biopsy, we showed that allele-specific expression of *MAPKAPK3* messenger RNA, corresponding to the risk allele for nonresponse, was significantly higher than that of the other allele. Luciferase reporter assay data indicated that overexpression of MAPKAPK3 inhibits IFN-alfa–induced gene transcription via IFN-stimulated response element and IFN-γ–activated site.** ***Conclusions:*** **The SNP rs3792323 in *MAPKAPK3* associates with the outcome of IFN therapy in patients with HCV genotype 1b. Our functional analyses indicate that MAPKAPK3 inhibits IFN-alfa–induced antiviral activity.**

Type I interferon (IFN), including IFN-alfa and IFN-β, has been used widely as an antiviral agent for chronic hepatitis C. However, even after the most effective combination therapy of pegylated-IFN-alfa plus ribavirin, more than 50% of patients infected with hepatitis C virus (HCV) genotype 1b and approximately 20% of patients with HCV genotype non-1b fail to eradicate the virus.[1–3]

The mechanisms of modulating the responsiveness to IFN therapy have been studied extensively. Both viral and host factors have been implicated in the resistance to IFN therapy. Viral factors, such as HCV genotype, serum HCV-RNA level, and the interferon sensitivity determining region, have been reported to be associated with the outcome of IFN therapy.[3–5] On the other hand, host factors including age, sex, race, liver fibrosis, and obesity have been shown to associate with the outcome of IFN therapy.[6,7] Furthermore, it has been reported that genetic polymorphisms of cytokines, chemokines, and IFN-stimulated genes are associated with the difference in response to IFN therapy.[7–12]

Recently, genetic polymorphism of type I IFN receptor-1 (IFNAR1) promoter region was reported to be associated with the outcome of IFN therapy in patients with HCV infection.[13] Although the mechanisms of this polymorphism for the different responsiveness to IFN therapy still are unclear, polymorphism of *IFNAR1* promoter region may influence the efficacy of IFN therapy, possibly through modulation of IFNAR1 expression level. Because type I IFN elicits antiviral activity by activation of signaling molecules downstream of type I IFN receptors, genetic polymorphisms in type I IFN signaling molecules

***Abbreviations used in this paper:*** **GAS, interferon-γ–activated site; IFN, interferon; IFNAR1, type I interferon receptor-1; ISRE, interferon-stimulated response element; JAK, Janus-activated kinase; MAP, mitogen-activated protein; MAPKAPK, mitogen-activated protein kinase–activated protein kinase; MAPKK, mitogen-activated protein kinase kinases; NR, nonresponders; SNPs, single nucleotide polymorphisms; SR, sustained responders; STAT, signal transducer and activator of transcription.**


0016-5085/09/$36.00
doi:10.1053/j.gastro.2009.01.061

also potentially could alter the responsiveness to IFN therapy. However, so far there has been no evidence of associations between polymorphisms of genes involved in type I IFN signal transduction and the efficacy of IFN therapy in patients with chronic hepatitis C.

In the present study, we examined whether single nucleotide polymorphisms (SNPs) in type I IFN signaling molecules are associated with the difference in response to IFN therapy in patients with chronic hepatitis C, using the tagging-SNP approach in a large case-control study. The tagging-SNP serves as a marker to detect associations between a particular gene region and the outcome of IFN therapy. A small set of tagging-SNPs is sufficient to capture genetic variation because polymorphisms that are physically close to each other have a tendency to be in linkage disequilibrium with each other.[14,15] The HapMap online database (http://www.hapmap.org) allows the tagging-SNP approach to be applied readily to many genes or regions.[16]

As for type I IFN signaling molecules, we focused on 2 signaling cascades downstream of type I IFN receptors. First, we examined the Janus-activated kinase (JAK)–signal transducer and activator of transcription (STAT) pathway, which is essential for type I IFN–induced antiviral activity.[17] We selected tagging-SNPs for 7 key genes in this pathway, including *IFNAR1*, *IFNAR2*, *JAK1*, tyrosine kinase 2, *STAT1*, *STAT2*, and IFN regulatory factor 9. Second, we also examined the p38 mitogen-activated protein (MAP) kinase pathway, which has been reported to cooperate with the JAK–STAT pathway in activation of type I IFN–induced antiviral activity.[18–23] We also selected tagging-SNPs for 6 key genes in this pathway, including ras-related C3 botulinum toxin substrate 1,[18] MAP kinase kinases 3 (MAPKK3),[19] *MAPKK6*,[19] p38 MAP kinase,[20,21] MAP kinase-activated protein kinase 2 (MAPKAPK2),[20–23] and *MAPKAPK3*.[20–22]

Here, we provide genetic evidence suggesting that 2 SNPs in *MAPKAPK3* are associated with the responsiveness to IFN therapy. The 2 SNPs may be useful as markers to predict the outcome of IFN therapy, which is very helpful clinically because IFN therapy is expensive and may cause serious adverse effects.[24] In addition, we provided functional evidence that suggests MAPKAPK3 influences IFN-alfa–induced antiviral activity.

## Patients and Methods

### *Study Subjects and DNA Preparation*

We enrolled 1055 patients with chronic HCV infection who were treated with IFN monotherapy before 2001, at the Department of Hepatology, Toranomon Hospital, Hiroshima University Hospital, and Hiroshima University affiliated hospitals. Each patient was treated with $6 \times 10^6$ units of IFN intramuscularly every day for 8 weeks, followed by the same dose twice a week for 16 weeks, with a total dose of 528 million units. The characteristics of participating patients are described in Table 1. All patients had abnormal serum alanine transaminase levels for more than 6 months, and were positive for both anti-HCV antibody and serum HCV RNA. All patients were negative for hepatitis B surface antigen, had no evidence of other liver diseases, and had not received immunosuppressive or antiviral therapy before enrollment in the study. Patients were classified into the following 2 groups: sustained responders (SRs) and nonresponders (NRs). SRs had normal alanine transaminase levels and no evidence of viremia at 6 months after completion of IFN therapy. Relapsed responders were excluded. NRs remained viremic at 6 months after completion of IFN therapy. HCV-RNA levels were determined by Amplicor-monitor assay (Roche Diagnostics, Basel, Switzerland) or branched-chain DNA assay and stratified into 2 categories according to cut-off values that have been reported previously.[25,26] It has been reported that having serum HCV-RNA levels higher than 1.0 mEq/mL by branched-chain DNA assay or 100 KIU/mL by Amplicor-monitor assay is a predictor of poor responsiveness to IFN therapy. Histologic staging was determined according to the previously described criteria using biopsy specimens of liver tissue.[27] All subjects in the present study were ethnically Japanese and gave written informed consent to participate in the study according to the process approved by the Ethical Committee at the SNP Research Center, The Institute of Physical and Chemical Research (RIKEN), Yokohama. Genomic DNA samples were obtained from peripheral blood of the participating pa-

**Table 1.** Characteristics of Patients With Chronic Hepatitis C

| | SRs | NRs | *P* value |
|---|---|---|---|
| Patients, n | 468 | 587 | — |
| Mean age ± SD, *y* | 54.6 ± 11.8 | 55.9 ± 10.3 | .1[a] |
| Sex | | | .002 |
| Male | 325 | 354 | |
| Female | 143 | 233 | |
| HCV genotype | | | <.0001[b] |
| 1a | 3 | 3 | |
| 1b | 208 | 434 | |
| 2a | 209 | 101 | |
| 2b | 48 | 49 | |
| HCV-RNA level[c] | | | <.0001[d] |
| High | 177 | 420 | |
| Low | 216 | 69 | |
| No data | 75 | 98 | |
| Fibrosis stage | | | .19[e] |
| F0 | 5 | 5 | |
| F1 | 213 | 254 | |
| F2 | 130 | 173 | |
| F3 | 24 | 43 | |
| F4 | 17 | 28 | |
| No biopsy | 79 | 84 | |

[a]Mann–Whitney U test.
[b]Chi-square test between HCV genotype 1b and non-1b.
[c]Low HCV-RNA level: <100 KIU/mL by Amplicor-monitor assay and <1.0 mEq/mL by branched-chain DNA assay.
[d]Chi-square test between HCV-RNA level high and low.
[e]Chi-square test between F0-1 and F2-4.

tients. DNA extraction was performed according to a standard phenol-chloroform protocol.[28]

### *Selection of Tagging-SNPs and Genotyping*

As shown in Supplementary Table 1, we selected 116 tagging-SNPs for a total set of 13 candidate genes related to the type I IFN pathway, using the HapMap database (public release 21a; phase II of the January 2007 National Center for Biotechnology Information build 35 assembly; dbSNP build 125) and the Haploview program (available: http://www.broad.mit.edu/mpg/haploview). With the selection criteria of $r^2$ greater than 0.8 and minor allele frequency of greater than 0.05 for the Japanese population, tagging-SNPs were selected from all bins that cover the entire gene region from approximately 2000 bp upstream of the transcription start site to 1500 bp of the 3′ untranslated region in each gene. The number of tagging-SNPs in each candidate gene were as follows: *IFNAR1*, 3; *IFNAR2*, 6; *JAK1*, 8; tyrosine kinase 2, 3; *STAT1*, 23; *STAT2*, 1; IFN regulatory factor 9, 5; ras-related C3 botulinum toxin substrate 1, 7; *MAPKK3*, 5; *MAPKK6*, 35; p38 MAP kinase, 10; *MAPKAPK2*, 6; and *MAPKAPK3*, 4 (Supplementary Table 1). SNPs were genotyped by using the Invader assay[29] and the TaqMan assay[30] as described previously. The probe sets for the Invader assay were designed and synthesized by Third Wave Technologies (Madison, WI) and those for the TaqMan assay by Applied Biosystems (Foster City, CA).

### *SNP Discovery*

To identify genetic polymorphisms within the coding region of *MAPKAPK3*, we amplified appropriate fragments of genomic DNA from 48 patients by polymerase chain reaction and sequenced the products to identify SNPs using previously described methods.[28,31]

### *Cells and Cell Culture*

Human hepatoma cell line, Huh7, was purchased from RIKEN Cell Bank (Tsukuba, Japan). Huh7 cells were cultured in Dulbecco's modified minimal essential medium (Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C under 5% $CO_2$.

### *Allele-Specific Transcript Quantification for* MAPKAPK3

Allele-specific transcript quantification was performed as described previously,[32,33] with some modifications. Liver biopsy samples were collected from 5 patients with informed consent before IFN therapy for chronic hepatitis C. Total RNA was isolated using the RNeasy Micro kit (Qiagen, Hilden, Germany) and treated with 5 IU/mL RNase-Free DNase I (Qiagen). First-strand complementary DNA (cDNA) was prepared using the SuperScript III Platinum Two-Step quantitative reverse-transcription polymerase chain reaction kit (Invitrogen, Carlsbad, CA). Genomic DNA from peripheral blood of the 5 patients was prepared as described earlier. Both cDNA and genomic DNA were amplified with specific primers for the 3′ untranslated region of *MAPKAPK3*. The primers were as follows: forward, 5′-CCTGTGAATGCTGAGTGAGCGAGTA-3′; reverse, 5′-AGTCACCCTTTGGGTCGGGAATAGT-3′.

For determination of allele-specific *MAPKAPK3* messenger RNA (mRNA) expression, probes for SNP rs1385025 (A/G) were designed and synthesized by Third Wave Technologies. The invader assays were performed in a 5-μL reaction volume containing 1 × signal buffer, 1 × FRET Mix (FRET22/FTRE7), 30 ng cleavase VIII enzyme, 0.3 μL of probe mixture (all reagents from Third Wave Technologies), and 2 ng polymerase chain reaction product in a 96-well plate format. The thermal profile was 95°C for 5 minutes, followed by 40 cycles at 63°C for 1 minute, and a real-time intensity of fluorescence (FAM for G allele, and VIC for A of rs1385025) was measured by use of the Mx3000P Multiplex Quantitative polymerase chain reaction system (Stratagene, La Jolla, CA). For the construction of standard curves for each allele, sequential dilution of an amplified product from genomic DNA of patients with double heterozygosity for rs1385025 and rs3792323 was used. Each experiment was performed in triplicate assay at least 3 times.

### *Luciferase Reporter Assay*

One day before transfection, $7 \times 10^3$ of Huh7 cells were seeded in a 96-well culture plate. We used 2 types of firefly luciferase expression vector that contain promoter element IFN-stimulated response element (ISRE) or IFN-γ-activated site (GAS). Huh7 cells were transfected with both 1 ng renilla luciferase expression vector pRL-TK (Promega, Madison, WI) and 10 ng firefly luciferase expression vector pISRE-TA-Luc or pGAS-TA-Luc (BD Biosciences, San Jose, CA), in conjunction with 40 ng expression plasmid pDEST51/mock (empty vector), pDEST51/*MAPKAPK3*, or pDEST51/suppressor of cytokine signaling 1, by use of the FuGENE 6 transfection reagent (Roche Applied Science, Indianapolis, IN). After 24 hours, cells were stimulated with IFN-alfa (Dainippon Sumitomo Pharma, Osaka, Japan) for 20 hours followed by Dual-Luciferase Assay (Promega). Firefly luciferase activity was normalized by renilla luciferase activity. Each experiment was performed at least 3 times. Data are expressed as mean ± SD in triplicate assay.

### *Statistical Analysis*

We calculated allele frequencies and tested fit to Hardy-Weinberg equilibrium by the chi-square test at each SNP, using Excel software (Microsoft, Redmond, WA).[34] Then, we compared differences in genotype distribution of each SNP between cases and controls with the chi-square test and the Cochran-Armitage trend test (Excel).[35] LD coefficients ($r^2$) were calculated as described previously (Excel).[36] The age of the SRs and NRs was

compared by the Mann-Whitney *U* test. Differences in categoric data of patients in the 2 groups were analyzed by the chi-square test. To evaluate internal consistency of the results shown by association study, a 2-stage replication design was simulated by the Monte Carlo method. We assessed population stratification by analyzing the data from 116 tagging-SNPs in patients with HCV genotype 1b, using a genomic control method that has been reported previously.[37] Multivariate logistic regression with stepwise forward selection was performed with a significance level of 0.05 for including variables, by use of the StatFlex 5.0 software package (Artec Inc., Osaka, Japan). For case-control haplotype analysis we estimated haplotype frequencies and tested for association by chi-square analysis, to detect differences in haplotype distribution between groups we used using Haploview 3.2 software. For allele-specific transcript quantification assay, statistical differences between allelic *MAPKAPK3* mRNA expression corresponding to haplotype 1 and haplotype 2 were analyzed by Mann-Whitney *U* test. For luciferase reporter assay, comparisons among the 3 groups were analyzed by the Kruskal-Wallis test, followed by the Scheffe test to evaluate statistical differences between the 2 groups (StatFlex 5.0 software package).

## Results

### *Association Between Tagging-SNPs in* MAPKAPK3 *and the Outcome of IFN Therapy*

We searched for the association between 116 tagging-SNPs for 13 candidate genes and the outcome of IFN therapy, using 468 SR and 587 NR subjects. We were successful in genotyping all 116 tagging-SNPs (Supplementary Table 2). The mean call rate was 99.4% across all tagging-SNPs. None of the tagging-SNPs showed a significant deviation from Hardy-Weinberg equilibrium. Because HCV genotype 1b, which is the most common in Japan, is associated with poor response to IFN treatment, we divided the patients into 2 subgroups according to the genotypes of HCV with which they were infected (1b vs non-1b), and performed the comparison separately.

We found that 2 SNPs, rs3792323 (A/T) and rs616589 (G/A), located in intron 2 of MAPKAPK3, are associated with the outcome of IFN therapy in patients with HCV genotype 1b; the T allele for rs3792323 was significantly more frequent in NRs than in SRs (Table 2; 33.4% vs 22.4%; $P = 5.2 \times 10^{-5}$; odds ratio, 0.57; 95% confidence interval, 0.44-0.75). Similarly, the A allele for rs616589 was significantly more frequent in NRs than in SRs (37.8% vs 26.4%; $P = 5.6 \times 10^{-5}$; odds ratio, 0.59; 95% confidence interval, 0.45-0.76).

In Table 2, the Cochran-Armitage trend test (assuming an additive model for minor alleles) revealed an allele dose-dependent association of rs3792323 with the outcome of IFN therapy ($P = 4.6 \times 10^{-5}$), with decreased

**Table 2.** Associations Between the Two SNPs in *MAPKAPK3* and the Outcome of IFN Therapy

| dbSNP ID | Allele 1/2 | HCV genotype | Patients, n | Allele frequency, % | | | | OR (95% CI) | Genotype (%) | | | Cochran-Armitage trend test, | OR (95% CI) | | Dominant model for allele 2 (11 vs 12, 22) | | Recessive model for allele 2 (11, 12 vs 22) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 1 | 2 | *P* value | | | 11 | 12 | 22 | | 11 vs 12 | 11 vs 22 | *P* value | OR (95% CI) | *P* value | OR (95% CI) |
| rs3792323 | A/T | total | SR (n = 468) | 71.8 | 28.2 | .088 | 0.85 (0.70–1.02) | | 242 (51.8) | 187 (40.0) | 38 (8.1) | .089 | 0.83 (0.65–1.08) | 0.74 (0.47–1.15) | .10 | 0.82 (0.64–1.04) | .32 | 0.80 (0.52–1.23) |
| | | | NR (n = 587) | 68.4 | 31.6 | | | | 273 (46.7) | 253 (43.3) | 58 (9.9) | | | | | | | |
| | | 1b | SR (n = 208) | 77.6 | 22.4 | .000052 | 0.57 (0.44–0.75) | | 124 (59.6) | 75 (36.1) | 9 (4.3) | .000046 | 0.58 (0.41–0.83) | 0.30 (0.14–0.63) | .00019 | 0.53 (0.38–0.74) | .0074 | 0.38 (0.18–0.79) |
| | | | NR (n = 434) | 66.6 | 33.4 | | | | 189 (43.9) | 196 (45.5) | 46 (10.7) | | | | | | | |
| | | non-1b | SR (n = 260) | 67.2 | 32.8 | .056 | 1.36 (0.99–1.86) | | 118 (45.6) | 112 (43.2) | 29 (11.2) | .06 | 1.40 (0.92–2.14) | 1.72 (0.83–3.57) | .067 | 1.45 (0.97–2.17) | .27 | 1.48 (0.73–2.99) |
| | | | NR (n = 153) | 73.5 | 26.5 | | | | 84 (54.9) | 57 (37.3) | 12 (7.8) | | | | | | | |
| rs616589 | G/A | total | SR (n = 468) | 68.0 | 32.0 | .034 | 0.82 (0.68–0.99) | | 215 (46.0) | 205 (43.9) | 47 (10.1) | .033 | 0.83 (0.64–1.07) | 0.67 (0.44–1.00) | .062 | 0.79 (0.62–1.01) | .12 | 0.74 (0.50–1.08) |
| | | | NR (n = 587) | 63.6 | 36.4 | | | | 235 (40.3) | 271 (46.5) | 77 (13.2) | | | | | | | |
| | | 1b | SR (n = 208) | 73.6 | 26.4 | .000056 | 0.59 (0.45–0.76) | | 111 (53.4) | 84 (40.4) | 13 (6.3) | .000048 | 0.59 (0.42–0.84) | 0.33 (0.17–0.62) | .00023 | 0.53 (0.38–0.75) | .0055 | 0.42 (0.23–0.79) |
| | | | NR (n = 434) | 62.2 | 37.8 | | | | 164 (38.0) | 209 (48.4) | 59 (13.7) | | | | | | | |
| | | non-1b | SR (n = 260) | 63.5 | 36.5 | .24 | 1.20 (0.89–1.61) | | 104 (40.2) | 121 (46.7) | 34 (13.1) | .25 | 1.33 (0.87–2.05) | 1.29 (0.68–2.46) | .18 | 1.32 (0.88–1.98) | .72 | 1.12 (0.61–2.06) |
| | | | NR (n = 153) | 67.5 | 32.5 | | | | 71 (47.0) | 62 (41.1) | 18 (11.9) | | | | | | | |

NOTE. *P* values were calculated from case-control analysis by the chi-square test and unadjusted for multiple testing. Odds ratios of having a sustained response to IFN therapy were calculated. Allele 1 and allele 2 denote a major and a minor allele, respectively.
OR, odds ratio; CI, confidence interval.

odds ratios of 0.58 and 0.30 for AT and TT genotypes, respectively (95% confidence interval, 0.41–0.83 for AT; 0.14–0.63 for TT). Under a dominant model for the T allele of rs3792323, a significant association also was seen in patients infected with HCV genotype 1b ($P = 1.9 \times 10^{-4}$; odds ratio, 0.53; 95% confidence interval, 0.38–0.74).

Similarly, an allele dose-dependent association of rs616589 with the responsiveness to IFN therapy was revealed in Table 2 ($P = 4.8 \times 10^{-5}$), with decreased odds ratios of 0.59 and 0.33 for GA and AA genotypes, respectively (95% confidence interval, 0.42–0.84 for GA; 0.17–0.62 for AA). Under a dominant model for the A allele of rs616589, a significant association also was seen in HCV genotype 1b-infected patients ($P = 2.3 \times 10^{-4}$; odds ratio, 0.53; 95% confidence interval, 0.38–0.75).

To adjust the *P* values for multiple testing, we applied a Bonferroni correction with each individual SNP as an independent variable (total, 116 SNPs). Despite this conservative adjustment, our results for rs3792323 and rs616589 about patients with HCV genotype 1b remained highly significant ($P < .05$). On the other hand, the other tagging-SNPs did not show significant associations with the outcome of IFN therapy after Bonferroni corrections (Supplementary Table 2).

### *Internal Validation of the Observed Associations*

To evaluate internal consistency of the results shown by the association study, 2-stage replication design was simulated by the Monte Carlo method. Half of the cases and half of the controls were selected randomly from HCV-1b-infected patients in this study, and used for the first-stage test with a significance level $\alpha 1$ (the probability of making a Type I error) in the allele-frequency model. Only the SNPs that were judged to be associated significantly with the phenotype then underwent the second-stage test. In the second stage, the remaining independent cases and controls were used to test the association between the selected SNPs and the phenotype with a significance level $\alpha 2$. We set $\alpha 1$ at 0.01, 0.02, and 0.05, and calculated $\alpha 2$ as $(0.05/116)/\alpha 1$ because the global significance level after Bonferroni's correction is 0.05/116 (total, 116 SNPs). The number of iterations was 100,000 for each condition. By this method, the results of the test in the first stage are validated by the test in the second stage.

When the level of significance was set at $\alpha 1 = .01$, .02, and .05, the proportions of significant results of SNP rs3792323 were 0.563, 0.595, and 0.557, respectively (Table 3). Similarly, those of SNP rs616589 were 0.554, 0.579, and 0.540, respectively when the same values of $\alpha 1$ were used. These results suggest that the results of the first-stage test could be replicated in many cases if 2 halves of the patients, the first-stage set and the second-stage set, were tested independently for the association.

**Table 3.** Internal Validation Analysis of the Observed Associations Between the Two SNPs in *MAPKAPK3* and the IFN Efficacy in Patients With HCV Genotype 1b

| SNP | $\alpha 1$ | Proportion of significant results | 95% CI |
|---|---|---|---|
| rs3792323 | 0.01 | 0.563 | 0.560–0.566 |
| | 0.02 | 0.595 | 0.591–0.598 |
| | 0.05 | 0.557 | 0.554–0.560 |
| rs616589 | 0.01 | 0.554 | 0.551–0.557 |
| | 0.02 | 0.579 | 0.576–0.582 |
| | 0.05 | 0.540 | 0.537–0.543 |

$\alpha 1$, a significance level for the first-stage test; CI, confidence interval.

### *Population Stratification Analysis*

We assessed population stratification by analyzing the data from 116 tagging-SNPs in patients with HCV genotype 1b by using the genomic control method. We estimated the inflation factor, which effectively can adjust for the confounding effect of population stratification regardless of its extent (inflation factor, 1.18; 95% confidence interval, 0.87–1.65). The corrected *P* values for rs3792323 and rs616589 in *MAPKAPK3* were .00017 and .00018, respectively. After a Bonferroni correction, the results for the 2 SNPs remained highly significant ($P < .05$). These results suggest that population stratification in our patients was negligible.

### *Haplotype Analysis*

We examined whether *MAPKAPK3* haplotypes show more significant associations with the effect of IFN therapy than single-marker analysis. Because the 2 SNPs rs3792323 and rs616589 were in strong linkage disequilibrium with an r-squared value of 0.82 in our genotype data of 1055 patients, we constructed *MAPKAPK3* haplotypes from 3 tagging-SNPs (rs3792323 A>T, rs3804628 G>A, and rs2040397 C>T), using the Haploview 3.2 software. As a result, 4 haplotypes with frequencies greater than 5% were deduced in patients with HCV genotype 1b: AGC 43.9%, TGC 29.8%, AGT 20.6%, and AAC 5.7%. Although haplotype TGC was associated the most significantly with the outcome of IFN therapy in 4 haplotypes ($P = .000051$), this *P* value was comparable with that for single-marker analysis ($P = .000046$ for rs3792323 in the Cochran–Armitage trend test).

### *Results of Multivariate Logistic Regression Analysis*

To determine independent factors on the outcome of IFN therapy in patients infected with HCV genotype 1b, we used multivariate logistic regression analysis with stepwise forward selection. We evaluated the following 6 factors: SNP rs3792323 (A allele vs T allele), rs616589 (G allele vs A allele), age (per year increase), sex (male vs female), fibrosis stage (F0–F1 vs F2–F4), and HCV-RNA level before treatment (low vs high).

**Table 4.** Predictive Factors Associated Independently With the Response to IFN Therapy in Patients With HCV Genotype 1b by Multivariate Logistic Regression Analysis

| | HCV genotype 1b | | |
|---|---|---|---|
| Variable | *P* value | OR | 95% CI |
| rs3792323 (T allele/A allele) | .0011 | 0.29 | 0.14–0.61 |
| Age (per year increase) | .0096 | 0.97 | 0.95–0.99 |
| Fibrosis stage (F0–F1/F2–F4) | .035 | 1.66 | 1.04–2.66 |
| HCV-RNA level (low/high)[a] | <.00001 | 8.25 | 5.05–13.50 |

OR, odds ratio; CI, confidence interval.
[a]Low HCV-RNA level: <100 KIU/mL by Amplicor-monitor assay and <1.0 mEq/mL by branched-chain DNA assay. Odds ratios of having a sustained response to IFN therapy were calculated.

We found that SNP rs3792323 is an independent factor associated with IFN efficacy (Table 4; $P = .0011$; odds ratio, 0.29; 95% confidence interval, 0.14–0.61). On the other hand, SNP rs616589 was removed from this model, suggesting that the 2 SNPs are not associated independently with the outcome of IFN therapy. This is consistent with the result that the 2 SNPs were in strong linkage disequilibrium in our genotype data.

Next, to eliminate the effect of confounding factors, we also tried running models by forcing in the earlier-mentioned 4 factors (age, sex, HCV-RNA level, and fibrosis stage) and SNP rs3792323 into multivariate logistic regression analysis in patients with HCV genotype 1b. We identified that SNP rs3792323 is associated with the outcome of IFN therapy ($P = .0014$; odds ratio, 0.30; 95% confidence interval, 0.14–0.63).

### *SNP Discovery Within the Coding Region of* MAPKAPK3

To investigate whether there is any genetic polymorphism in *MAPKAPK3* that results in amino acid substitution, we sequenced the coding region of *MAPKAPK3* from genomic DNA isolated from 48 patients. We did not find any nonsynonymous allelic variants in *MAPKAPK3*.

### *Allele-Specific Transcript Quantification of* MAPKAPK3

Next, we examined the possibility that SNP rs3792323 associates with *MAPKAPK3* expression in liver biopsy specimens from patients with chronic hepatitis C. Because rs3792323 in *MAPKAPK3* intron 2 was not present in mRNA, we selected SNP rs1385025 (A/G) in the 3′ untranslated region as a marker SNP. We confirmed that rs1385025 showed complete linkage disequilibrium to rs3792323 ($D'$ values = 1), using HapMap data and the Haploview program. We selected 5 patients who were doubly heterozygous with genotype rs1385025 A/G and rs3792323 A/T for this assay. Haplotype pairs of these patients theoretically were specified to be haplotype 1 (rs1385025A rs3792323A) and haplotype 2 (rs1385025G rs3792323T).

We measured the relative contribution of each haplotype to *MAPKAPK3* transcription in these patients, using probes that detect each allele of rs1385025. As shown in Figure 1, allele-specific *MAPKAPK3* mRNA expression corresponding to haplotype 2 was 1.15- to 1.76-fold higher than that of haplotype 1 ($P = .009$). This result indicated that SNP rs1385025 and rs3792323 associate with the expression level of *MAPKAPK3*.

### *Effects of MAPKAPK3 on IFN-Alfa–Induced Gene Transcription Via ISRE and GAS Elements*

We tested whether transient overexpression of MAPKAPK3 influences IFN-alfa-induced gene transcription via ISRE and GAS elements, which are essential promoter elements for type I IFN-induced antiviral activity, by use of luciferase reporter assay. When MAPKAPK3 was overexpressed in Huh7 cells, IFN-alfa-induced luciferase activities via ISRE and GAS elements were suppressed significantly by various doses of IFN-alfa, compared with the negative control (Figure 2). Similar results were obtained in suppressor of cytokine signaling 1, which has been reported to suppress IFN-alfa-induced gene expressions. In addition, we also overexpressed β-galactosidase gene as a negative control. In comparison with this control, MAPKAPK3 also significantly inhibited IFN-alfa-induced luciferase activities via ISRE and GAS elements (data not shown). These results suggest that MAPKAPK3 can inhibit IFN-alfa-induced gene transcription via ISRE and GAS elements.

## Discussion

We identified that SNP rs3792323 (A/T) and rs616589 (G/A), located in *MAPKAPK3*, are associated

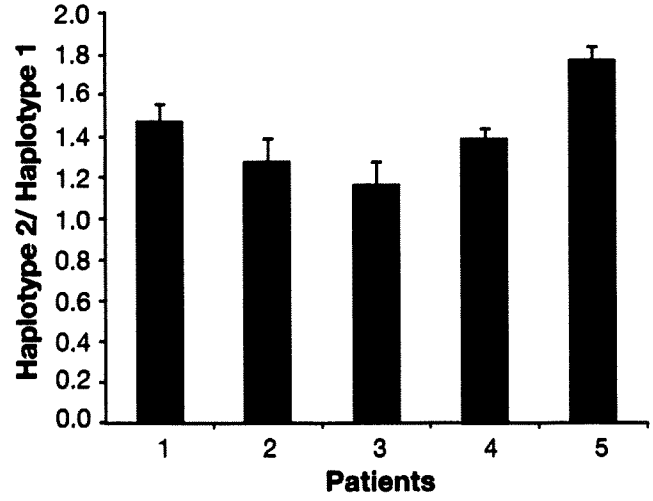


**Figure 1.** Allele-specific transcript quantification of *MAPKAPK3*. The allele-specific *MAPKAPK3* mRNA expression ratio for haplotype 2 to haplotype 1 is shown. Individual data from liver biopsies from 5 patients are indicated. Each experiment was performed in triplicate assay at least 3 times. Data represent the mean ± SD.