isoboles in combinations using each nutrient and IFN-α or FLV graphed nearly straight lines in each case, indicating that the suppressive effects of these cotreatments on HCV RNA replication were additive (Fig. 7A and B). Similar additive effects were obtained in combination with IFN-β (data not shown). It was noteworthy that all cotreatments with each nutrient and CsA resulted in curved, concave plots of 50% isoboles, thus suggesting that these combinations with CsA exerted synergistic inhibitory effects on HCV RNA replication (Fig. 7C). These results indicate that these three nutrients, administered as a supportive nutritional therapy, could potentially improve the SVR rate associated with IFN therapy alone.

The anti-HCV activities of BC, VD2, and LA, as well as that of CsA but not those of IFN and FLV, were completely canceled by VE. Among the 46 nutrients tested, BC and VD2 exhibited inhibitory effects on HCV RNA replication up to 70%, and LA exhibited inhibitory effects up to 90%, without exhibiting any cytotoxicity (Fig. 5A). In contrast, most of the liposoluble vitamins enhanced HCV RNA replication in OR6 cells. We used VE in the following studies because VE is one of the most common vitamins in the daily diet and it exerts a strong enhancing effect on HCV RNA replication. To clarify the mechanism of these opposing effects, we investigated whether the anti-HCV effects of BC, VD2, and LA were canceled by the addition of VE. We also tested representative anti-HCV compounds (i.e., CsA, IFN-α, IFN-β, and FLV) in combination with VE. We first examined the influence of 10 μM VE on the nutrients and compounds at the 70% inhibitory concentration level (Fig. 8A and B). The inhibitory effects of IFN-α, IFN-β, and FLV were hardly influenced by cotreatment with VE, whereas the anti-HCV effects of BC, VD2, LA, and CsA were canceled to a significant level by VE in the OR6 cells (Fig. 8A, upper panel). These results were also confirmed using authentic HCV RNA-replicating cells (Fig. 8A, lower panel). To normalize these results, we divided the luciferase value observed in the presence of VE by that in the absence of VE, and we considered this value to represent the effects of VE. When this value was larger than the value obtained in the absence of anti-HCV reagent (2.0; column 1 in Fig. 8B), we interpreted it as indicative of a reagent whose anti-HCV effects were canceled by VE. According to this criterion, BC (4.1), VD2 (4.9), LA (5.6), and CsA (4.0) were evaluated to be reagents for which the anti-HCV effects were canceled by VE (columns 2, 3, 4, and 5 in Fig. 8B). The anti-HCV effects of IFN-α, IFN-β, and FLV were not affected by VE (columns 6, 7, and 8 in Fig. 8B). We next examined the influence of 10 μM VE on the anti-HCV nutrients and compounds at the 90% inhibitory concentration level (Fig. 8C and D). BC and VD2 were not assessed in this experiment, because the maximum inhibitory effect was 70% in the case of these nutrients (Fig. 5A). Similar results were obtained in this experiment. LA (10.8) and CsA (7.7) were evaluated to be reagents for which the anti-HCV effects were canceled by VE (compare columns 2 and 3 to column 1 in Fig. 8D), although IFN-α (2.8) and IFN-β (2.7) were slightly affected by VE at this concentration (Fig. 8D, compare columns 4 and 5 to column 1). Judging by these results, it appears that BC, VD2, LA, and CsA may share some mechanism by which VE negated their anti-HCV activities.

DISCUSSION

The differential effects of BC and VA, as well as those of VD2 and VD3, which belong to the same categories as VA and VD, respectively, are of interest. We observed that whereas BC and VD2 inhibited HCV RNA replication, VA enhanced it, and VD3 exhibited basically no effect. The mechanism governing how these vitamins from the same category exert different effects on HCV RNA remains to be elucidated. However, liposoluble vitamins have been reported to exhibit various physiological activities with each nuclear receptor, consequently acting as hormone-like substances (19, 20, 27, 35). Differences in the gene products induced by each of these vitamins may lead to differences in the effects on HCV RNA replication. Another explanation might be considered in the light of findings suggesting that VA is an antioxidant, and yet recently, BC has been reported to induce oxidative stress (32, 43). This diversity of activities among vitamins in the same category, VA, might result in a variety of influences on HCV RNA replication. Further studies are still needed to account for why these different consequences are generated.

Previous studies have demonstrated that PUFAs such as AA, EPA, and DHA inhibit HCV RNA replication in cell culture systems (17, 21). However, saturated and mono-unsaturated fatty acids have been shown to enhance HCV RNA replication (17). In the prior studies, the cells tolerated the presence of PUFAs at concentrations of up to 50 μM. In contrast, in our study, 50 μM PUFAs were toxic, with the exception of LA. Furthermore, saturated and mono-unsaturated fatty acids hardly exhibited any effects on HCV RNA replication in our OR6 cell culture system. These discrepancies might be due to differences in both the clonalities of the cells and the HCV strains used in each experiment.

FIG. 8. VE canceled the anti-HCV activities of BC, VD2, LA, and CsA. (A and B) Effects of VE on the nutrients and compounds at the 70% inhibitory concentration. Both OR6 cells and OR6c cells, into which authentic HCV RNA was introduced, were treated with control medium [(−)], 10 μM BC, 5 μM VD2, 30 μM LA, 0.6 μg/ml of CsA, 0.6 IU/ml of IFN-α, 0.4 IU/ml of IFN-β, or 1.2 μM FLV in either the absence or presence of 10 μM VE for 72 h. After treatment, an RL assay of harvested OR6 cell samples was performed, and then the relative RL activity was calculated as described in the legend to Fig. 2. Subsequently, the production of HCV core antigen in OR6c cells was analyzed by immunoblotting using antibody specific to HCV core antigen. β-Actin was used as a control for the amount of protein loaded per lane (A). Then, the ratio of RL activity in the presence of 10 μM VE (+) to the RL activity in the absence of VE (−) was calculated. The horizontal line indicates the promotive effect of 10 μM VE alone on HCV RNA replication as a baseline (B). (C and D) Effects of VE on the nutrients and compounds at the 90% inhibitory concentration. Both OR6 cells and OR6c cells were treated with control medium, 50 μM LA, 1 μg/ml of CsA, 1 IU/ml of IFN-α, 0.6 IU/ml of IFN-β, and 2 μM FLV in either the absence (−) or presence (+) of 10 μM VE for 72 h. After treatment, the RL assay and Western blot analysis were performed (C), and then the ratio of RL activity in the presence of 10 μM VE to the RL activity in the absence of VE was calculated in the same manner as that described above (D).

Here, we demonstrated that three nutrients, BC, VD2, and LA, exhibited anti-HCV effects in polyclonal genome-length and subgenomic HCV RNA (strain O of genotype 1b)-replicating cells. These results indicated that the inhibitory activities of at least three anti-HCV nutrients are not limited to a specific cell clone (OR6).

Moreover, IFN or FLV exhibited additive anti-HCV effects when the cells were cotreated with each of the three anti-HCV nutrients. However, CsA showed synergistic anti-HCV effects in combination with each of these three nutrients. Interestingly, these results coincided with the experiment using VE, as VE canceled the anti-HCV effects of CsA but not those of IFN or FLV. It was recently demonstrated that the anti-HCV effects of CsA are related to the inhibition of cyclophilin (31, 42). CsA is also known as an oxidant that can cause renal or vascular dysfunction, and interestingly, antioxidants, including VE, attenuate these CsA-induced side effects (16, 22). Furthermore, we confirmed that another antioxidant, Se, also weakened the anti-HCV effects of BC, VD2, and LA (data not shown). Therefore, BC, VD2, and LA may possess an anti-HCV mechanism similar to that of CsA, and oxidative stress may be involved in these anti-HCV effects to some extent. Among the nutrients tested, VA, VC, VE, and Se enhanced HCV RNA replication, and these nutrients functioned as antioxidants. In contrast, four PUFAs inhibited HCV RNA replication, and they served as oxidants (29, 44). These results are further evidence of the involvement of oxidative stress in HCV RNA replication.

CH C patients may take excessive doses of VE during the course of interferon therapy, because as an antioxidant, VE has been expected to prevent injury to hepatocytes caused by oxidative stress. However, our results suggest that the potentially negative effects of VE on therapy for CH C patients should be carefully considered. To date, the significance of the role played by ordinary nutrients in viral infections has not been well characterized and has even been underestimated. We believe that our findings will shed light on the field of viral infection from the perspective of the nutritional sciences.

It is difficult to determine the blood concentrations of the nutrients tested in this study because the administration conditions may affect the concentrations in the blood. Rühl et al. (35) reported that the concentrations of BC in human serum are between 0.34 to 0.54 μM and that the average concentration in the human liver is 4.4 μM. Hagenlocher et al. (12) reported that the concentration of LA in human serum is 0.8 to 11.9 μg/100 μl. Armas et al. (3) reported that the concentration of VD2 in human serum at 24 h after a 50,000-IU administration is about 50 nM. The concentration of the nutrient in this study is higher than that in those reports. Therefore, monotreatment of the nutrient may not eliminate HCV. However, these nutrients may boost the effect of IFN treatment in combination like ribavirin does.

It is worth trying to examine the effects of BC, VD2, and LA on the recently developed JFH1 infectious virus production system in a future study. Here, it remains unclear whether these three nutrients affect the production of the virus. Furthermore, the comparison of the effects of these three nutrients between HCV genotypes 1 and 2 will provide useful information for the HCV therapy, as HCV genotypes 1 and 2 respond differentially to IFN treatment.

The precise mechanism underlying the anti-HCV activities of the nutrients has not been clarified in this study. The nutrients may inhibit viral RNAs and proteins, including the internal ribosome entry site, NS3-4A serine protease, and NS5B polymerase. Further in vitro study will be needed to clarify the targets of the nutrients responsible for their anti-HCV activities. Another possibility is that the nutrients inhibit the cellular proteins required for HCV RNA replication. We are now planning a study to clarify the mechanism underlying the nutrients' anti-HCV activities.

In conclusion, we found that three nutrients, BC, VD2, and LA, inhibited HCV RNA replication in a cell culture system and that Se, tryptophan, and various vitamins (A, C, E, and K) enhanced HCV RNA replication. The anti-HCV effects of BC, VD2, and LA were reversed by VE. These results are expected to provide useful information for the improvement of the SVR rates of patients receiving the currently standard IFN therapy. In addition, these findings may contribute to the development of a nutritional supplement specific to the treatment of people with CH C.

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DDX3 DEAD-Box RNA Helicase Is Required for Hepatitis C Virus RNA Replication▽

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DDX3, a DEAD-box RNA helicase, binds to the hepatitis C virus (HCV) core protein. However, the role(s) of DDX3 in HCV replication is still not understood. Here we demonstrate that the accumulation of both genome-length HCV RNA (HCV-O, genotype 1b) and its replicon RNA were significantly suppressed in HuH-7-derived cells expressing short hairpin RNA targeted to DDX3 by lentivirus vector transduction. As well, RNA replication of JFH1 (genotype 2a) and release of the core into the culture supernatants were suppressed in DDX3 knockdown cells after inoculation of the cell culture-generated HCVcc. Thus, DDX3 is required for HCV RNA replication.

DEAD-box RNA helicases are involved in various RNA metabolic processes, including transcription, translation, RNA splicing, RNA transport, and RNA degradation (9, 20). DDX1 and DDX3, DEAD-box RNA helicases, have been implicated in the replication of human immunodeficiency virus type 1 (HIV-1). Both DDX1 and DDX3 interact with HIV-1 Rev and enhance Rev-dependent HIV-1 RNA nuclear export (10, 24).

On the other hand, DDX3 binds to the hepatitis C virus (HCV) core protein (17, 19, 25), and DDX3 expression is deregulated in HCV-associated hepatocellular carcinoma (HCC) (7, 8). However, the biological function of DDX3 in HCV replication is still not understood. To address this issue, we first used lentivirus vector-mediated RNA interference to stably knock down DDX3 in three HuH-7-derived cell lines: O cells, harboring a replicative genome-length HCV RNA (HCV-O, genotype 1b) (13); sO cells, harboring its subgenomic replicon of HCV RNA (14); or RSc cured cells, which cell culture-generated HCV (HCVcc) (JFH1, genotype 2a) (23) could infect and effectively replicate in (M. Ikeda et al., unpublished data). Oligonucleotides with the following sense and antisense sequences were used for the cloning of short hairpin RNA (shRNA)-encoding sequences against DDX3 in the lentivirus vector: for DDX3i#3, 5′-GATCCCCGGAGGA AATTATAACTCCCTTCAAGAGAGGGAGTTATAATTT CCTCCTTTTTGGAAA-3′ (sense) and 5′-AGCTTTTCCAA AAAGGAGGAAATTATAACTCCCTCTCTTGAAGGGA GTTATAATTTCCTCCGGG-3′ (antisense); for DDX3i#7, 5′-GATCCCCGGTCACCCTGCCAAACAAGTTCAAGAG ACTTGTTTGGCAGGGTGACCTTTTTGGAAA-3′ (sense) and 5′-AGCTTTTCCAAAAAGGTCACCCTGCCAAACAA GTCTCTTGAACTTGTTTGGCAGGGTGACCGGG-3′ (antisense). These oligonucleotides were annealed and subcloned into the BglII-HindIII site, downstream from an RNA polymerase III promoter of pSUPER (6). To construct pLV-DDX3i#3 and pLV-DDX3i#7, the BamHI-SalI fragments of the corresponding pSUPER plasmids were subcloned into the BamHI-SalI site of pRDI292 (5), an HIV-1-derived self-inactivating lentivirus vector containing a puromycin resistance marker allowing for the selection of transduced cells. The vesicular stomatitis virus G protein (VSV-G)-pseudotyped HIV-1-based vector system has been described previously (18). We used the second-generation packaging construct pCMV-ΔR8.91 (26) and the VSV-G-envelope plasmid pMDG2. The lentivirus vector particles were produced by transient transfection of 293FT cells with FuGene 6 (Roche).

Western blot analysis of the lysates demonstrated the only trace of DDX3 protein in DDX3 knockdown O cells (DDX3i#3) (Fig. 1A). In this context, the HCV core expression level was significantly decreased in the DDX3 knockdown O cells (Fig. 1A). To further confirm this finding, we examined the level of HCV RNA in these cells. We found that accumulation of genome-length HCV-O RNA was notably suppressed in DDX3 knockdown O cells (Fig. 1B). Furthermore, the efficiency of colony formation in DDX3 knockdown Oc cells (created by eliminating genome-length HCV RNA from O cells by interferon treatment) transfected with the genome-length HCV-O RNA with an adapted mutation at amino acid (aa) position 1609 in the NS3 helicase region (K1609E) (13) was also notably reduced compared with that in control cells (Fig. 1C). In contrast, highly efficient knockdown of an unrelated host factor, poly(ADP-ribose) polymerase 1 (PARP-1) (4), had no observable effects on HCV RNA replication, the efficiency of colony formation, or the core expression level (data not shown), suggesting that our finding was not due to a nonspecific event. Interestingly, accumulation of the subgenomic replicon RNA (HCV-sO) was also suppressed in DDX3 knockdown sO cells (Fig. 1D). Moreover, we examined the potential role of DDX3 in an HCV infection and produc-

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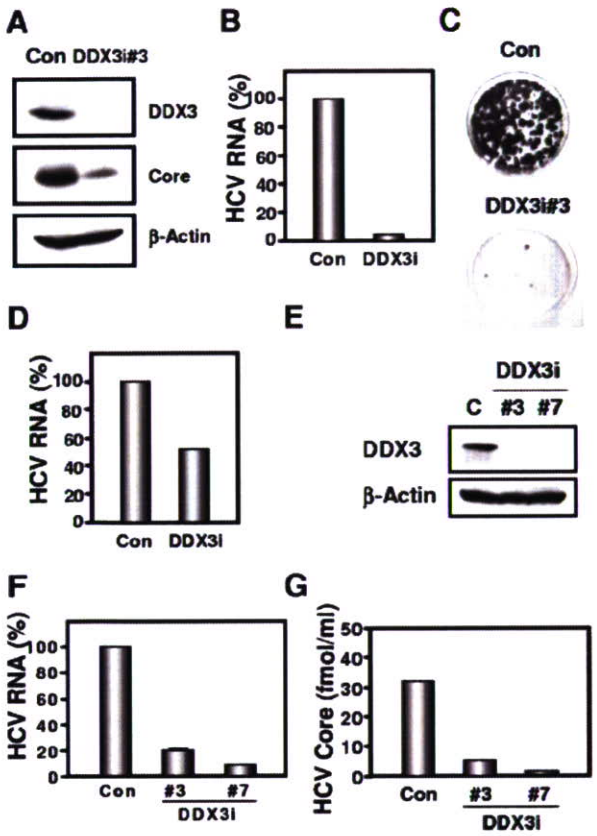

FIG. 1. Requirement of DDX3 for HCV replication. (A to D) Effect of DDX3 knockdown on HCV RNA replication. (A) Inhibition of DDX3 expression by shRNA-producing lentivirus vector. The results of Western blot analysis of cellular lysates with anti-DDX3 (ProSci), anti-HCV core (CP-9; Institute of Immunology), or an anti-β-actin antibody (Sigma) in O cells expressing shRNA against DDX3 (DDX3i#3) as well as in O cells transduced with a control lentivirus vector (Con) are shown. (B) The level of genome-length HCV RNA was monitored by real-time LightCycler PCR (Roche). Experiments were done in duplicate, and bars represent the mean percentages of HCV RNA. (C) Efficiency of colony formation in DDX3 knockdown cells. In vitro-transcribed ON/C-5B K1609E RNA (2 μg) was transfected into the DDX3 knockdown Oc cells (DDX3i#3) or the Oc cells transduced with a control lentivirus vector (Con). G418-resistant colonies were stained with Coomassie brilliant blue at 3 weeks after electroporation of RNA. Experiments were done in duplicate, and representative results are shown. (D) The level of subgenomic replicon RNA was monitored by real-time LightCycler PCR. Experiments were done in duplicate, and bars represent the mean percentages of HCV RNA. (E to G) Effect of DDX3 knockdown on HCV infection. (E) Inhibition of DDX3 expression by shRNA-producing lentivirus vector. The results of Western blot analysis of cellular lysates with anti-DDX3 or an anti-β-actin antibody for RSc cells expressing the shRNA DDX3i#3 or DDX3i#7 and for RSc cells transduced with a control lentivirus vector (Con) are shown. (F) The level of genome-length HCV (JFH1) RNA was monitored by real-time LightCycler PCR after inoculation of the cell culture-generated HCVcc. Results from three independent experiments are shown. (G) The levels of the HCV core in the culture supernatants were determined by an enzyme-linked immunosorbent assay (Mitsubishi Kagaku Bio-Clinical Laboratories). Experiments were done in duplicate, and bars represent the mean HCV core protein levels.

tion system (23). We found 80 to 90% reductions in the accumulation of JFH1 RNA and 82 to 94% reductions in the release of the core into the culture supernatants in DDX3 knockdown HuH-7-derived RSc cured cells at 4 days after inoculation of HCVcc (Fig. 1E to G). Thus, DDX3 seems to be required for HCV RNA replication.

Previously, DDX3 was identified as an HCV core-interacting protein by yeast two-hybrid screening. This interaction required the N-terminal domain of the core (aa 1 to 59) and the C-terminal domain of DDX3 (aa 553 to 622) (17, 19, 25). To determine whether the core can interact with DDX3 regardless of the HCV genotype, we used the HCV-O core (genotype 1b) and the JFH1 core (genotype 2a) (Table 1). We first examined their subcellular localization by confocal laser scanning microscopy as previously described (3). Consistent with previous reports (17, 19, 25), both the HCV-O core and JFH1 core mostly colocalized with DDX3 in the perinuclear region (Fig. 2A). Then we immunoprecipitated lysates from 293FT cells in which hemagglutinin (HA)-tagged DDX3 and HCV-O core, JFH1 core, or their 40-aa N-truncated mutants were overexpressed with an anti-HA antibody. Cells were lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 4 mM EDTA, 0.5% NP-40, 10 mM NaF, 0.1 mM Na_3VO_4, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Lysates were precleared with 30 μl of protein G-Sepharose (GE Healthcare Bio-Sciences). Precleared supernatants were incubated with 1 μg of anti-HA antibody (3F10; Roche) at 4°C for 1 h. Following absorption of the precipitates

TABLE 1. Primers used for construction of the HCV core-expressing plasmids[a]

Plasmid name	Direction	Primer sequence
pCXbsr/core(HCV-O)	Forward	5′-GGAATTCCCACCATGAG CACGAATCCTAAACCTC-3
	Reverse	5′-ATAAGAATGCGGCCGCC TATCAAGCGGAAGCTGG GATGGT-3′
pcDNA3/core(HCV-O)	Forward	5′-CGGGATCCAAGATGAGC ACGAATCCTAAACCTCAA AGA-3′
pcDNA3/FLAG-core(HCV-O)	Reverse	5′-CCGCTCGAGTCAAGCGG AAGCTGGGATGGTCAAA CA-3′
pcDNA3/Δcore(HCV-O)	Forward	5′-CGGGATCCAAGATGGGC CCCAGGTTGGGTGTGCG C-3′
pcDNA3/FLAG-Δcore(HCV-O)	Reverse	5′-CCGCTCGAGTCAAGCGG AAGCTGGGATGGTCAAA CA-3′
pcDNA3/core(JFH1)	Forward	5′-CGGGATCCAAGATGAGC ACAAATCCTAAACCTCAA AGA-3′
pcDNA3/FLAG-core(JFH1)	Reverse	5′-CCGCTCGAGTCAAGCAG AGACCGGAACGGTGATG CA-3′
pcDNA3/Δcore(JFH1)	Forward	5′-CGGGATCCAAGATGGGC CCCAGGTTGGGTGTGCG C-3′
pcDNA3/FLAG-Δcore(JFH1)	Reverse	5′-CCGCTCGAGTCAAGCAG AGACCGGAACGGTGATG CA-3′

[a] To construct pCXbsr/core(HCV-O), a DNA fragment encoding the core was amplified by PCR from pON/C-5B (13) with the indicated primers. The PCR product was digested with EcoRI-NotI and subcloned into the same site of pCX4bsr (1). To construct pcDNA3/core(HCV-O), pcDNA3/FLAG-core(HCV-O), pcDNA3/Δcore(HCV-O), and pcDNA3/FLAG-Δcore(HCV-O), DNA fragments encoding the core were amplified by PCR from pON/C-5B (13) with the indicated primer sets. To construct pcDNA3/core(JFH1), pcDNA3/FLAG-core(JFH1), pcDNA3/Δcore(JFH1), and pcDNA3/FLAG-Δcore(JFH1), DNA fragments encoding the core were amplified by PCR from pJFH1 (23) with the indicated primer sets. The PCR products were digested with BamHI and XhoI and then subcloned into the same site of pcDNA3 (Invitrogen) or pcDNA3-FLAG (2).

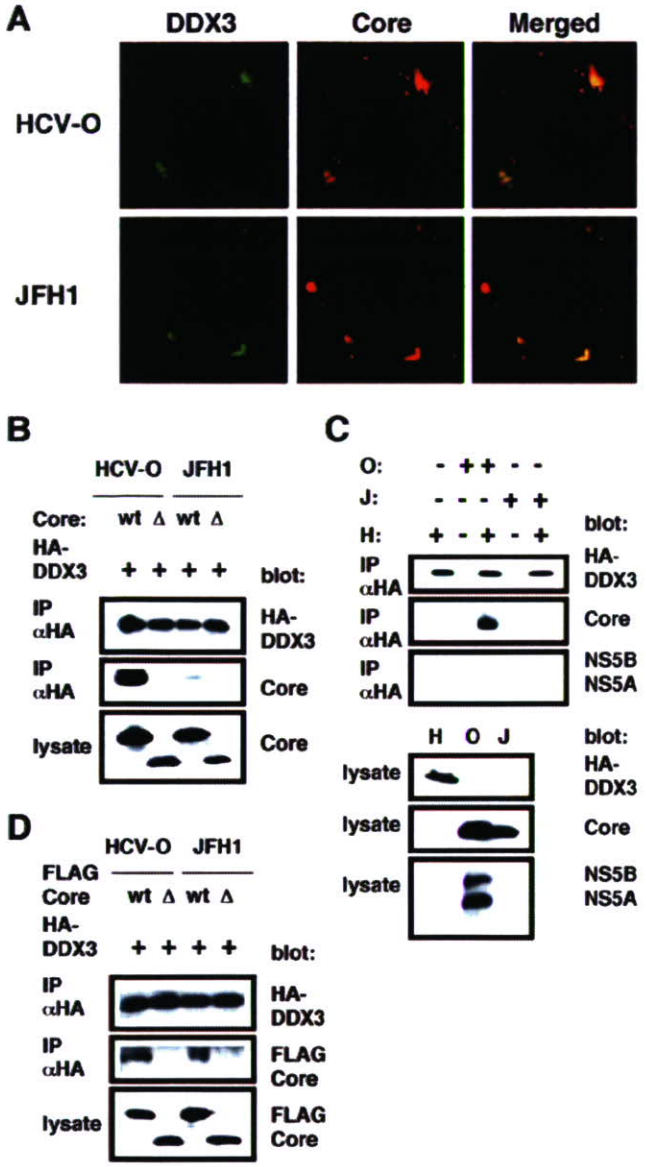

FIG. 2. Interaction of the HCV core with DDX3. (A) The HCV core colocalizes with DDX3. 293FT cells cotransfected with 100 ng of pCXbsr/core(HCV-O) or pcDNA3/core(JFH1) and 200 ng of pHA-DDX3 were examined by confocal laser scanning microscopy. Cells were stained with anti-HCV core (CP-9 and CP-11 mixture) and anti-DDX3 antibodies and were then visualized with fluorescein isothiocyanate (DDX3) or Cy3 (core). Images were visualized using confocal laser scanning microscopy (LSM510; Carl Zeiss). The right panels exhibit the two-color overlay images (Merged). Colocalization is shown in yellow. (B) The core binds to DDX3. 293FT cells were cotransfected with 4 μg of pHA-DDX3 and 4 μg of pCXbsr/core(HCV-O) (wt), pcDNA3/Δcore(HCV-O) (Δ), pcDNA3/core(JFH1) (wt), or pcDNA3/Δcore(JFH1) (Δ). The cell lysates were immunoprecipitated with an anti-HA antibody (3F10; Roche), followed by immunoblot analysis using either anti-HA (HA-7; Sigma) or anti-HCV core antibody (CP-9 and CP-11 mixture). (C) 293FT cells transfected with 4 μg of pHA-DDX3 (H), O cells (O), or RSc cells 3 days after inoculation of HCVcc (JFH1) (J) were lysed and immunoprecipitated (IP) with 1 μg of anti-HA antibody (3F10), followed by immunoblotting with anti-HA (HA-7), anti-core (CP-9 and CP-11 mixture), or anti-HCV NS5A (no. 8926) and anti-HCV NS5B. (D) 293FT cells transfected with 4 μg of pHA-DDX3 and 4 μg of pcDNA3/FLAG-core(HCV-O) (wt), pcDNA3/FLAG-Δcore(HCV-O) (Δ), pcDNA3/FLAG-core(JFH1) (wt), or pcDNA3/FLAG-Δcore(JFH1) (Δ) were lysed and immunoprecipitated with 1 μg of an anti-HA antibody (3F10), followed by immunoblotting with an anti-HA (HA-7) or anti-core (CP-9 and CP-11 mixture) antibody.

on 30 μl of protein G-Sepharose resin for 1 h, the resin was washed four times with 700 μl lysis buffer. Proteins were eluted by boiling the resin for 5 min in 1× Laemmli sample buffer. The proteins were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by immunoblot analysis using either anti-HA (HA-7; Sigma) or anti-HCV core antibody (CP-9 and CP-11 mixture). We observed that the HCV-O core but not its N-truncated mutant could strongly bind to HA-tagged DDX3 (Fig. 2B). In contrast, the binding activity of the JFH1 core to HA-tagged DDX3 seemed to be fairly weak (Fig. 2B). As well, immunoprecipitation of lysates of 293FT cells expressing HA-tagged DDX3, O cells, or JFH1-infected RSc cells, or mixtures of these lysates, with an anti-HA antibody revealed that HCV-O core but not JFH1 core could bind strongly to DDX3 (Fig. 2C). The anti-HCV core antibody we used could detect both HCV-O core and JFH1 core (Fig. 2), while both anti-HCV NS5A and anti-NS5B antibodies failed to detect JFH1 NS5A and NS5B (Fig. 2C). At least, we failed to detect an interaction between DDX3 and either HCV-O NS5A or NS5B under experimental conditions that permitted the core to interact with DDX3 by immunoprecipitation (Fig. 2C). In contrast, the FLAG-tagged JFH1 core could bind to HA-tagged DDX3 just as efficiently as the FLAG-tagged HCV-O core could (Fig. 2D). Thus, the binding affinity or stability of the complex formed between the JFH1 core and DDX3 might be weaker than that between the HCV-O core and DDX3.

Since DDX3 is required for HIV-1 and HCV replication, we hypothesized that the HCV core might affect the function of HIV-1 Rev when both proteins were coexpressed. To test this hypothesis, we used the Rev-dependent luciferase-based reporter plasmid pDM628, harboring a single intron that includes both the Rev-responsive element (RRE) and the luciferase coding sequences (Fig. 3A) (10). In this system, Rev binds to RRE on the unspliced reporter mRNA, allowing its export from the nucleus for luciferase reporter gene expression, while the intron containing the luciferase gene is excised during RNA splicing when cells are transiently transfected with pDM628 alone. As previously reported (10), the luciferase activity in 293FT cells transfected with this reporter plasmid was stimulated by Rev, which induced a fourfold increase in the reporter signal (Fig. 3B). Luciferase activity was increased eightfold by the combination of Rev and DDX3, whereas neither the HCV-O core nor the JFH1 core had any effect on this Rev function (Fig. 3B). Since the Rev-binding domain (the N-terminal domain) and the core-binding domain (the C-terminal domain) do not overlap in DDX3, the HCV core might not compete with HIV-1 Rev for binding with DDX3. However, the development of a novel DDX3 inhibitor might provide a powerful antiviral agent against both HIV-1 and HCV (15).

Taking these results together, this study has shown for the first time that DDX3 is required for HCV RNA replication.

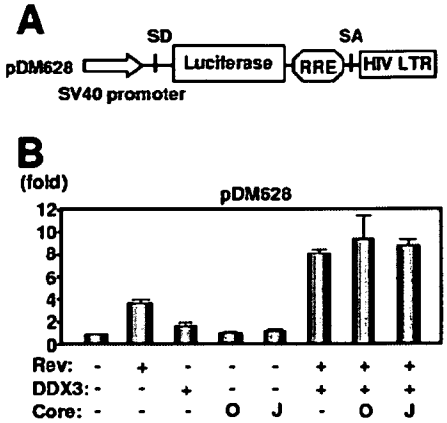

FIG. 3. HCV core does not affect the DDX3-mediated synergistic activation of Rev function. (A) Schematic representation of HIV-1 Rev-dependent luciferase-based reporter plasmid pDM628 harboring a splicing donor (SD), splicing acceptor (SA), and RRE. (B) 293FT cells were cotransfected with 100 ng of pDM628, 200 ng of pcRev, 200 ng of pHA-DDX3, and/or 100 ng of either pcDNA3/core(HCV-O) (O) or pcDNA3/core(JFH1) (J). A luciferase assay was performed 24 h later. All transfections utilized equal total amounts of plasmid DNA owing to the addition of the empty vector pcDNA3 to the transfection mixture. The relative stimulation of luciferase activity (*n*-fold) is shown. The results shown are means from three independent experiments.

Since helicases are motor enzymes that use energy derived from nucleoside triphosphate hydrolysis to unwind double-stranded nucleic acids, the DDX3–core complex might unwind the HCV double-stranded RNA and separate the RNA strands or might contribute to the function of HCV NS3 helicase. Since the replication of subgenomic replicon RNA was also partially suppressed in DDX3 knockdown cells (Fig. 1D), DDX3 might be associated with an HCV nonstructural protein(s) or HCV RNA itself. Indeed, Tingting et al. recently reported that DDX1 bound to both the HCV 3′ untranslated region (3′ UTR) and the HCV 5′ UTR and that short interfering RNA-mediated knockdown of DDX1 caused a marked reduction in the replication of subgenomic replicon RNA (22). Furthermore, Goh et al. demonstrated that DDX5/p68 associated with HCV NS5B and that depletion of endogenous DDX5 correlated with a reduction in the transcription of negative-strand HCV RNA (11). However, we failed to observe an interaction between DDX3 and NS5A or NS5B by immunoprecipitation under our experimental conditions in which the core could interact with DDX3 (Fig. 2C). Importantly, our DDX3 knockdown study demonstrated a more significant reduction in the accumulation of genome-length HCV RNA (95% reduction) than in the accumulation of subgenomic replicon RNA (52% reduction) (Fig. 1B and D). To date, it has been demonstrated that the 5′ UTR, the 3′ UTR, and the NS3-to-NS5B coding region are sufficient for HCV RNA replication (16); however, the core might be partly involved in the replication of genome-length HCV RNA. Importantly, DDX1 and DDX3 were specifically detected in the lipid droplets of core-expressing Hep39 cells by proteomic analysis (21), suggesting that DDX3 might be associated with HCV assembly or might incorporate into the HCV virion through interaction with the core to act as an RNA chaperone.

Recent studies have suggested a potential role of DDX3 and DDX5 in the pathogenesis of HCV-related liver diseases. DDX3 expression is deregulated in HCV-associated HCC (7, 8), and Huang et al. identified single-nucleotide polymorphisms in the DDX5 gene that were significantly associated with an increased risk of advanced fibrosis in patients with chronic hepatitis C (12). Interestingly, DDX3 might be a candidate tumor suppressor. DDX3 inhibits colony formation in various cell lines, including HuH-7, and up-regulates the $p21^{waf1/cip1}$ promoter (8). If DDX3 could in fact suppress tumor growth, then the core might overcome DDX3-mediated cell growth arrest and down-regulate $p21^{waf1/cip1}$ through interaction with DDX3, and it might also be involved in HCC development.

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Identification of Novel Epoxide Inhibitors of Hepatitis C Virus Replication Using a High-Throughput Screen[▽]†§

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Using our high-throughput hepatitis C replicon assay to screen a library of over 8,000 novel diversity-oriented synthesis (DOS) compounds, we identified several novel compounds that regulate hepatitis C virus (HCV) replication, including two libraries of epoxides that inhibit HCV replication (best 50% effective concentration, < 0.5 μM). We then synthesized an analog of these compounds with optimized activity.

Hepatitis C virus (HCV) infects over 170 million people worldwide and frequently leads to cirrhosis, liver failure, and hepatocellular carcinoma (1). Currently, the best therapy for the treatment of chronic hepatitis C is a combination of pegylated interferon and ribavirin, which has suboptimal efficacy and has an unfavorable side effect profile (14). The identification of more-effective and better-tolerated agents is therefore a high priority.

We have recently reported the successful adaptation of the Huh7/Rep-Feo replicon cell line (18) to a high-throughput screening assay system (8). Using this system, we previously screened a library of 2,568 well-known compounds whose biological activity is fully characterized (8). In order to discover novel regulators of HCV replication, we then screened a library of 8,064 diversity-oriented synthesis (DOS) compounds (15, 16). This library, known as the DOS set, is a

TABLE 1. Hits by library from the primary high-throughput screening with the DOS set[a]

Library	Increased luciferase signal hit libraries			Antiviral hit libraries		
	Hits	Members	Reference(s)	Hits	Members	Reference(s) or sources
FPA	11	319	5			
BUCMLD	4	880	10, 17	4	880	10, 17, Fig. 1, Table 2
JMM	4	544	13			
UGISS	1	319	2			
BUCMLD epoxyquinol				12	34	10, 17, Fig. 1 and 2, Table 2
SM				9	27	Fig. 1 and 2, Table 2
SpOx				6	612	6, 12
BEA				3	238	3
ICCB6				3	352	4
YKK				2	281	9
RTE				2	159	19

[a] The total number of compounds which comprise each library is listed in the "Members" columns.

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† This publication is dedicated to Yoshito Kishi on the occasion of his 70th birthday.

§ Supplemental material for this article may be found at http://aac.asm.org/.

‡ L.F.P. and S.S.K. contributed equally to this project.

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TABLE 2. Results of secondary screening with antiviral hit compounds from the SM and BUCMLD libraries[a]

Compound name	EC_{50}	CC_{50}
BUCMLD-B10A11	<0.5 (<0.5–0.5)	19.5 (19.4–22.4)
BUCMLD-B10A3	0.7 (<0.5–5.2)	9.0 (7.1–10.0)
BUCMLD-XL-184	1.4 (0.8–3.9)	>50
BUCMLD-B10A5	1.5 (<0.5–5.4)	39.3 (28.6–>50)
BUCMLD-XL-190	2.5 (<0.5–10)	>50
BUCMLD-B10A1	2.6 (1.0–5.0)	18.0 (15.9–19.5)
SM_A14B5	3.5 (2.7–4.4)	27.1 (18.0–44.6)
BUCMLD-XL-189	3.8 (2.2–7.0)	>50
SM_A6B5_2P100	6.6 (4.0–15.3)	>50
BUCMLD-B10A8	7.0 (0.9–30.0)	>50
BUCMLD-B10A10	7.0 (5.4–30.0)	>50
BUCMLD-B10A14	7.6 (1.0–23.0)	>50
BUCMLD-B10A7	7.75 (1.0–30.0)	35.3 (33.6–36.7)
SM_A4B6_2P123	8.0 (6.3–10.0)	>50
SM_A5B5_2P118	9.1 (2.5–16.7)	>50
SM_A7C2_2P155	12.7 (7.0–24.0)	>50
BUCMLD-B13A2	14.2 (6.4–45.0)	>50
SM_A1B2_1P32	19.6 (11.7–28.2)	>50
SM_A1B5_2P24	19.7 (6.25–50.0)	>50
BUCMLD-B13A1	21.1 (7.5–36.7)	>50
SM_A5B3_2P141	25.7 (18.3–39.8)	>50
SM_A5B2_2P142	26.7 (9.1–50.0)	>50
BUCMLD-NTM-EN2-67A	30.0 (0.7–46.1)	>50
SM_A12B3	>30	>50
BUCMLD-B10A13	42.9 (25.2–59.4)	>50
BUCMLD-XL-130	>100	>50

[a] Note that structure-activity relationship SM library compounds are also included. The EC_{50} and 50% cytotoxic concentration (CC_{50}) are reported in μM with 95% confidence intervals in parentheses. A value of <0.5 indicates a concentration of less than 0.5 μM; >30 indicates a concentration of greater than 30 μM; >50 indicates a concentration of greater than 50 μM; and >100 indicates a concentration of greater than 100 μM.

meta-library comprised of DOS libraries from chemists throughout the United States and Canada. Information about the DOS set is available at http://www.broad.harvard.edu/chembio/platform/screening/compound_libraries/index.htm.

The high-throughput primary screen and the secondary validation assays were performed as described in our previous publication (8).

Computational data analysis of the primary screening results was performed as previously described (8) except for the hit criteria. As the characteristics of this data set are different from those generated by our previous screen (8), different threshold values were chosen to assure optimal hit selection. Compounds were considered hits for inhibiting replication if they had a composite Z score of <-2.57 in the reporter gene screen, a reproducibility of >0.9 or <-0.9 in that screen, and a composite Z score of >-2.00 in the cell viability screen. Compounds were considered hits for stimulating luciferase production if they had a composite Z score of >2.50 in the reporter gene screen, a reproducibility of >0.9 or <-0.9 in that screen, and a composite Z score of <1.00 in the cell viability screen.

Full synthetic experimental procedures and spectroscopic data for the SM library compounds discussed in this publication are provided in the supplemental material. The synthesis of the full SM library, including compounds not discussed here, will be the subject of an upcoming report.

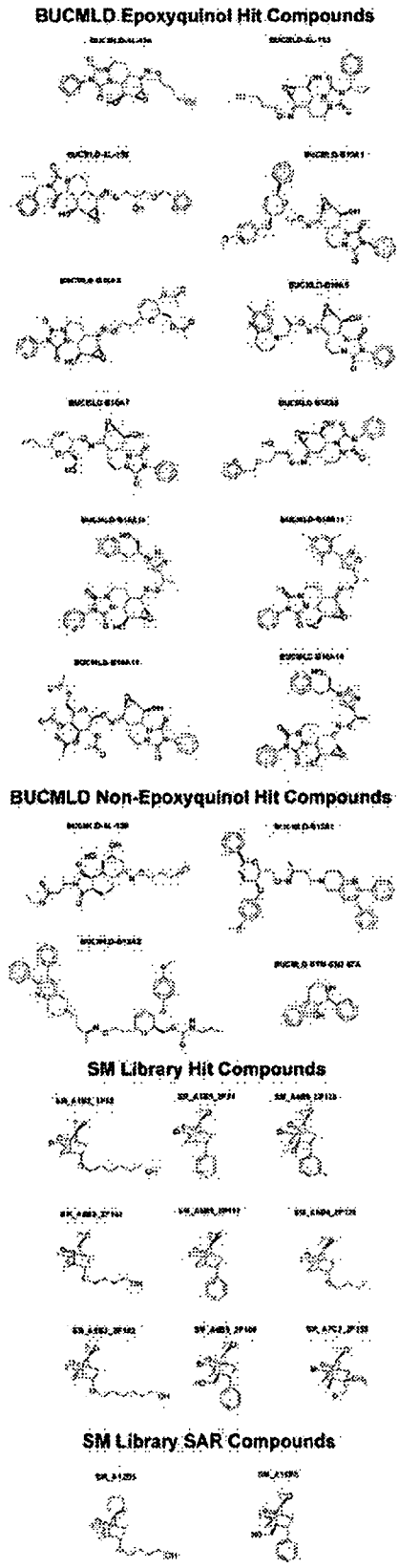

FIG. 1. Structures of antiviral hit compounds from the BUCMLD and SM libraries. SAR, structure-activity relationship.

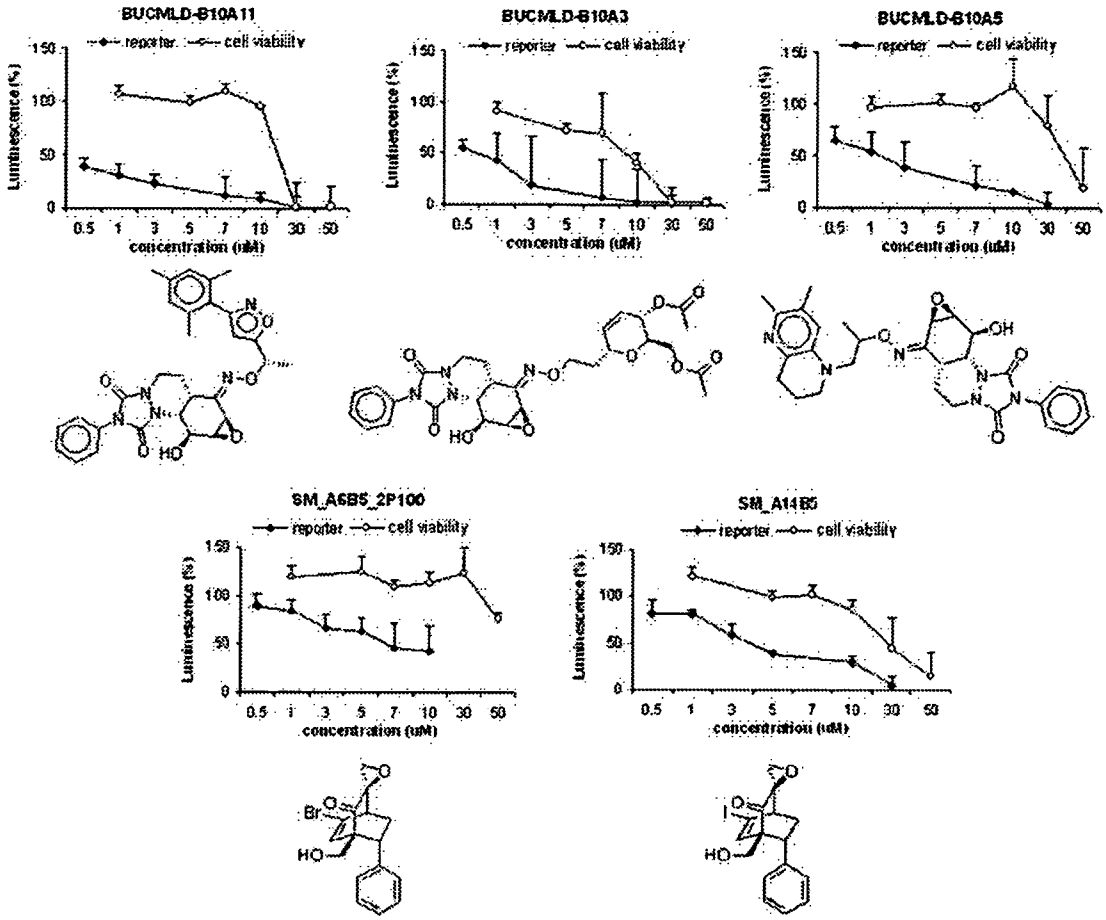

FIG. 2. Selected graphical results of secondary screening with antiviral hit compounds from the SM and BUCMLD epoxyquinol libraries. Luciferase activity for HCV RNA replication levels is shown as a percentage of control. Cell viability is also shown as a percentage of control. Each point represents the average of triplicate data points with standard deviation represented as the error bar.

The synthesis of the BUCMLD epoxyquinol library has been previously described (10, 17).

Full experimental details regarding the JFH1 HCVcc system (11) are provided in the supplemental material. We identified 41 antiviral compounds that inhibited HCV replication and 20 proviral compounds that increased luciferase production (Table 1). In our analysis of the antiviral hit compounds from the DOS set, a striking finding was that 21 of the 41 compounds contained an epoxide moiety. Moreover, the most potent of these compounds were epoxides. Further analysis revealed that these epoxides came from only two DOS libraries, SM and BUCMLD epoxyquinol (10, 17), with very high sublibrary hit rates of 35% and 33%, respectively (Table 1). Of note, the non-hit members of these two libraries did exhibit antiviral activity but failed to meet the formal hit criteria.

As we were especially intrigued by these epoxide-bearing compounds, we restricted our hit validation to these compounds (Table 2 and Fig. 1). SM_A6B5_2P100 was the most active member of the SM library, while BUCMLD-B10A11 was the most potent member of the BUCMLD epoxyquinol library (Table 2 and Fig. 2).

Structure-activity relationship analysis of the SM library reveals the structural elements most important for antiviral activity (Table 2 and Fig. 1). Comparing SM_A5B5_2P118 to SM_A1B5_2P24, iodinated compounds are more active than brominated ones. Comparing SM_A5B5_2P118 to SM_A5B3_2P141 and SM_A5B2_2P142, compounds with a phenyl substituent are more active than those with aliphatic chains. Finally, the most active compounds, SM_A4B6_2P123 and SM_A6B5_2P100, have a bridgehead substituent. Thus, we hypothesized that the most active compound should bear an iodine, a phenyl substituent, and a bridgehead substituent.

SM_A14B5, which incorporates all of these elements, was therefore synthesized, as it was reasoned to be the most active SM library compound. Indeed, SM_A14B5 had a 50% effective concentration (EC_{50}) of approximately 3.5 μM, which is about half that of SM_A6B5_2P100 (Table 2 and Fig. 2).

The most potent compounds from each library, SM_A14B5 and BUCMLD-B10A11, underwent further validation in the infectious JFH1 HCVcc system (11). They were tested at concentrations of 5 μM and 1 μM, respectively, and inhibited HCV replication 48.4% ± 5.9% and 45.1% ± 5.2%, respectively, relative to the level of inhibition achieved by interferon at a concentration of 1 ng/ml. These data roughly approximate the EC_{50} validation data derived from the OR6 system (7) in

which inhibition was also measured relative to that of interferon at a concentration of 1 ng/ml.

Our observations suggest that the epoxide moiety is essential for potent antiviral activity. Analyzing the BUCMLD compounds, those compounds that bear an epoxide moiety are, in general, more-potent antivirals than those that do not (Table 2 and Fig. 1). Furthermore, all of the compounds from the SM library bear epoxides. SM_A12B3, an analog of SM_A5B3_2P141, which bears a tetrahydrofuran moiety in place of an epoxide, was therefore synthesized to further test this hypothesis. SM_A12B3 had negligible antiviral activity (Table 2), while SM_A5B3_2P141 displayed modest antiviral activity. Other analogs of SM compounds bearing tetrahydrofuran rings in place of epoxides showed similar attenuation of antiviral activity relative to their parent compounds. Unfortunately, attempts to synthesize the tetrahydrofuran analog of the most potent SM compound, SM_A14B5, have so far been unsuccessful.

It is interesting to note that it is the urazole-containing epoxyquinol constituents of the BUCMLD epoxyquinol library, rather than the maleimide-derived ones, that demonstrated anti-HCV activity in the primary screen. It is therefore likely that the combination of a urazole with the epoxide is necessary for the activity of the BUCMLD epoxyquinol compounds.

Although none of our most potent antiviral DOS compounds showed significant cytotoxicity at their EC_{50}s, all of them ultimately proved to be cytotoxic at higher concentrations (Table 2 and Fig. 2). Therefore, future modifications should not only aim to improve anti-HCV activity but should also attempt to decrease cytotoxicity, in order to widen the therapeutic window.

It is tempting to hypothesize that these epoxides exert their antiviral effects through a common pathway. Presumably, they act as electrophiles, with the nucleophilic target making a covalent bond by attacking and opening the epoxides. Studies to elucidate their mechanism of action are under way.

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Potential role of vitamin K_2 as a chemopreventive agent against hepatocellular carcinoma

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Vitamin K, a cofactor necessary for the production of several antihemorrhagic factors, can inhibit the growth of various types of cells derived from neoplasms. In hepatoma cells, vitamin K_2 causes cell-cycle arrest and apoptosis. Vitamin K_2 is widely used in Japan to treat osteoporosis. The safety, relatively low cost and ease of use of vitamin K_2 have led to good compliance with treatment. The result of preliminary clinical trials in patients with chronic liver diseases are intriguing and suggest that vitamin K_2 might reduce the risk of hepatocellular carcinoma (HCC) in patients with liver cirrhosis as well as prevent disease recurrence after curative therapy in patients with HCC. This article reviews the potential role of vitamin K_2 as a chemopreventive agent against HCC and discusses future directions for clinical trials.

Key words: hepatocellular carcinoma, vitamin K_2, viral hepatitis, liver cirrhosis, chemoprevention

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) arises almost exclusively in patients with chronic liver disease, especially hepatic cirrhosis. The annual incidence of HCC in patients with cirrhosis ranges 5–7%.[1–3] The rate of recurrence after curative treatment of primary HCC is high.[4–6] Epidemiological studies estimate that the number of deaths from HCC will increase by 2010–2015.[7] Decreased mortality from HCC requires preventive therapy. Prospective studies have been performed to evaluate the chemopreventive properties of interferon (IFN), "Sho-saiko-to", and an acyclic retinoid.[8–11]

The vitamin K family is known to inhibit the growth of human cancer cell lines. However, the mechanisms of this effect have yet to be fully explored. Recently, vitamin K_2 has attracted attention as a new chemopreventive agent against HCC.

BACKGROUND OF VITAMIN K

VITAMIN K IS a cofactor for the enzyme γ-glutamyl-carboxylase, which converts glutamate residues into γ-carboxy-glutamate. Vitamin K-dependent proteins include coagulation factors II (prothrombin), VII, IX, and X, protein C and S, osteocalcin, surfactant-associated proteins, and bone matrix protein. The vitamin K family of molecules comprises the natural forms vitamin K_1 (phylloquinone) and vitamin K_2 (menaquinones) as well as the synthetic form vitamin K_3 (menadione). These naphthoquinone-containing molecules inhibit tumor cell growth in culture, with vitamin K_3 being more potent than either vitamin K_1 or K_2. Vitamin K_2 inhibits growth of human cancer cell lines and suppresses induction of differentiation in various human myeloid leukemia cell lines.[12–14] Clinically, myelodysplastic syndrome has been successfully treated with vitamin K_2.[14]

A number of findings indicate that vitamin K may have a role in controlling cell growth. Underlying mechanisms may involve redox cycling (as known for vitamin K_3), proteins with growth-inhibitory properties induced by vitamin K, such as prothrombin,[15] previously unidentified pathways involving arylation,[16] or growth arrest genes such as *gas* 6.[17] Geranylgeraniol (GGO), a side chain of vitamin K_2, strongly induces apoptosis of tumor cells, suggesting that GGO might inhibit cell growth.[18]

Recently, microarray analysis has shown that several genes are induced by treatment with vitamin K_2.[19] Protein kinase A (PKA) is a common activator of related

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Table 1 Baseline characteristics[21]

	Treatment ($n = 21$)	Control ($n = 19$)	*P*-value
Average age (years)	59.8 ± 8.7	61.4 ± 7.1	0.54
HBV/HCV	1/20	1/18	0.94
Albumin (g/dL)	3.9 ± 0.3	3.9 ± 0.3	0.87
Platelets (10^4/mm^3)	14.7 ± 5.4	12.1 ± 5.2	0.13
Total bilirubin (mg/dL)	0.8 ± 0.2	0.9 ± 0.4	0.47
ALT (IU/mL)	81.7 ± 42.7	70.4 ± 33.4	0.36
AFP (ng/mL)	13.4 ± 17.7	13.3 ± 8.7	0.99
IFN (+/−)	4/17	3/16	0.79

Mann–Whitney *U*-test for age, serum albumin, platelets, total bilirubin, alanine transferase (ALT) and α-fetoprotein (AF); χ^2 test for hepatitis B and C virus (HBV/HCV). IFN (+/−): Patents who received interferon(IFN) prior to enrollment; +, yes; −, no.

signaling pathways, identified by microarray analysis. Vitamin K_2 is thought to activate PKA, which inhibits RhoA activation. Alterations caused by high-dose treatment with vitamin K_2 result in cell-cycle arrest at the G1 and G2/M phases, accompanied by inhibition of tumor invasion. The effects of vitamin K_2 in doses used to treat osteoporosis are poorly understood, especially in the liver. However, the results of *in vitro* studies suggest that vitamin K_2 is one of the most promising agents for the chemoprevention of HCC.

PRIMARY CHEMOPREVENTION

WE PREVIOUSLY REPORTED a 2-year study showing that vitamin K_2 helps to prevent bone loss in women with viral cirrhosis of the liver.[20] Most of the subjects agreed to participate in an extended study designed to clarify the long-term effects of vitamin K_2 on bone loss associated with cirrhosis. The incidence of HCC was found to differ between women who received vitamin K_2 and those who did not.[21] In detail, the subjects of the initial 2-year study were 50 women with viral liver cirrhosis who were admitted to our department between 1996 and 1998. If the results of abdominal dynamic computed tomography and abdominal ultrasonography suggested the presence of HCC, abdominal angiography or needle biopsy was performed to confirm the diagnosis. Three patients in the treated group and four in the control group were confirmed to have HCC and were excluded from further study. The remaining 43 patients were randomly assigned by means of sealed envelopes to receive 45 mg/day of vitamin K_2 (Glakay; Eisai, Tokyo, Japan) p.o. (treated group) or no vitamin K_2 (control group). At the end of the first study (after 2 years of treatment), 21 patients in the treated group and 19 in the control group consented to participate in a longer trial. In a longer trial, all but one patient in each group had hepatitis C virus (HCV) infection; two other patients had hepatitis B infection. Seven patients, four in the control group and three in the treated group, had previously received IFN-α for their HCV infections, but HCV was not eradicated. No patient was given IFN therapy after study entry. Surveillance for HCC was done according to detailed guidelines for the follow up of patients with liver cirrhosis in Japan.[8] Compliance with vitamin K_2 in the treated group was good; no patient had adverse reactions or dropped out of the study. The two groups were similar with respect to age, virus type, platelets, alanine aminotransferase (ALT), α-fetoprotein (AFP) and other clinical findings (Table 1). After the first study commenced, HCC was detected in two of the 21 patients given vitamin K_2 and nine of the 19 controls; the cumulative proportion of patients with HCC was smaller in the treated group (log-rank test, $P = 0.024$; Fig. 1). On univariate analysis, the risk ratio for the development of HCC in the treated group versus the control group was 0.195 (0.042–0.913; $P = 0.038$). On multivariate analysis with adjustment for age, ALT activity, serum albumin, total bilirubin, platelet count, AFP, and history of treatment with IFN-α, the risk ratio for the development of HCC in patients given vitamin K_2 was 0.126 (0.016–0.992; $P = 0.049$) (Table 2).

The original goal of our trial was to assess the long-term effects of vitamin K_2 on bone loss in women with viral liver cirrhosis. Our trial thus had several important limitations when the data were used to assess the value of vitamin K_2 for the primary prevention of HCC in patients with liver cirrhosis. Factors limiting the value of our findings included the small study group, the inclusion of only women and the participation of only one

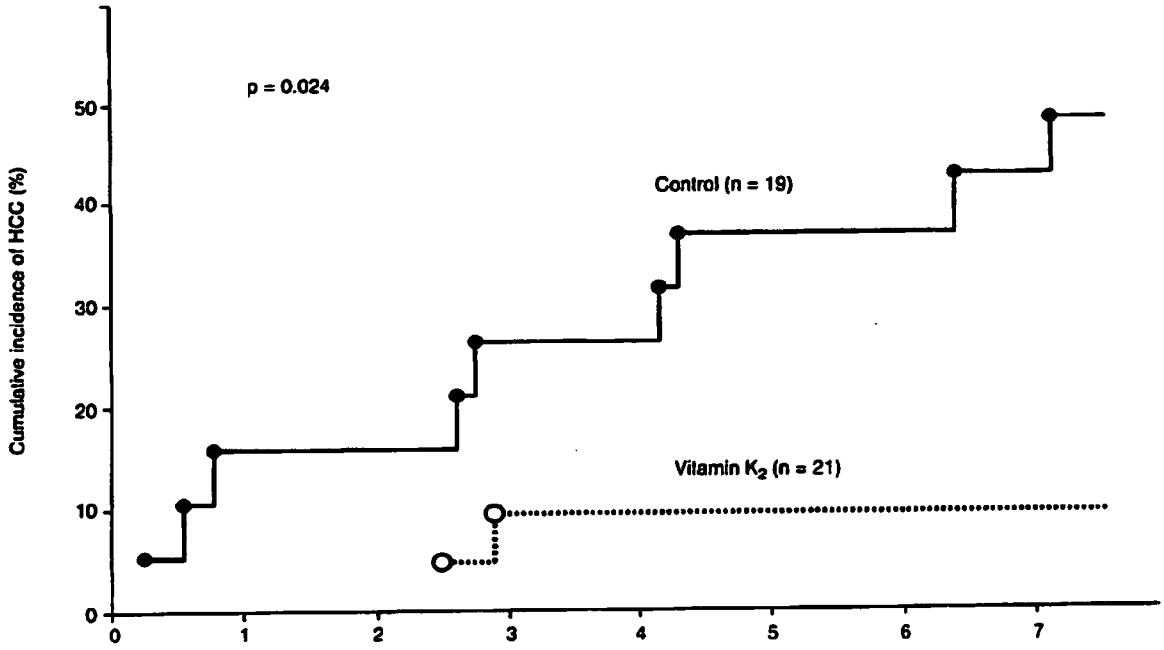

Figure 1 Cumulative incidence of hepatocellular carcinoma (HCC) diagnosed in patients treated with vitamin K_2 and in a control group.[21] All patients were followed up for at least 6 years. Vertical marks on curves show the latest follow-up to date for the 15 patients monitored for less than 7 years.

center. However, similar to previously reported randomized controlled studies of cirrhosis in which the primary end-point was the development of HCC, patients with evidence of HCC on highly sensitive imaging studies were excluded, and the two study groups were similar with respect to risk factors for HCC, including age, severity of cirrhosis, history of IFN therapy and type of hepatitis virus infection. Our results indicate that vitamin K_2

Table 2 Adjusted odds ratios for the development of hepatocellular carcinoma (HCC)[21]

	Odds ratio	95% CI	*P*-value
VK_2/control	0.126	0.016–0.992	0.0491
Total bilirubin (mg/dL) (1.0+/<1.0)	0.294	0.042–2.044	0.2161
Albumin (g/dL) (<3.5/3.5+)	33.434	2.362–473.352	0.0094
Platelets (10^4/mm^3) (<100/100+)	2.235	0.458–10.900	0.3200
ALT (IU/mL) (<80/80+)	0.393	0.071–2.164	0.2831
AFP (ng/mL) (20+/<20)	1.689	0.306–9.335	0.5477
IFN (+/–)	1.260	0.201–7.903	0.8053

Adjusted for age and all other variables in this table. IFN (+/–): Patents who received IFN prior to enrollment; +, yes; –, no. CI, confidence interval; VK_2, vitamin K_2.

decreases the risk of HCC to approximately 20% as compared with control, suggesting that vitamin K_2 may delay the onset of hepatocarcinogenesis.

SECONDARY CHEMOPREVENTION

THE RATE OF recurrence after curative therapy for HCC is high. Improved outcomes in HCC require inhibition of tumor recurrence. Before our study on primary chemoprevention, vitamin K_2 has been used to prevent the development of second primary malignancies after curative therapy for HCC. Koike *et al.* showed that the administration of vitamin K_2 to patients with HCC who have high levels of des-γ-carboxy prothrombin decreased the risk of portal vein invasion by tumor.[22] Preliminary results of a study being conducted by Mizuta *et al.* suggest that vitamin K_2 inhibits the recurrence of HCC, especially in patients with HCV (unpubl. results). However, this study is in progress; its results remain to be published.

COMBINATION THERAPY

PREVIOUS STUDIES HAVE evaluated the effectiveness of single agents for preventing HCC in patients with chronic liver diseases. To our knowledge, studies assessing the value of combination therapy for chemoprevention have not been reported. One of the reasons for the lack of studies evaluating combined treatment is concern about adverse effects associated with different agents. For example, adverse effects of IFN therapy include fever, leukopenia and thrombocytopenia. In contrast, vitamin K_2 has not been associated with serious side-effects in patients with osteoporosis. Vitamin K_2 may therefore be able to be used concomitantly with other chemopreventive agents, without increasing the risk of adverse reactions. Yoshiji *et al.* reported that a combination of vitamin K_2 and perindopril, an angiotensin-converting enzyme (ACE) inhibitor, was more effective for chemoprevention than either agent alone in a diethylnitrosamine-induced rat hepatocarcinogenesis model.[23] The number and size of enzyme-altered preneoplastic lesions were both significantly reduced, and the expression of CD31, a marker of neovascularization, was decreased in rats given combination treatment. Their findings suggested that a low dose of vitamin K_2 (1 μM) inhibits the proliferation of endothelial cells. Clinical trials examining whether vitamin K_2 plus an ACE inhibitor prevents HCC in patients with chronic liver diseases thus appear to be warranted.

CONCLUSION

AVAILABLE EVIDENCE SUGGESTS that vitamin K plays a role in controlling cell growth. The mechanisms responsible for the vitamin K_2-mediated inhibition of cell growth remain unexplained. Clinical studies have suggested that treatment vitamin K_2 reduces the incidence of HCC in patients with chronic liver diseases. Indeed, the annual incidence of HCC in control patients was 8.8%, similar to the incidence of HCC (7.9%; 32/107) in patients with liver cirrhosis in Japan,[1] as compared with only 1.6% in patients who received vitamin K_2 in our study. However, previous clinical studies of vitamin K_2 have focused on patients with specific characteristics or risk factors for HCC, including only women or patients with high levels of des-γ-carboxy prothrombin. Future investigations should attempt to define which patients would optimally benefit from chemopreventive therapy with vitamin K_2. The safety, relatively low cost and ease of use of vitamin K_2 have led to good compliance with treatment. These properties make vitamin K_2 a suitable candidate for clinical trials assessing the value of combination treatment for chemoprevention or chemotherapy in patients at risk for, or with a confirmed diagnosis of, HCC.

The results of preliminary trials are intriguing and suggest a potential role for vitamin K_2 in the prevention of primary and secondary hepatocarcinogenesis in patients with hepatic cirrhosis. However, currently available results must be verified by multicenter randomized controlled studies in which the primary end-point is the prevention of HCC by vitamin K_2.

CONFLICT OF INTEREST

NO CONFLICT OF interest has been received from the authors.

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CLINICAL STUDIES

Development of hepatocellular carcinoma in patients with chronic hepatitis C who had a sustained virological response to interferon therapy: a multicenter, retrospective cohort study of 1124 patients

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Abbreviations:
ALT, alanine aminotransferase; CH-C, chronic hepatitis C; CT, computed tomography; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; PIVKA II, protein-induced vitamin K absence or antagonist-II; SVR, sustained virological response; US, ultrasonography.

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Abstract

Background: Interferon (IFN) improves hepatic inflammation/fibrosis and reduces the risk of hepatocellular carcinoma (HCC) in patients with chronic hepatitis C (CH-C). However, HCC develops in some patients who have a sustained virological response (SVR) to IFN therapy. We designed this study to establish a follow-up protocol for patients with CH-C who have SVR to IFN therapy. *Methods:* We retrospectively studied 1124 patients with CH-C who received IFN. *Results:* HCC developed in 3.5% of patients with SVR to IFN. As compared with SVR patients without HCC, SVR patients with HCC were predominantly male ($P = 0.003$), older at the initiation of IFN therapy ($P = 0.002$), and at a more advanced histologic stage of disease ($P < 0.001$). However, three of the 13 SVR HCC patients had mild fibrosis. The mean interval from IFN therapy to the detection of HCC in SVR HCC patients was 5.8 years and did not differ significantly from that in non-SVR HCC patients ($P = 0.304$). Although most patients with HCC received curative therapy, the prognosis of some SVR HCC patients was poor, probably because of insufficient follow-up, resulting in delayed detection of HCC. *Conclusions:* SVR patients with CH-C who are elderly, male, or have an advanced histologic stage are at a high risk for the development of HCC after IFN therapy. We recommend that SVR patients should be observed carefully for more than 10 years after the completion of IFN therapy, even if they only have early fibrosis.

Hepatitis C virus (HCV) infection is a common cause of chronic hepatitis worldwide. It is also a major risk factor for hepatocellular carcinoma (HCC) (1, 2). Interferon (IFN) was first used as an antiviral therapy for HCV by Hoofnagle et al. in 1986 (3). It has been shown to be the only generally approved therapy that can eradicate the virus. IFN therapy improves hepatic inflammation and attenuates the progression of hepatic fibrosis in patients with chronic hepatitis C (CH-C) and may therefore reduce the risk of HCC (4–9). In fact, several studies have demonstrated that IFN therapy not only reduces inflammation and fibrosis scores but also lowers the incidence of HCC, especially in patients who have a sustained virological response (SVR) (8–13).

However, the development of HCC in patients with SVR has been reported (14–22). The incidence of the development of HCC in SVR patients during long-term follow-up remains poorly understood. The characterization of patients in whom HCC develops after having SVR to IFN therapy is required because of the high attendant risks, despite the permanent clearance of HCV.

This study was designed to establish a follow-up protocol for patients with CH-C who have SVR to IFN therapy. We investigated the incidence of and risk factors for HCC in patients with CH-C who responded to IFN therapy.

Methods

Patients

This was a retrospective cohort study. The study group comprised 1124 patients with CH-C who had received IFN therapy from 1992 to 2003 [684 males and 440 females; mean age, 52 years (range, 17–79 years)]. The medical records of these patients were obtained from Osaka City University Hospital and affiliated hospitals. All patients were seropositive for anti-HCV antibody, positive for serum HCV RNA, and seronegative for hepatitis B surface antigen. No patient had evidence of HCC on ultrasonography (US) or computed tomography (CT). We excluded patients who had coexisting liver diseases, such as autoimmune hepatitis or primary biliary cirrhosis. No patient was infected with the human immunodeficiency virus. In principle, liver biopsy was performed within 6 months before the start of IFN therapy. The histologic diagnosis was made according to the classification of Desmet et al. (23).

Treatment

The patients received various types of IFN, such as natural IFN-α, recombinant IFN-α-2a, recombinant IFN-α-2b, or IFN-β. In general, the dosage and duration of treatment with IFN was in accordance with Japanese National Health Insurance guidelines, i.e., 3–10 MU of IFN for 24 weeks (daily for 2 weeks, followed by three times per week for 22 weeks). In some patients, treatment with IFN was continued for 48 weeks, or IFN was withdrawn within < 24 weeks because of severe adverse effects. Patients who received a total dose of < 200 MU of IFN were excluded from the study. Eighty-five patients received a combination of recombinant IFN-α-2b and ribavirin. Ribavirin was given orally twice a day at a total daily dose of 600 mg for patients who weighed 60 kg or less and 800 mg for patients who weighed more than 60 kg. The duration of treatment with ribavirin was 24–48 weeks. Laboratory studies were performed at the start of therapy, at the end of therapy, and 6 months after the end of therapy. Patients with SVR were defined as those who remained negative for HCV RNA for 6 months after finishing IFN therapy. Other patients were defined to be non-SVR patients. The study group comprised 373 SVR patients (234 males and 139 females; age, 49 ± 12 years) and 751 non-SVR patients (450 males and 301 females; age, 52 ± 10 years).

Follow-up

We followed up all patients at appropriate intervals for at least 1 year after the end of IFN therapy. The median duration of follow-up was 66 months (range, 12–197 months). We performed biochemical examinations, sometimes including α-fetoprotein and/or protein-induced vitamin K absence or antagonist-II (PIVKA-II), every 3–12 months after the end of IFN therapy. US, CT, or both were performed at least once annually. HCC was diagnosed on the basis of imaging studies (hepatic angiography, dynamic CT, or magnetic resonance imaging). If the results of imaging studies were equivocal, suspected liver lesions underwent targeted biopsy. Patients in whom HCC was diagnosed within 1 year after the end of IFN therapy were excluded from the study to rule out the possibility that HCC was present (but too small to be detected) before the start of IFN therapy.

Among the 1124 patients studied, the clinical characteristics were compared among patients in whom HCC developed after having SVR to IFN therapy (SVR HCC patients), patients in whom HCC did not develop after having SVR (SVR non-HCC patients), and patients in whom HCC developed after failing to have SVR (non-SVR HCC patients).

Statistical analysis

The statistical significance of differences in categorical variables between groups was assessed with the use of the χ^2 test or Fisher's exact test, as appropriate. The rates of HCC development were calculated by the Kaplan–Meier method and compared with the use of the log-rank test. Multivariate analysis of factors potentially associated with the interval from the end of IFN therapy to the detection of HCC was performed using a Cox proportional-hazards model. All statistical analyses were performed with the Statistical Analysis System (SAS Institute Inc., Cary, NC). P values of less than 0.05 were considered to indicate statistical significance.

Table 1. Comparison of baseline characteristics between SVR patients with and without HCC

	SVR HCC	SVR non-HCC	*P* value
No. of patients	13	360	
Sex (male:female)	13:0	221:139	0.003
Age (years, mean ± SD)	58 ± 6	49 ± 12	0.002
Fibrosis stage			
F0,1	3	105	
F2	0	61	
F3	7	25	
F4	2	4	< 0.001

SD, standard deviation; HCC, hepatocellular carcinoma; SVR, sustained virological response.

Table 2. Comparison of baseline characteristics between SVR and non-SVR patients with HCC

	SVR HCC	non-SVR HCC	*P* value
No. of patients	13	61	
Sex (male:female)	13:0	38:23	0.155
Age (years, mean ± SD)	58 ± 6	58 ± 5	0.801
Intervals (months, mean ± SD)	69.1 ± 39.1	66.2 ± 33.4	0.304
Fibrosis stage			
F0,1	3	10	
F2	0	18	
F3	7	20	
F4	2	10	0.138

SD, standard deviation; HCC, hepatocellular carcinoma; SVR, sustained virological response.

Results

Comparison of baseline characteristics between SVR patients with and those without HCC

During observation, HCC was detected in 74 of 1124 patients more than 1 year after the end of IFN therapy. According to subgroup, HCC developed in 13 (3.5%) of the 373 SVR patients, and 61 (8.1%) of the 751 non-SVR patients. The clinical characteristics of the 13 SVR HCC patients are compared with those of the 360 SVR non-HCC patients in Table 1. The SVR HCC patients included a higher proportion of males ($P = 0.003$) and were older ($P = 0.002$) than the SVR non-HCC patients. Among all SVR patients, SVR HCC patients had a more advanced stage of fibrosis ($P < 0.001$). However, three SVR HCC patients had mild fibrosis (F1).

Comparison of baseline characteristics between SVR and non-SVR patients with HCC

We compared the clinical characteristics of the 13 SVR HCC patients with those of the 61 non-SVR HCC patients (Table 2). The sex, age, and fibrotic score of the SVR HCC patients did not differ significantly from those of the non-SVR HCC patients. The mean interval from the end of IFN therapy to the detection of HCC was 69.1 ± 39.1 months (about 5.8 years) in the SVR HCC patients, which was similar to that in the non-SVR HCC patients (66.2 ± 33.4 months). Two SVR HCC patients and 13 non-SVR HCC patients died from recurrence of HCC.

The interval from the end of IFN therapy to the detection of HCC did not differ significantly between the SVR and non-SVR patients with HCC ($P = 0.176$, Fig. 1). Cox proportional-hazards analysis showed no significant association of sex, age, response to IFN therapy, histologic stage, or HCV serotype with the interval from the end of IFN therapy to the detection of HCC (data not shown).

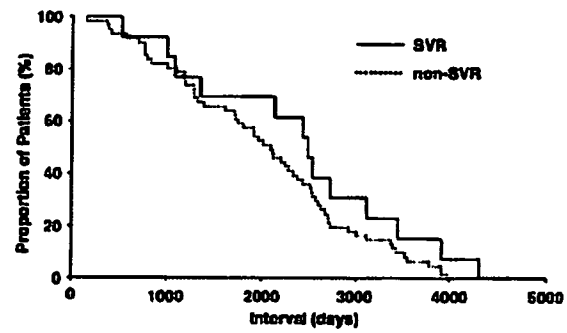

Fig. 1. Kaplan–Meier curve showing hepatocellular carcinoma (HCC)-free intervals in sustained virological response (SVR) and non-SVR patients after Interferon (IFN) therapy. The interval from the end of IFN therapy to the detection of HCC did not differ significantly between SVR patients and non-SVR patients ($P = 0.176$).

Characteristics of SVR patients with HCC at the time of diagnosis

Table 3 shows the characteristics of the 13 SVR HCC patients at the time of diagnosis. In eight of the 13 patients, HCC was detected more than 5 years after the end of IFN therapy. Two patients abused alcohol (cases 8 and 11). No patient was exposed to environmental carcinogens such as aflatoxin B1. At the time of diagnosis of HCC, all patients were negative for HCV RNA. Adequate follow-up was not possible in two patients (cases 11 and 12) because they did not return to the outpatient clinic for scheduled examination. Five patients had abnormal alanine aminotransferase (ALT) levels at the detection of HCC, but most patients had a good hepatic reserve after the elimination of HCV. Seven patients underwent curative treatment, i.e., partial hepatectomy, and five patients received transcatheter arterial embolization, percutaneous