

In conclusion, the results of the present study suggest that HU may be an effective anti-HCV reagent that can be used either alone or in combination with IFN- α to treat chronic hepatitis C.

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Short communication

Hydroxyurea suppresses HCV replication in humans: a Phase I trial of oral hydroxyurea in chronic hepatitis C patients

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Background: HCV is the main causative agent of chronic liver disease, which could progress to liver cirrhosis and hepatocellular carcinoma. By using a recently developed genome-length HCV RNA replication reporter assay system, we found that hydroxyurea (HU), an inhibitor of DNA synthesis, inhibited HCV RNA replication.

Methods: To test the hypothesis that HU suppresses HCV replication in humans, we conducted a Phase I trial involving Japanese patients with chronic hepatitis C (CHC) and investigated the safety and effectiveness of a 4-week course of oral HU.

Results: A total of nine patients were treated with an HU dose level of 500 mg three times daily. Dose-limiting toxicity was not observed at this dose level. Of the nine patients, eight exhibited a moderate decrease in serum HCV RNA levels during the trial. A decrease in HCV RNA levels to nadir levels was achieved for the eight patients (median $-0.27 \log_{10}$ IU/ml [range -0.08 – -0.44]) at various times during the 4 weeks after therapy initiation.

Conclusions: The results of this Phase I trial suggest that HU has potential as an anti-HCV agent that could be effective for the treatment of CHC patients.

Introduction

HCV is the main causative agent of chronic hepatitis C (CHC), which can progress to liver cirrhosis or hepatocellular carcinoma [1–3]. HCV infection is a global health problem with >170 million individuals infected worldwide [4]. HCV genotype 1 is the major genotype found in Japan, the United States and many other countries. Unfortunately, <50% of the patients infected with HCV genotype 1 respond to the standard combination therapy of pegylated interferon (PEG-IFN) and ribavirin [5,6].

In order to develop a more effective therapy, especially for genotype-1-infected patients, we recently developed a genome-length HCV RNA (strain O of genotype 1b) replication reporter system (OR6), which has served as an effective screening tool [7,8]. By application of this system, we found that hydroxyurea (HU), an inhibitor of DNA synthesis, inhibited HCV

RNA replication [9]. Furthermore, HU is an inhibitor of the enzyme ribonucleoside diphosphate reductase, which is involved in DNA synthesis and exerts cytostatic effects via this mechanism. HU is primarily used to treat patients with chronic leukaemia, melanoma and other solid tumours [10]. In addition, HU activity against viruses such as HIV and herpes simplex virus (HSV) has been recently reported [11,12]. Several clinical trials of HU in combination with a protease inhibitor have been performed in HIV-infected patients [10,13]. In these studies, the HU dose levels were set between 500 mg once daily and 400 mg three times daily. Moreover, in humans, the maximum tolerated dose (MTD) of HU was estimated as 800 mg/m² when administered orally every 4 h; at this dose, the mean peak plasma concentration increased to 2,480 µmol/l [14]. The MTD of HU is substantially higher than its

Table 1. Characteristics of patients in the trial

Patient number	Age, years	Gender	Genotype	Fibrosis ^a	Pretreatment		Response to HU treatment	
					IFN therapy (duration)	Maximal HCV RNA decrease ^b	Maximal HCV RNA decrease ^b	Range ^c
1	67	F	1b	F3	PEG-IFN/RBV (48W)	-2.66 (6.68-4.02)/36W	-0.37 (7.15-6.78)/3W	-0.37-0.18
2	71	F	1b	F1	PEG-IFN/RBV (48W)	-5.41 (6.96-1.55)/48W	-0.26 (6.83-6.57)/2W	-0.26--0.10
3	64	F	1b	F3	PEG-IFN/RBV (12W)	-1.26 (6.46-5.20)/12W	-0.28 (6.74-6.46)/3W	-0.28-0.02
4	59	F	1b	F1	PEG-IFN/RBV (48W)	-0.19 (6.21-6.02)/8W	-0.15 (7.31-7.16)/4W	-0.15-0.04
5	67	M	1b	F1	None	-	-0.08 (6.95-6.87)/4W	-0.08--0.02
6	69	F	1b	F1	IFN- α monotherapy	Unknown	-0.18 (6.78-6.60)/2W	-0.18--0.03
7	60	F	1b	F1	PEG-IFN/RBV (20W)	-0.54 (6.12-5.58)/12W	-0.32 (6.45-6.13)/4W	-0.32-0.01
8	63	F	1b	Unknown	None	-	0.10 (6.09-6.19)/2W	0.10-0.25
9	63	F	1b	F1	PEG-IFN/RBV (32W)	-1.61 (6.55-4.94)/8W	-0.44 (5.82-5.38)/4W	-0.44-0.36

^aMetavir score. ^bPresented as the maximal HCV RNA decrease in log₁₀ IU/ml (HCV RNA range) per number of treatment weeks (W) shown. ^cHCV RNA decrease range. F, female; HU, hydroxyurea; IFN, interferon; M, male; PEG-IFN, pegylated interferon; RBV, ribavirin.

50% effective concentration (EC₅₀), which was determined in our previous study [9]; thus, these observations prompted us to conduct a Phase I clinical trial of HU in CHC patients. This is the first report on the anti-HCV activity of HU in CHC patients.

Methods

Patients

From January 2009 to March 2009, nine Japanese CHC patients (one male and eight female, mean age 64 years [range 59-71]) were enrolled in the trial. These patients, who satisfied the inclusion criteria, were recruited from the outpatient Hepatitis Clinic, Yokohama City University Medical Center (Yokohama, Japan; Table 1). According to the inclusion criteria, the patients were required to have the following haematological laboratory values at entry: absolute neutrophil count >1,500 cells/ μ l, absolute platelet count >70,000 cells/ μ l and haemoglobin >10.0 g/dl. The following patients were excluded: lactating or pregnant patients and those with unstable angina pectoris, cardiac insufficiency (New York Heart Association Classes III and IV), uncontrolled arrhythmia, uncontrolled hypertension and uncontrolled diabetes mellitus at the time of acquisition of consent.

HCV RNA was detected in the serum of all the patients at 6 months of follow-up or later. Six patients had previously received the standard combination therapy of PEG-IFN- α 2b and ribavirin, and one patient had received IFN monotherapy; however, none of these patients showed response to treatment. The remaining two patients had refused IFN treatment. HCV genotype analyses revealed that all the patients were infected with HCV genotype 1b.

None of the patients had previously received corticosteroid or immunosuppressive treatment. Furthermore, none of the patients had a history of alcohol or

drug abuse, and there was no evidence of any metabolic or autoimmune disorders. The trial was approved by the institutional Ethics Committee (Yokohama City University Medical Center) and complied with the guidelines of Good Clinical Practice, the Helsinki Declaration and Japanese laws. Informed consent was obtained from all the patients.

Study design

We conducted a Phase I study to investigate the safety and effectiveness of a 4-week course of oral HU in order to determine the recommended dose (RD) for a subsequent Phase II study. The primary aim was to evaluate the safety of HU by determining the RD; this was performed by sequential monitoring of toxicity in three patients in each cohort. The secondary aim was to assess the anti-HCV effect of HU. One cycle consisted of consecutive oral administration of HU (Hydrea[®]; Bristol-Myers Squibb, Tokyo, Japan) for 28 days followed by an observation period. The HU dose levels were set between 500 mg once daily and 500 mg four times daily, with a starting dose of 500 mg three times daily because these dose levels were previously reported for patients with sickle cell anaemia [15]. Any Grade 4 haematological toxicity and Grade 3 non-haematological toxicity observed at any point during the therapy was considered as dose-limiting toxicity (DLT). The criteria for dose de-escalation were as follows: absolute neutrophil count <750 cells/ μ l, absolute leukocyte count <1,500 cells/ μ l and absolute platelet count <50,000 cells/ μ l during the therapy. The toxicity was assessed according to the Common Terminology Criteria for Adverse Effects (CTCAE) version 3.0 (Public Health Service, National Institutes of Health, National Cancer Institute, Bethesda, MD, USA).

DLT data were continually monitored to enable decisions regarding HU dose escalation and de-escalation

based on a new dose-finding algorithm using a Bayesian study monitoring method [16,17].

Laboratory measurements

Blood tests, including measurement of serum alanine aminotransferase (ALT) and HCV RNA concentrations, were performed immediately before the treatment, every week during the treatment and 4 weeks after cessation of the HU treatment. The serum HCV RNA levels including stored serum during prior IFN and PEG-IFN therapy were measured using the TaqMan® PCR assay (COBAS® TaqMan® HCV test; Roche Diagnostics, Tokyo, Japan; Table 1). The serum ALT concentrations and other parameters were measured using a sequential multiple autoanalyser.

Results

A total of nine patients were treated with an HU dose level of 500 mg three times daily. DLT was not observed at this dose level; however, two patients (number 5 and number 6) required dose de-escalation at 3 weeks after therapy initiation because they developed Grade 3 neutropaenia. After dose de-escalation, their neutropaenia resolved at 4 weeks after therapy initiation (Figure 1A). For all nine patients, dose escalation was not performed according to the clinical judgement of the investigators because two patients experienced adverse events (AEs), the levels of which were close to Grade 4; thus, 500 mg three times daily of HU was estimated to be an acceptable dose. Discontinuation of treatment was not required for any of the patients. All the patients experienced ≥ 1 treatment-related AEs. The most frequently observed treatment-related AE was neutropaenia, which was observed in all 9 (100%) patients (Figure 1A), followed by leukopaenia (Figure 1B) and thrombopaenia (Figure 1C), each in 6 (67%) patients, and brown discolouration of the nails in 4 (44%) patients. Most of these treatment-related AEs were CTCAE Grade 1 or 2, except neutropaenia, which was Grade 2 or 3; however, severe AEs were not observed. Furthermore, a decrease in serum HCV RNA concentrations during HU treatment was apparent in eight of the nine patients (Figure 1D); however, the decreases were modest and were not statistically significant. In patient 9, the maximum decrease in the serum HCV RNA concentration was $-0.44 \log_{10}$ IU/ml at 4 weeks after therapy initiation, and the mean decrease in the serum HCV RNA concentration at the end of the treatment was $-0.12 \log_{10}$ IU/ml. A reduction in HCV RNA levels up to nadir levels was achieved in eight patients (median $-0.27 \log_{10}$ IU/ml [range -0.08 – -0.44]) at various times during the 4 weeks after therapy initiation (Figure 1D). The serum HCV RNA concentration increased in six of the nine patients at 4 weeks after cessation of the HU treatment; however, significant changes

in the serum ALT concentrations during this trial were not observed in any of the patients (Figure 1E).

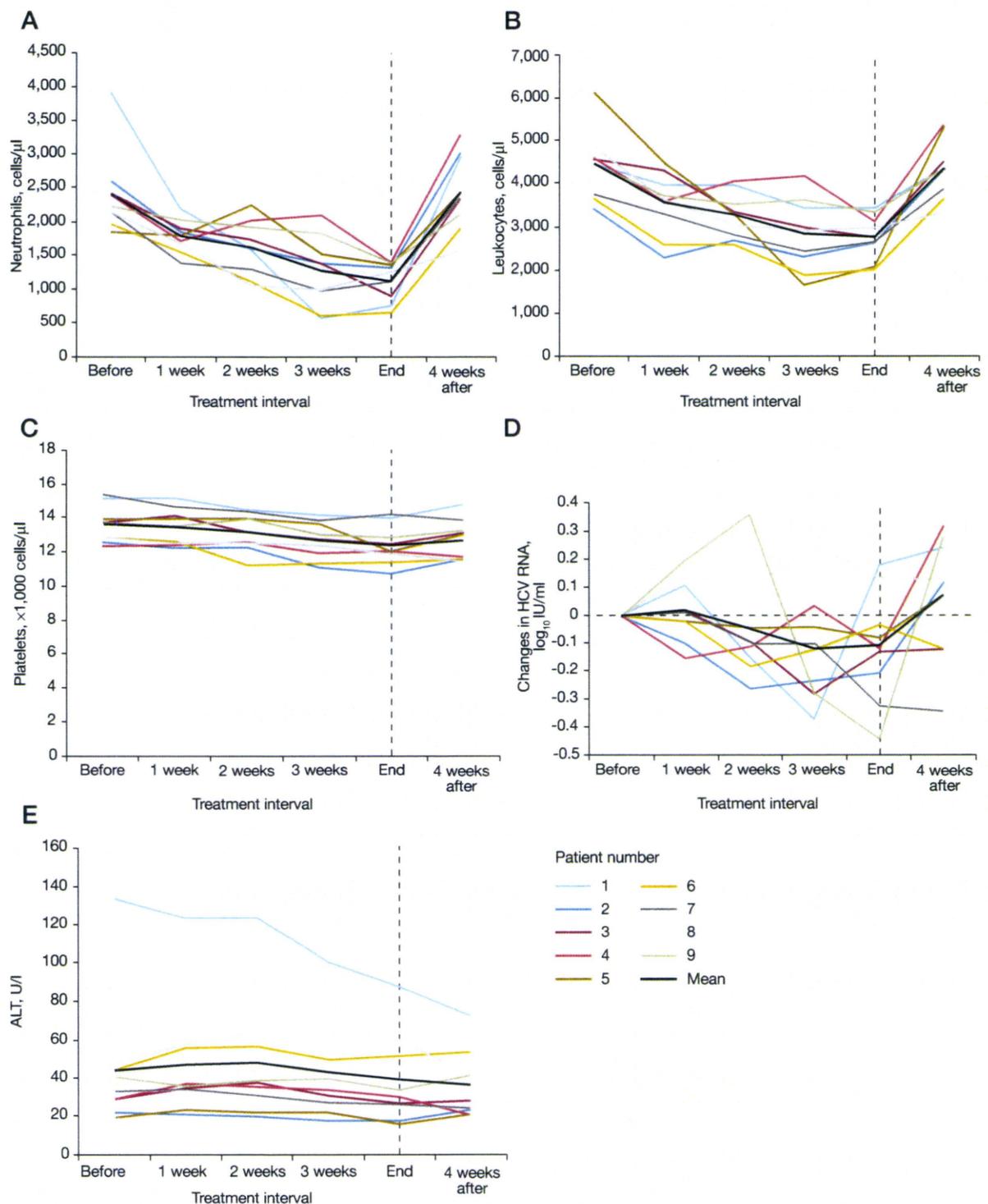
Discussion

HU is an inhibitor of the enzyme ribonucleoside diphosphate reductase that catalyses the conversion of ribonucleotides to deoxyribonucleotides, which are used during DNA synthesis. For decades, HU has been used as an anti-neoplastic agent for the treatment of patients with chronic granulocytic leukaemia and several types of cancers [10]. Currently, it is used to treat patients with leukaemia and other malignancies, such as sickle-cell anaemia, HIV infection, thrombocythaemia and psoriasis [18]. Recently, by using the OR6 system, we found that HU effectively inhibited HCV RNA replication. The median EC_{50} of HU ($60 \mu\text{mol/l}$ with a 50% cytotoxic dose of 150 – $200 \mu\text{mol/l}$) was considerably lower than the clinically achievable concentration (approximately $2,480 \mu\text{mol/l}$) [9]. Furthermore, in this trial, the mean plasma concentration of HU was estimated as 272 – $990 \mu\text{mol/l}$, as previously reported [14]. In this trial, we have shown, for the first time, that oral HU treatment might be associated with a decrease in the serum HCV concentration. These results strongly suggest that HU exhibits antiviral activity in patients with CHC infection. However, CTCAE Grade 3 neutropaenia was observed during this 4-week trial; therefore, reduction in the HU dose is required for longer treatment periods.

HU is thought to reduce serum HCV RNA concentration in CHC patients via two distinct mechanisms. The first mechanism, which has been reported for HU-induced HIV inhibition, involves prevention of G1/S transition by HU, which could play an important role in HCV inhibition [19,20]. Recently, cyclophilin B, a host factor, was identified as a crucial factor for the efficient replication of the HCV genome [21]; however, other unidentified host factors could also be crucial in this regard. The second mechanism might be that in which HU inhibits HCV RNA replication by interacting with these important host factors. Drugs that target such host cellular factors instead of the virus are less likely to induce drug resistance in HCV. Although the anti-HCV activity of HU alone might not be satisfactory in this trial, HU could be considered as a component of a new combination therapy involving IFN- α or other anti-HCV agents, such as ribavirin, protease inhibitors and polymerase inhibitors for CHC patients. Indeed, ribavirin, a cornerstone of the current standard therapy, alone did not exhibit sufficient anti-HCV activity, as previously reported [22,23].

In conclusion, this Phase I trial revealed that HU suppresses HCV replication in CHC patients. Further clinical trials including a new combination therapy

Figure 1. Response to HU treatment



(A) Changes in absolute neutrophil count during the trial. A decrease in leukocyte count was observed in all patients; this condition was alleviated 4 weeks after cessation of hydroxyurea (HU) treatment. (B) Changes in absolute leukocyte count during the trial. A decrease in leukocyte count was observed in all patients; this condition was alleviated 4 weeks after cessation of HU treatment. (C) Changes in absolute platelet count during the trial. A mild decrease in platelet count was observed in all patients; this condition was alleviated 4 weeks after cessation of HU treatment. (D) Changes in HCV RNA concentrations during the trial. During HU treatment, a decrease in serum HCV RNA concentrations was apparent in eight of the nine patients. (E) Changes in the serum levels of alanine aminotransferase (ALT) during the trial. No significant change in the serum ALT concentrations was observed in any of the nine patients during HU treatment.

involving IFN- α or other anti-HCV agents should clarify whether HU is a potential anti-HCV agent that will be effective for the treatment of CHC patients.

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Disclosure statement

The authors declare no competing interests.

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An antioxidant resveratrol significantly enhanced replication of hepatitis C virus

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Abstract

AIM: To elucidate the effect of antioxidants, resveratrol (RVT) and astaxanthin (AXN), on hepatitis C virus (HCV) replication.

METHODS: We investigated the effect of recent popular antioxidant supplements on replication of the HCV replicon system OR6. RVT is a strong antioxidant and a kind of polyphenol that inhibits replication of various viruses. AXN is also a strong antioxidant. The replication of HCV RNA was assessed by the luciferase reporter assay. An additive effect of antioxidants on antiviral effects of interferon (IFN) and ribavirin (RBV) was investigated.

RESULTS: This is the first report to investigate the effect of RVT and AXN on HCV replication. In contrast to other reported viruses, RVT significantly enhanced HCV RNA replication. Vitamin E also enhanced HCV RNA replication as reported previously, although AXN did not affect replication. IFN and RBV significantly reduced HCV RNA replication, but these effects were dose-dependently hampered and attenuated by the addition of RVT. AXN did not affect antiviral effects of IFN or RBV.

CONCLUSION: These results suggested that RVT is not suitable as an antioxidant therapy for chronic hepatitis C.

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Key words: Replicon system; Luciferase assay; Ribavirin; Interferon; Polyphenol; Astaxanthin

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INTRODUCTION

Chronic liver disease develops in over 70% of those infected with hepatitis C virus (HCV), and HCV is now the most common cause of liver cirrhosis and also hepatocellular carcinoma (HCC), especially in Japan. It has been said that the median time for progression to cirrhosis is 30-40 years, but other factors such as male gender, the age at infection, co-infection with hepatitis

B virus or human immunodeficiency virus (HIV), and alcohol consumption accelerate progression of this chronic disease. Oxidative stress has been postulated to be one of the deleterious factors of chronic hepatitis, and it was reported that antioxidant levels were significantly reduced in chronic hepatitis and cirrhosis^[1]. Moreover, it was also found that HCV proteins themselves generate oxidative stress, and the additive effect of oxidative stress caused by the inflammatory process in hepatitis and that induced by HCV proteins may further advance the disease stage of chronic hepatitis C^[2]. Therefore, it is thought that antioxidant therapy has a role in slowing disease progression to cirrhosis and subsequent HCC. In fact, there are studies suggesting a beneficial effect of antioxidant therapy for patients who did not respond to interferon (IFN) therapy, and that a combination of antiviral and antioxidant therapies may enhance the overall response rate of patients with chronic hepatitis C^[3,4].

Current recommended therapy for previously untreated and relapsed patients is a combination of pegylated interferon (Peg-IFN) and ribavirin (RBV), resulting in a sustained virological response in around 50% of genotype 1 patients with high viral load^[5]. Recent studies have shown that protease and polymerase inhibitors possess a strong additive effect on antiviral therapy of Peg-IFN/RBV and seem to be promising^[6]. However, these regimens are expensive and adverse effects are sometimes severe and frequent, meaning that large numbers of patients give up treatment for a variety of reasons^[7]. These conditions have led patients with chronic hepatitis C to use complementary and alternative medicine (CAM) including various supplements, and a previous survey found that about 40% of patients of liver disease outpatient clinics in the US used CAM at least once during the preceding month^[8]. Moreover, a large number of supplements are used by patients universally to maintain their health condition or improve quality of life even if they are cared by medical doctors and they always do not tell doctors whether they used CAM and/or supplements. The most frequent CAM or supplements taken by patients with chronic hepatitis C were antioxidants, which may be beneficial for this disease as described above.

Resveratrol (RVT) was discovered to be a strong activator of *sirtuin*, a gene for longevity^[9], and has been implicated as the most important polyphenol responsible for the beneficial effects of red wine consumption, which has been called as the "French Paradox"^[10]. Polyphenols contained in red wine have shown a strong antioxidative effect on cardioprotection, anti-atherosclerosis and relaxation of vascular endothelium through nitric oxide release. *Sirtuin* is activated when a person undergoes calorie restriction, and RVT is thought to be a surrogate for calorie restriction, which induces stabilization of DNA and also increases longevity by 70%. RVT is also known to improve liver lesions such as acetaminophen-induced hepatic injury and liver fibrosis in the mouse. When RVT was administered to mice fed a high-fat diet, fatty liver induced by this high-

calorie diet was significantly improved^[11]. In addition to these favorable reactions, it has been reported that RVT inhibited viral replication of several major viruses, such as cytomegalovirus (CMV), varicella-zoster, influenza A, and herpes simplex virus (HSV)^[12-15]. This supplement is popular and is thought to be one of candidates for the supplemental treatment of chronic hepatitis C.

Another candidate is astaxanthin (AXN: 3,3'-dihydroxy-b, b-carotene-4,4'-dione), which also showed a strong antioxidative effect^[16]. AXN is the carotenoid responsible for the pink pigmentation in the flesh of salmon, lobster, krill and other aquatic animals and plants. Recent studies have indicated that AXN is more powerful than its carotenoid cousin, β carotene, at neutralizing singlet oxygen^[17]. This supplement is known to improve the condition of so-called metabolic syndrome^[18,19], and is therefore popular. The antiviral effect of this supplement has not been examined so far.

These reports suggested that RVT and AXN might be good candidates for an antioxidative as well as an anti-HCV agent. However, we have no information whether these antioxidants affect HCV replication or not and if they are suitable for patients with chronic hepatitis C. In this study, we tried to assess the effect of these antioxidants on HCV replication using the HCV replicon system as an *in vitro* tool^[20]. This is the first report to investigate the effect of RVT and AXN on HCV replication *in vitro*.

MATERIALS AND METHODS

Cells and virus

OR6 cells, a cell line cloned from ORN/C-5B/KE cells^[21] supporting genome-length HCV RNA (strain O of genotype 1b) encoding the luciferase reporter gene, were used. This cell line was originally derived from a hepatoma cell line, HuH-7, as described elsewhere^[21]. The schematic organization of the ORN/C-5B/KE gene is shown in Figure 1A. This cell line was cultured and maintained as previously reported^[22]. Another cell line used was sKAH-5R^[23], which was established from a patient with acute hepatitis C, having subgenomic HCV RNA encoding the luciferase reporter gene (Figure 1B). The latter cell line was cultured under the same conditions as the OR6 cells, which includes the gene without a structural region of HCV RNA from the ORN/C-5B/KE gene.

Chemicals

We evaluated RVT and AXN as new supplements, and vitamin E (VE) was used as a control because its effect on the HCV replicon system was already reported elsewhere^[24]. RVT (3,5,4'-trihydroxystilbene), RBV (1- β -D-ribofuranosyl-1H-1,2,4-triazole-3-carboxamide), AXN (3,3'-dihydroxy-b,b-carotene-4,4'-dione), VE and IFN-2b were purchased from Sigma-Aldrich Japan (Tokyo, Japan). AXN, RVT and VE were prepared as 10-20 mg/mL stock solutions in dimethylsulfoxide (DMSO) and stored at -80°C until used. This stock solution was diluted with culture medium. The final

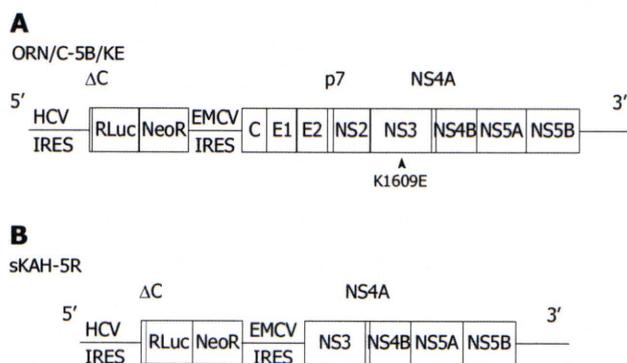


Figure 1 Organization of hepatitis C virus (HCV) RNA used in the replicon systems. A: Genome-length HCV RNA generated in OR6 cells; B: Subgenomic HCV RNA used in sKAH-5R and s1B-4R cells. Structural region of HCV RNA was deficient in the replicated RNA of sKAH-5R and s1B-4R cells.

concentration of DMSO was 0.2%, which did not interfere with viral replication, the highest concentration of RVT used in this study was 100 $\mu\text{mol/L}$, that of AXN was 50 $\mu\text{mol/L}$, and that of VE was 15 $\mu\text{mol/L}$. To monitor the anti-HCV effects of IFN and RBV on replication, OR6 cells (1.5×10^4 /well) were plated onto 24-well plates at least in triplicate for each assay and cultured for 4 h. Then the cells were treated with IFN at a final concentration of 1, 2, 4, 10, or 20 U/mL or RBV at a final concentration of 10 or 25 $\mu\text{mol/L}$ for 72 h, harvested with renilla lysis reagent (Promega, Madison, WI), and assayed for luciferase activity according to the manufacturer's protocol. The same protocol was applied for sKAH-5R cells. The additive effect of RBV, RVT and AXN on the antiviral effect of IFN (1 U) was studied and compared using luciferase activity.

Luciferase reporter assay

Approximately 1 to 4.5×10^4 cells were plated onto 6-well plates and cultured for 24 h. Cells were treated with each agent for 72 h. The cells were then harvested with Renilla lysis reagent and subjected to the Renilla luciferase (RL) assay according to the manufacturer's protocol.

Cell viability

We tested the toxic effect of RBV as described elsewhere^[22]. The effect of RVT (5-100 $\mu\text{mol/L}$) and AXN (1-50 $\mu\text{mol/L}$) on cell viability was investigated. To examine the cytotoxic effect of RVT and AXN on cells with replicon RNA, the cells were seeded at a density of 2×10^5 cells per dish onto 6-well plates. After 24 h culture, the cells were treated with RVT at final concentrations of 25 and 50 $\mu\text{mol/L}$ in the absence of G418. After incubation for 72 h, the number of viable cells was counted in an improved Neubauer-type hemocytometer after trypan blue dye (Invitrogen, Carlsbad, CA) treatment.

Statistical analysis

The difference in relative luciferase activity was tested using the Student's *t*-test and the Mann-Whitney *U*-test as appropriate. *P*-values < 0.05 were considered statistically

significant. Every experiment was performed in triplicate and two independent experiments were done.

RESULTS

RVT dose-dependently enhanced HCV RNA replication but AXN inhibited replication

The effect of RVT and AXN was examined in comparison to that of VE, using the OR6 assay system, in which genomic length HCV RNA replication is represented by RL fluorescence activity.

After treatment of OR6 cells with various concentrations of RVT for 72 h, the luciferase activity was dose-dependently increased up to 20 $\mu\text{mol/L}$ (Figure 2A). The activity gradually decreased at higher concentrations, but at 100 $\mu\text{mol/L}$ it was still higher than that without RVT. Since it has been reported that the proliferation of the HCV subgenomic replicon is dependent on host-cell growth, we examined the effect of RVT on cell number and viability of OR6 cells by the trypan blue dye exclusion test. As shown in Figure 2B, RVT did not increase OR6 cells until 15 $\mu\text{mol/L}$, and the cell viability decreased at higher concentrations than 20 $\mu\text{mol/L}$ of RVT. This decrease seen in higher concentrations paralleled the luciferase activity shown in Figure 2A, and it seemed that RVT further enhanced HCV RNA replication at concentrations higher than 20 $\mu\text{mol/L}$ when estimated by the number of viable cells. Different from the effect of RVT, AXN did not enhance luciferase activity (Figure 2C). The luciferase activity decreased at a concentration more than 10 $\mu\text{mol/L}$, and this decrease seemed to be due to decrease in cell viability (Figure 2D).

VE dose-dependently increased luciferase activity up to a concentration of 15 $\mu\text{mol/L}$ (Figure 3). This result indicated that VE also upregulates HCV RNA replication in OR6 cells. The cytotoxic effect of VE was not observed in the cell viability test. This result was compatible to the data already reported elsewhere^[24].

Proliferative effect of RVT was also observed in the subgenomic replicon

Next, we further investigated the effect of RVT on other clones of HCV RNA. We tested the effect of RVT on the subgenomic HCV RNA-replicating cell, sKAH-5R. RVT also enhanced the replication of sKAH-5R subgenomic HCV RNA at 2-10 $\mu\text{mol/L}$ (Figure 4A). This concentration is not toxic to the sKAH-5R cells, but more than 20 $\mu\text{mol/L}$ of RVT was toxic to sKAH-5R cells (Figure 4B). Thus, subgenomic replicons showed the same results observed in the full-genome length replicon.

Anti-viral effects of IFN and RBV

The effects of IFN-2b and RBV were independently applied to these cells to demonstrate that anti-viral agents reduce HCV RNA replication in this cell line. Figure 5A shows the time course of luciferase activity after administration of IFN. Luciferase activity was

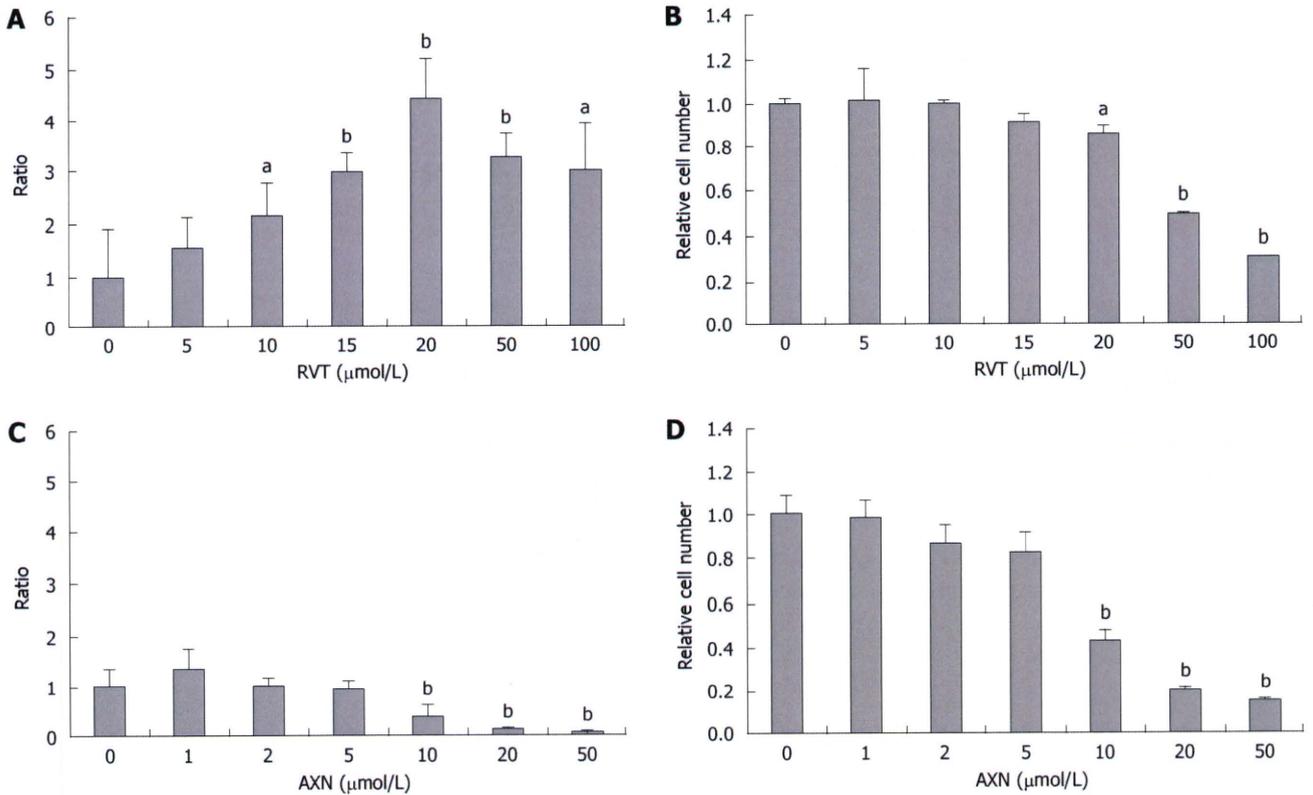


Figure 2 Luciferase activity and cell viability of OR6 cells after addition of resveratrol (RVT) and astaxanthin (AXN) for 72 h. The indicated concentrations of RVT or AXN were added to the culture medium of OR6 cells, and after 72 h of culture, cells were harvested with Rnilla lysis buffer and the lysate was subjected to the luciferase assay. Cell viability was evaluated by a trypan blue dye exclusion assay. A: Luciferase activity after addition of RVT; B: Cell viability after addition of RVT; C: Luciferase activity after addition of AXN; D: Cell viability after addition of AXN. ^a*P* < 0.05, ^b*P* < 0.01.

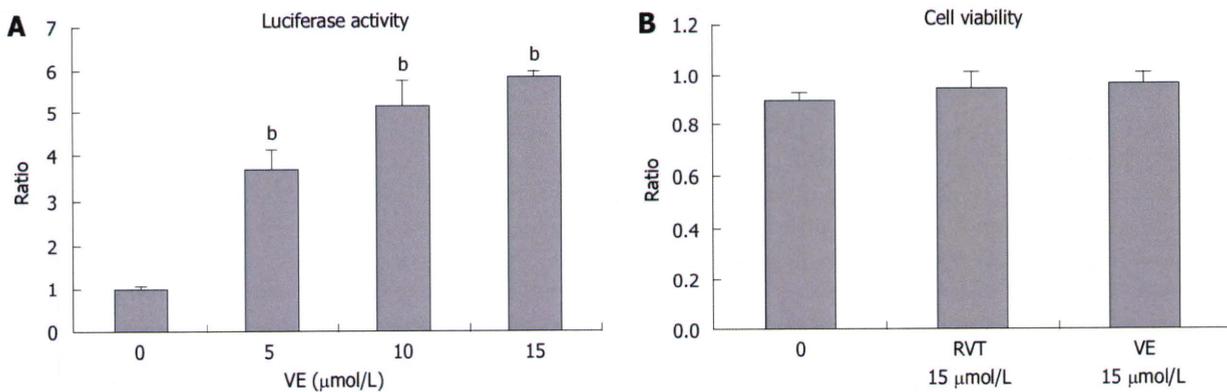


Figure 3 The effect of vitamin E (VE) on luciferase activity and cell viability of OR6 cells (72 h). Cells were treated with the indicated concentrations of VE for 72 h. A: Luciferase activity after addition of VE; B: Cell viability after addition of RVT and VE. ^b*P* < 0.01.

dose-dependently inhibited by IFN. Figure 5B shows the effect of RBV at concentrations of 10 and 25 μmol/L on luciferase activity of OR6 cells 72 h after addition of RBV in comparison to that of IFN (1 U/mL). RBV also reduced luciferase activity of OR6 cells dose-dependently, but the effect was smaller than that of IFN. Anti-viral effects of IFN and RBV were thus confirmed in this cell line.

RVT reversed anti-viral activity of IFN and RBV, but AXN did not affect it

We then investigated whether RVT reverses the anti-viral

effects of RBV and IFN. Luciferase activity of OR6 cells 72 h after treatment with RBV or IFN was compared to treatment with RBV plus RVT or IFN plus RVT (Figure 6A). The effect of treatment with 10 μmol/L of RVT alone on OR6 cells showed a 2.1-fold increase of luciferase activity. The addition of RVT (10 μmol/L) to RBV- or IFN-treated cells reversed the anti-proliferative effect of RBV on HCV RNA even when the cells were treated with 25 μmol/L of RBV plus 1 U/mL of IFN, which was normally enough to reduce HCV RNA to 1/5 (Figure 5B). Ten μmol/L RVT upregulated luciferase activity in RBV- or IFN-treated OR6 cells around 2-fold

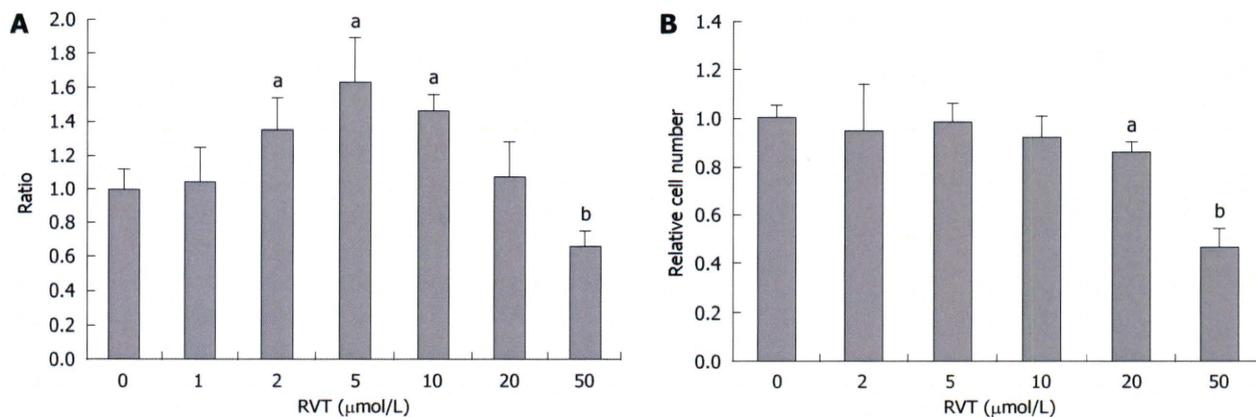


Figure 4 The effect of resveratrol on subgenomic replicon cells, sKAH-5R. RVT was added to the cells for 72 h and luciferase activity was assayed as was indicated in Figure 2. A: Luciferase activity after addition of RVT; B: Cell viability after addition of RVT. ^a*P* < 0.05, ^b*P* < 0.01.

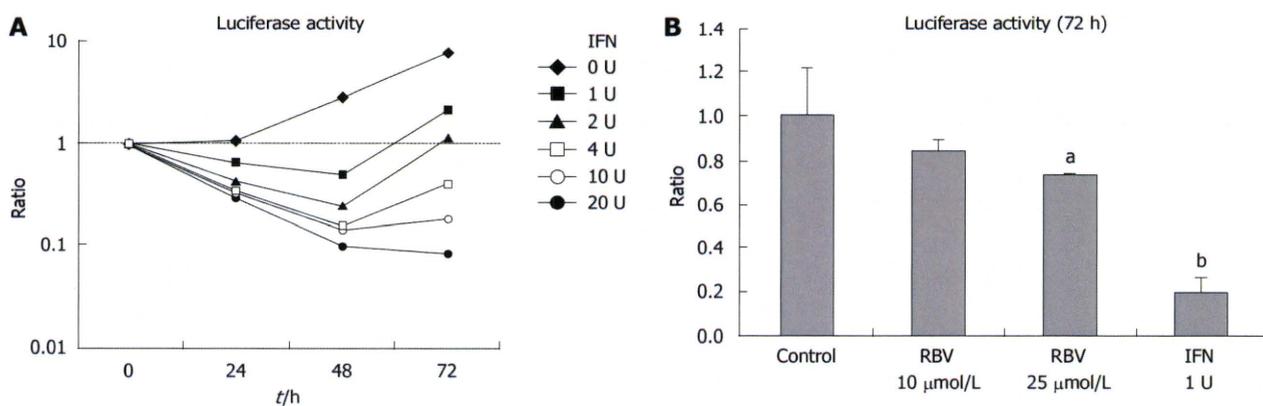


Figure 5 Sequential change of luciferase activity of OS6 cells after addition of interferon (IFN). A: IFN was added for the indicated duration and the ratio to the luciferase activity at time 0 was shown. Statistical significance (^a*P* < 0.05, ^b*P* < 0.01) is shown below: at 24 h 0 vs 1^a, 2^b, 4^b, 10^b and 20^b; at 48 h, 0 vs 1^b, 2^b, 4^b, 10^b and 20^b; 1 vs 2^b, 4^b, 10^b and 20^b; 20 vs 2^b, 4^b and 10^b; at 72 h, 0 vs 1^b, 2^b, 4^b, 10^b and 20^b; 1 vs 2^b; 1 vs 4^b, 10^b and 20^b; 2 vs 4^b, 10^b and 20^b; 4 vs 10^b and 20^b; 10 vs 20^b; B: Luciferase activity after addition of RBV and IFN for 72 h was shown as the ratio to that without antiviral agents. ^a*P* < 0.05, ^b*P* < 0.01.

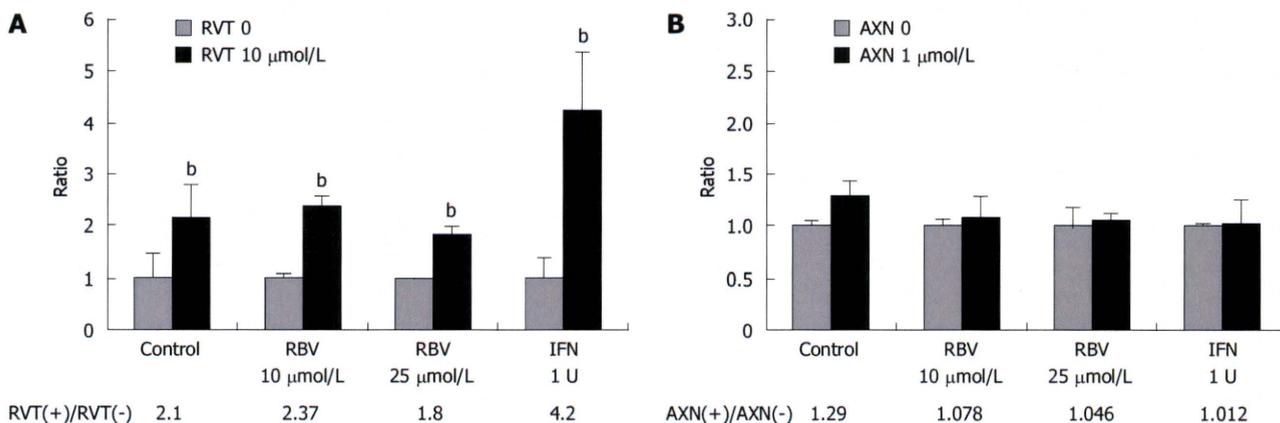


Figure 6 The enhancing effect of RVT on replication of HCV RNA, even when treated with ribavirin (RBV) and IFN. RVT was simultaneously added to RBV or IFN. A: The ratio was calculated as the ratio of luciferase activity with RVT to that without RVT. ^b*P* < 0.01 vs RVT 0; B: The same procedure was applied in AXN.

and 4.2-fold, respectively (Figure 6A). On the other hand, AXN did not affect the effect of RBV and IFN (Figure 6B), indicating that AXN has no disadvantageous effect on antiviral activity of IFN and RBV.

This proliferative effect of RVT was further emphasized by comparison to the additive effects of RBV

with IFN. We compared the dose-dependent effect of RBV, RVT and AXN on the anti-proliferative effect of IFN (Figure 7). RBV (A), RVT (B) and AXN (C) were added to OS6 cells at concentrations of 0, 1, 2, 5, 10 or 15 μmol/L with 1 U/mL of IFN for 48 h. The ratio between luciferase activity of RBV-, RVT- or AXN-treated

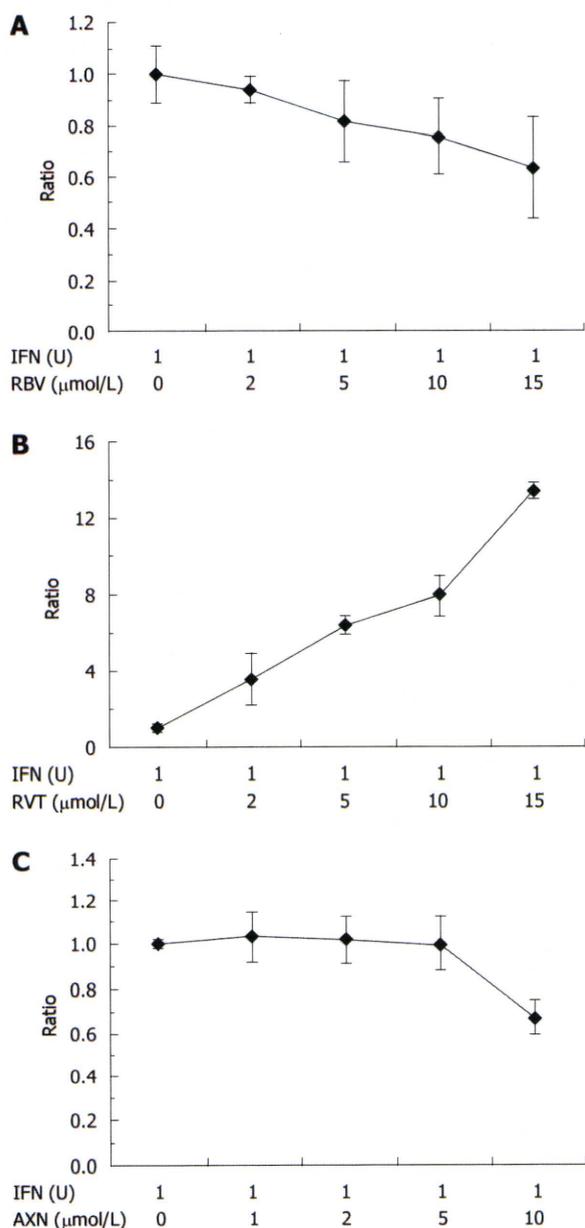


Figure 7 The comparative effect of RBV (A), RVT (B) and AXN (C) on the luciferase activity of IFN-treated cells. Cells were simultaneously treated with 1 U/mL of IFN and the indicated concentrations of RBV, RVT and AXN were added with IFN.

cells and that without co-treatment is shown. RBV further reduced the IFN-induced decrease in luciferase activity (Figure 7A), while RVT reversed this decrease, and further increased luciferase activity (Figure 7B). This effect was dose-dependent and it is noted that the enhanced ratio was strikingly large. On the other hand, AXN did not affect antiviral effect of IFN (Figure 7C).

DISCUSSION

We have shown that RVT, a natural polyphenol contained in red wine and peanuts, enhanced *in vitro* replication of HCV RNA without producing significant proliferation of host cells. This is the first report demonstrating a proliferative effect of RVT on HCV. RVT inhibited

replication of HSV-1, HSV-2^[25-27] human CMV^[13], Influenza A and orthomyxo virus^[15]. Moreover, it was reported that RVT inhibited replication of HIV-1 synergistically with nucleoside analogues^[28]. These reports suggest that RVT has a broad spectrum of anti-viral activities, and that RVT may selectively target the host, rather than the virus, as a mode of action for inhibiting viral replication. In spite of these inhibitory effects on viral replication, the mechanism of enhancing replication of HCV RNA is unclear. These results suggested that RVT is not suitable for antioxidant therapy of chronic hepatitis C. We also examined the effect of VE on replication of HCV RNA in OS6 cells, and VE enhanced its replication as effectively as RVT. On the other hand, AXN did not enhance replication of HCV RNA and had no effect on antiviral activity of IFN and RBV. These results indicated that we could recommend patients with chronic hepatitis C do not take RVT, especially when they receive antiviral therapy.

RVT is a non-flavonoid polyphenol and exerts anti-oxidative, anti-neoplastic and anti-inflammatory properties^[11]. Moreover, RVT has received much attention as an agent for prolongation of lifespan by activating silent information regulator 2 proteins, or sirtuins^[25], which are implicated in influencing aging and regulating transcription, apoptosis and stress resistance^[29]. These are causes for the popularity of this supplementation. Therapeutic intervention in liver injury with RVT has been suggested in various liver diseases^[30], such as alcohol-induced liver disease^[31], drug-induced liver injury^[32], ischemia-reperfusion injury^[33], and fatty liver diseases^[11,34]. Furthermore, RVT has been implicated to be favorable for prevention of hepatic fibrosis^[35,36]. These observations in combination with anti-viral effects indicated that RVT might be therapeutically beneficial or suitable for chronic hepatitis C. However, the direct effect of RVT on HCV RNA replication has not been studied thus far. In spite of our expectation, RVT did not inhibit replication of HCV, and on the contrary, it enhanced replication. Moreover, RVT hampered the anti-viral effect of IFN or RBV, and HCV RNA replication was enhanced even when enough concentration of IFN or RBV was administered to OR6 cells to reduce HCV replication. This condition was quite different from that observed in HIV-1 replication, in which the effect of RVT was synergistic with anti-viral effect of nucleotide analogues. Unlike RVT, AXN did not affect HCV replication and IFN-based antiviral activity, while it possesses strong antioxidant power.

An immunological response against virus-infected cells is an important pathogenic mechanism of chronic viral hepatitis. Reactive oxygen species (ROS) produced by activated macrophages and a consequent rise of lipid peroxidation cause direct activation of hepatic stellate (Ito) cells, leading to hepatic fibrosis and cirrhosis^[37]. Moreover, HCV core protein directly increases ROS as well as lipid peroxidation products and antioxidant gene expression^[38]. HCV infection is also associated with liver

iron accumulation^[39], which further produces ROS in the liver. These observations suggested that anti-oxidant therapy has an important role in slowing disease progression to cirrhosis in chronic hepatitis C. In consequence of this theory, the use of CAM is common in patients with chronic liver disease^[8]. Liu *et al.*^[40] reviewed medicinal herbs for HCV infection and concluded that some agents may have an effect on liver enzymes, but there is no firm evidence supporting efficacy of CAM. However, few studies have investigated the effect of antioxidants on HCV itself. Yano *et al.*^[24] investigated the effect of ordinary nutrients on HCV RNA replication using the replicon system, and found that some antioxidants such as β -carotene, vitamin D₂ and linoleic acid inhibited replication. They also showed an effect of VE on HCV RNA replication that was the same as in our study. In our study, AXN did not affect replication of HCV RNA. Thus, there is a group of antioxidants which inhibit replication of HCV, while there is another group of antioxidants which enhance its replication. The precise mechanism of this difference has not been clarified, but the investigation of this mechanism may provide new insights into anti-viral mechanisms. Recently it has been demonstrated that anti-HCV nutrients induce activation of the MEK-ERK1/2 signaling pathway through phosphorylation of ERK1/2^[41]. Study of this phenomenon may provide clues for a new therapeutic strategy in anti-viral treatment of HCV.

RVT has been shown to have a large number of regulatory biological functions, and Docherty *et al.*^[25-27] extensively studied the mechanism by which RVT inhibits the replication of HSV. However, even though it has been extensively studied, the molecular mechanism of RVT's action is not clear. Our results were quite different from those of Docherty's. In our study, not all antioxidants but 2 of 3 antioxidants increased the replication of HCV suggesting that the molecular mechanism of each agent is likely variable depending on viruses when we speculate in combination with studies of Docherty *et al.*^[25-27] and Yano *et al.*^[24]. On the other hand, reports suggesting a correlation between HCV replication and lipid metabolism have accumulated recently. It has been demonstrated that the cellular lipid droplet is an important structure for replication or assembly of viral components of HCV, especially HCV core protein^[42]. The inhibitory effect of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors on HCV have also been reported^[43]. Moreover, the success of peg-IFN plus RBV combination therapy, that resulted in the disappearance of HCV, affected lipid metabolism thereafter *in vivo*^[44]. Thus, it is conceivable that HCV genomic structure as well as the intracellular lipid is indispensable for viral replication of HCV. It is thought that RVT and VE affect intracellular lipid metabolism because they are lipid-soluble antioxidants. It is also interesting that HCV itself produces ROS, and that antioxidants affect the replication of HCV.

Bechmann *et al.*^[45] recently demonstrated that RVT

in response to free fatty acid administration deteriorates fibrogenic activation of human hepatic stellate cells. They showed that RVT upregulated the expression of key mRNAs associated with activated, fibrogenic stellate cells, and also demonstrated that the combined presence of free fatty acids and RVT significantly reduced the hepatic stellate cells' susceptibility to apoptosis. This report was controversial since previous reports^[35,36] demonstrated favorable effects of RVT on prevention of fibrosis progression. Bechmann *et al.*^[45] pointed out that the concentration of RVT was different from the previous study, and species' differences (employing rat *vs* human hepatic stellate cells) might be significant. Thus, RVT may have different therapeutic effects at various concentrations, and further investigation is needed to clarify a role of RVT in chronic liver diseases. Their result also suggested that patients with chronic hepatitis C should not take RVT as an additive nutrient, especially when they receive IFN-based antiviral therapy. Further investigations focusing on the enhancing mechanism of RVT on HCV RNA and different responses between RVT and AXN is necessary, and these approaches may develop a new strategy of anti-HCV agents.

In conclusion, we recommend patients with chronic hepatitis C who receive IFN-based antiviral therapy not to take RVT as an antioxidant supplement, although AXN may not affect anti-viral therapy.

COMMENTS

Background

Antiviral therapy for chronic hepatitis C has been developing, but the current standard therapy with pegylated interferon (Peg-IFN) and ribavirin combination therapy for 12 mo has achieved around 50% of patients who are infected with genotype 1 hepatitis C virus (HCV). Patients who have not attained viral clearance tend to take several supplementations for this chronic disease with an expectation for retardation of disease progression. A previous survey found that about 40% of patients of liver disease outpatient clinics in the US used complementary and alternative medicine (CAM) at least once during the preceding month. Among CAM, antioxidants have been popularly used by patients with chronic hepatitis C because it is said that oxidative stress deteriorates chronic hepatitis. However, the information about the use of supplementations for chronic hepatitis C was insufficient.

Research frontiers

Resveratrol (RVT) was discovered to be a strong activator of *sirtuin*, a gene for longevity, and the most important polyphenol responsible for the beneficial effects of red wine, which has been called the "French Paradox". RVT showed a strong antioxidative effect on cardioprotection, anti-atherosclerosis and relaxation of vascular endothelium through nitric oxide release. This information resulted in the popularity of this supplementation for people who suffered from chronic diseases. Since RVT inhibits the replication of other viruses, it is thought that RVT also inhibits HCV replication. However they revealed RVT enhanced HCV replication.

Innovations and breakthroughs

The investigation on HCV replication has been enabled by using the replicon system, in which HCV RNA replicates but unfortunately viral particles were not produced. Recently, the cell culture system in which HCV particles are produced was developed by Dr. Wakita T, and many new insights of HCV virology have been discovered. This study focused on the effect of taking daily supplementations on viral replication and antiviral therapy of HCV.

Applications

The authors recommend patients with chronic hepatitis C who receive IFN-

based antiviral therapy not to take RVT as an antioxidant supplement, although astaxanthin (AXN) may not affect anti-viral therapy.

Peer review

In this study, Nakamura *et al* tried to show the efficacy of antioxidants, RVT and AXN, on HCV replication. Since RVT inhibits the replication of other viruses, it was thought that RVT also inhibited HCV replication. However they revealed RVT enhanced HCV replication. These results are very interesting.

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Original Article

Deregulation of miR-92a expression is implicated in hepatocellular carcinoma development

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MicroRNAs (miRNAs) belong to a class of the endogenously expressed non-coding small RNAs which primarily function as gene regulators. Growing evidence suggests that miRNAs have a significant role in tumor development and may constitute robust biomarkers for cancer diagnosis and prognosis. The miR-17-92 cluster especially is markedly overexpressed in several cancers, and is associated with the cancer development and progression. In this study, we have demonstrated that miR-92a is highly expressed in hepatocellular carcinoma (HCC). In addition, the proliferation of HCC-derived cell lines was enhanced by miR-92a and inhibited by the anti-miR-92a antagomir. On the other hand, we have found that the relative amount of miR-92a in the plasmas from HCC patients is decreased compared with that from the healthy donors. Interestingly, the amount of miR-92a was elevated after surgical treatment. Thus, although the physiological significance of the decrease of miR-92a in plasma is still unknown, deregulation of miR-92 expression in cells and plasma should be implicated in the development of HCC.

Key words: hepatocellular carcinoma, microRNA, miR-638, miR-92a, plasma

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MicroRNAs (miRNAs) are small endogenous non-coding RNAs that regulate gene expression and have a critical role in many biological and pathological processes.¹ Recent studies have shown that deregulation of miRNA expression contributes to the multistep processes of carcinogenesis, and have shown promise as tissue-based markers for cancer classification and prognostication.^{2,3} However, biological roles of only a small fraction of known miRNAs have been elucidated to date.

The miR-17-92 cluster at 13q31.3 consists of six miRNAs: miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a-1, and plays an important role for development of lung cancer,⁴ B-cell lymphomas,⁵ chronic myeloid leukemia,⁶ medulloblastomas,⁷ colon cancer⁸ and hepatocellular carcinoma (HCC).⁹ In addition, mice deficient in the miR-17-92 cluster died shortly after birth with lung hypoplasia, and B-cell development was impaired in the mice.¹⁰ It has been reported, however, that miR-92a increases cell proliferation by negative regulation of an isoform of the cell-cycle regulator p63.¹¹ Furthermore, miR-92a regulates angiogenesis.¹² Thus, it is clear that the miR-92a has some oncogenic characteristics. However, the specific biological role of miR-92a in the processes of human cancer development has remained unclear.

Here, we have revealed that miR-92a is implicated in human HCC development. Furthermore, we have demonstrated that miR-92a in human blood has the potential to be a noninvasive molecular marker for diagnosis of human HCC.

MATERIALS AND METHODS

In situ hybridization of miR-92a

Locked nucleic acid (LNA)-modified probes for miR-92a and negative control (miRCURY-LNA detection probe, Exiqon, Vedbaek, Denmark) were used. The probe sequences were as follows; *miR-92a*, 5'-ACAGGCCGGACAAGTGCAATA-3'; and a scrambled oligonucleotides used for negative control, 5'-GTGTAACACGTCTATACGCCCA-3'. *In situ* hybridization was performed using the RiboMap *in situ* hybridization kit (Ventana Medical Systems, Tucson, AZ, USA) on the Ventana Discovery automated *in situ* hybridization instrument (Ventana Medical Systems). The *in situ* hybridization steps were performed as previously described.¹³ Staining was evaluated by two investigators and graded as follows: negative (-), no or occasional (<5%) staining of tumor cells; positive (+), mild to strong (>5%) staining of tumor cells. Paraffin-embedded tissue samples of hepatocellular carcinoma (HCC) and adjacent non-tumorous liver

cirrhosis (LC) were obtained from HCC patients at Ogaki Municipal Hospital (Ogaki, Japan). Details of the clinical data are provided in Table 1.

Plasma collection, RNA isolation and quantitative RT-PCR

Whole blood samples were collected from healthy donors and the patients with HCC at Ogaki Municipal Hospital. This study was approved by the institutional review board (IRB) of Tokyo Medical University, and all subjects provided written informed consent under the institutional review board. Details of clinical data are provided in Table 1. Diagnoses were confirmed using the post-operated tissues. Blood samples of the patients (Cases 1–10) were collected one day before the operation and then properly stored. One week after operation, blood samples of the patients were collected again. Whole blood was separated into plasma and cellular fractions by centrifugation at 1600 g for 15 min. Total RNA in the

Table 1 Summary of clinical details of hepatocellular carcinoma (HCC) used for *in situ* hybridization and serum analysis

	Year	Sex	Virus type	Histologic type	Stage	Child-Pugh	miR-92a
Case 1	53	Male	HBV	Poorly	I	A	+
Case 2	59	Male	HBV	Moderate	II	A	+
Case 3	79	Male	NBNC	Moderate	III	A	+
Case 4	73	Male	HCV	Well	I	A	+
Case 5	76	Female	HCV	Moderate	IV-A	A	+
Case 6	59	Male	HCV	Moderate	II	A	+
Case 7	69	Female	HCV	Moderate	I	A	+
Case 8	71	Male	HCV	Moderate	I	A	+
Case 9	59	Female	HBV	Well	I	A	-
Case 10	69	Male	NBNC	Moderate	IV-A	A	-
Case 11	61	Female	HBV	Poorly	IV-A	B	+
Case 12	73	Male	NBNC	Moderate	II	A	+
Case 13	67	Male	NBNC	Moderate	IV-A	A	+
Case 14	61	Male	NBNC	Moderate	III	A	+
Case 15	45	Male	HBV	Moderate	I	A	+
Case 16	68	Female	HCV	Moderate	III	A	+
Case 17	70	Male	NBNC	Poorly	II	A	+
Case 18	59	Male	HCV	Moderate	III	A	+
Case 19	43	Male	HBV	Moderate	II	A	+
Case 20	69	Male	HCV	Moderate	II	A	-
Case 21	76	Male	HCV	Moderate	III	A	-
Case 22	53	Male	HCV	Moderate	II	A	-

HCV, hepatitis C virus; HBV, hepatitis B virus; NBNC, non-B non-C virus.

Table 2 Summary of clinical details of hepatocellular carcinoma (HCC) used for qPCR analysis

Code no.	Year	Sex	Virus type	Histologic type	Non-tumorous tissue	AFP	PIVKA-II
91	53	Male	HCV	Moderate	LC	5	0.06
160	59	Male	HCV	Moderate	LC	NI	NI
O89	68	Male	HCV	Moderate	LC	8	25
O90	70	Male	HCV	Moderate	LC	686	962
K89	51	Male	HCV	Moderate	LC	NI	NI

LC, liver cirrhosis; HCV, hepatitis C virus; NI, no information.

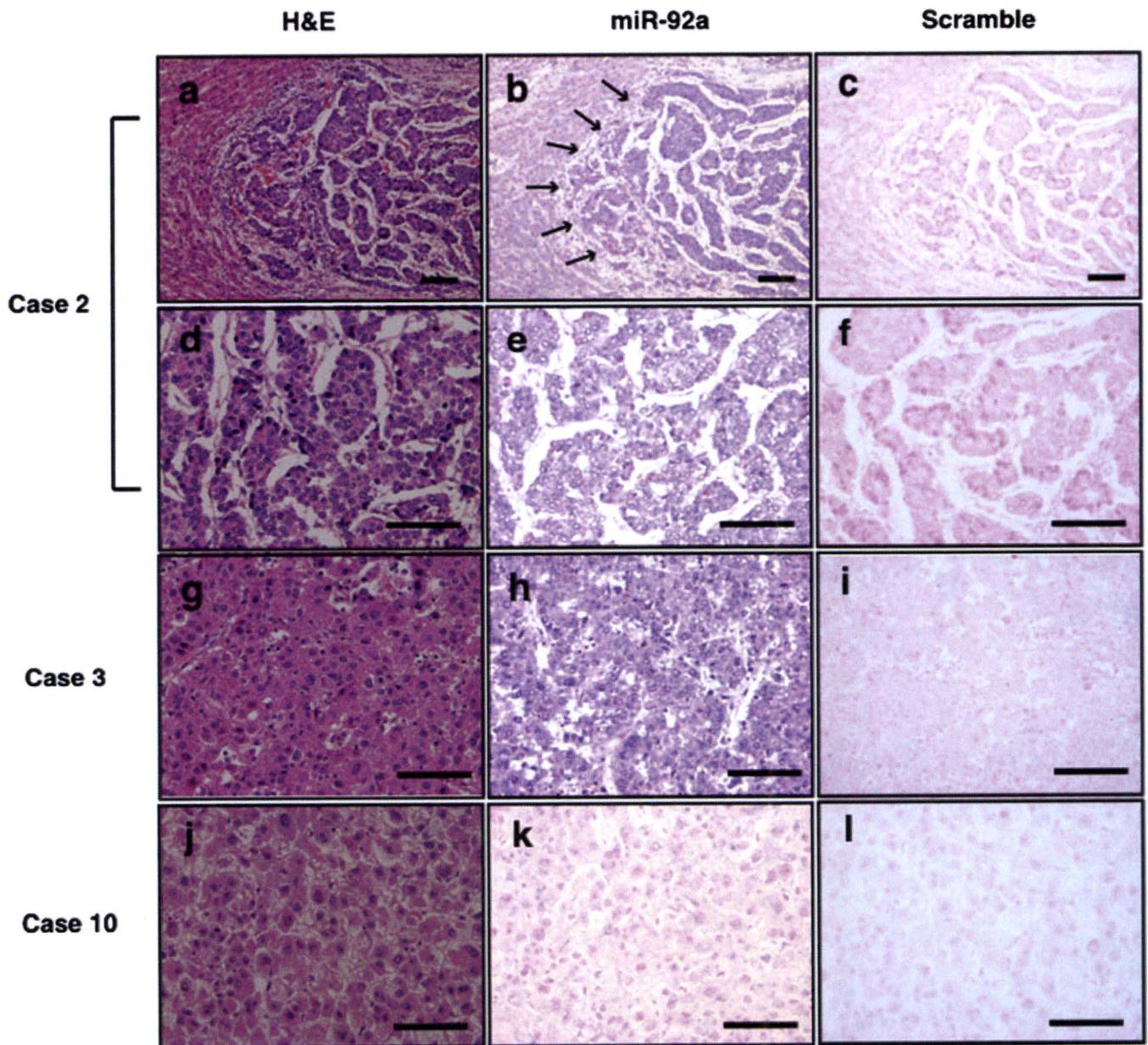


Figure 1 MiRNA expression in hepatocellular carcinoma (HCC). *In situ* hybridization was performed using Locked nucleic acid (LNA)-modified probes for miR-92a and negative control. Case 2 and Case 3 were positive cases for miR-92a. Case 10 was a negative case for miR-92a. (a–c) Low power field of boundary of HCC and non-tumor lesion. Arrowheads indicated a border. Only HCC regions were positive for miR-92a. (d–l) High power field of HCC. Blue signals represent positive for miR-92a. Bars indicate 100 μ m.

plasma was isolated using Isogen-LS (NIPPON GENE, Tokyo, Japan) according to the manufacturer's instructions. The RNA sample was suspended in 20 μ L of nuclease free water. In general, we obtained 400 ng of RNA from 1 mL of plasma. MiRNAs were quantified using TaqMan MiRNA Assays (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA) as previously described.¹³

For miR-92a quantification in tissue samples, five pairs of fresh HCC and non-tumorous LC samples were surgically resected from HCC patients (Table 2). All the patients or their

guardians provided written informed consent, and the Ethics Committee of the Kyoto University Graduate School and Faculty of Medicine approved all aspects of this study. The amounts of miR-92a were normalized to RNU48 that is one of rRNAs (Applied Biosystems).

Cell culture and transfection

Hepatocellular carcinoma (HCC) cell lines HepG2, OR6 and SN1a were cultured in Dulbecco's modified Eagle's medium

(DMEM) (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS). OR6 and SN1a are derived from the Huh7 HCC cell line and maintain hepatitis C virus (HCV) replicon.^{14–16} The miR-92a oligonucleotide used in the transfection experiments is a synthetic double-strand 19 nucleotide RNA oligonucleotide (5'-UUGCACUUGUCCC GGCCUG-3') purchased from B-Bridge International (Tokyo, Japan). The scrambled oligonucleotide represents a mix of two different frames of the miR-92 sequence (5'-UAUUGC ACUUGUCCC GGCCUGUCCC GGCC-3' and 5'-AUUGCAC UUGUCCC GGCCUTT-3'). Locked nucleic acid (LNA) oligonucleotide miR-92 knockdown (antagomir) was obtained from Exiqon (Vedbaek, Denmark, <http://www.exiqon.com>). The oligonucleotides were individually transfected by HiperFect (QIAGEN K. K., Tokyo, Japan) into the cells at a final concentration of 100 nM.

In vitro proliferation assays

The effects of miR-92a and the anti-miR-92a antagomir on the growth of HepG2, OR6 and SN1a were evaluated using the MTT cell growth assay kit (Cell Count Reagent SF, Nacalai tesque, Kyoto, Japan). The cells were transfected with miR-92a or the antagomir. The cell numbers were then assessed with MTT assay at 48 or 72 h after the transfection. The MTT assay was performed according to the manufacturer's recommendation. The reagents were added to each well and incubated at 37°C for 4 h. The MTT reduced by living cells into a formazan product was assayed with a multiwell scanning spectrophotometer at 450 nm.

RESULTS

Highly expression of miR-92a in HCC cells

We first examined whether or not miR-92a is expressed in hepatocellular carcinoma (HCC). We performed *in situ* hybridization using locked nucleic acid (LNA)-modified probes digoxigenin (DIG) labelled. We found that miR-92a was strongly expressed in cancer cells of 17 out of 22 HCC cases (Table 1 and Fig. 1). No significant differences were observed in age, sex, virus type, clinical stage and tumor differentiation of the clinical samples. In contrast, we did not detect miR-92a expression in non-cancerous hepatocytes around the HCCs.

Furthermore, we quantified miR-92a levels in HCC sections ($n = 5$) and their adjacent non-tumorous liver cirrhosis (LC) sections ($n = 5$) by TaqMan qRT-PCR (Table 2 and Fig. 2). The levels of miR-92a expression in HCC sections were higher than that in adjacent LC sections (Fig. 2).

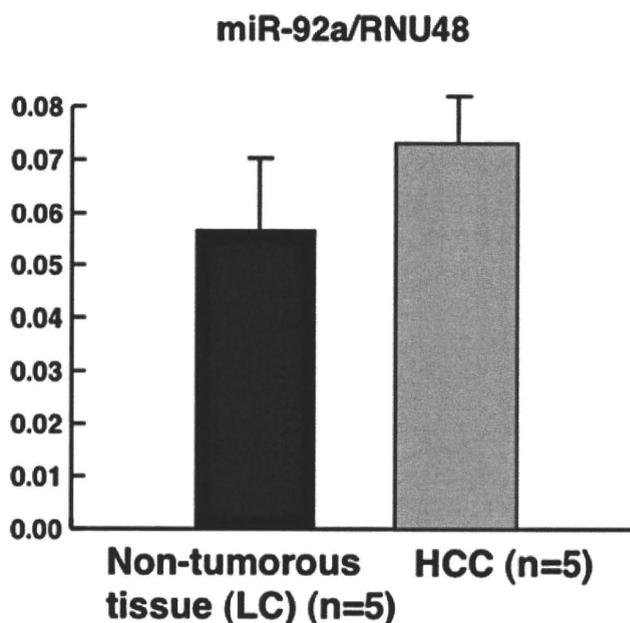


Figure 2 Quantification of miR-92a expression in hepatocellular carcinoma (HCC) tissue samples. The ratios of miR-92a to RNU48 in HCC tissues and their adjacent non-tumorous liver cirrhosis (LC) tissues were analyzed by TaqMan qRT-PCR. Bars, s.d.

Effects of miR-92a on a Hepatoma cell lines HepG2, OR6 and SN1a

Next, we investigated whether miR-92a affects cell proliferation of human HCC cell lines, HepG2, OR6 and SN1a. We transiently transfected either miR-92a or the anti-miR-92a antagomir into the cells. Antagomirs are single-stranded RNAs that are complementary to a specific miRNA and cause the depletion of the miRNA.¹⁷ After the transfection, we found that all of the cells transfected with the anti-miR-92a antagomir showed lower proliferation rate than the cells transfected with a control RNA oligonucleotide (Fig. 3a). In contrast, the cells except for HepG2 showed increased proliferation rate when miR-92a was transfected (Fig. 3a). We also confirmed the amounts of miR-92a in the cells by quantitative real time PCR (Fig. 3b).

The ratio of miR-92a to miR-638 serves as a biomarker for HCC

Finally, we sought to determine whether the expression level of miR-92a in blood sera could discriminate HCC patients from healthy individuals. Previously, we have revealed that miR-92a is dramatically reduced in the plasmas of acute leukemia patients although in leukemic cells it is strongly expressed.¹³ We analyzed the miR-92a levels in the plasma samples from normal individuals ($n = 10$) and HCC patients

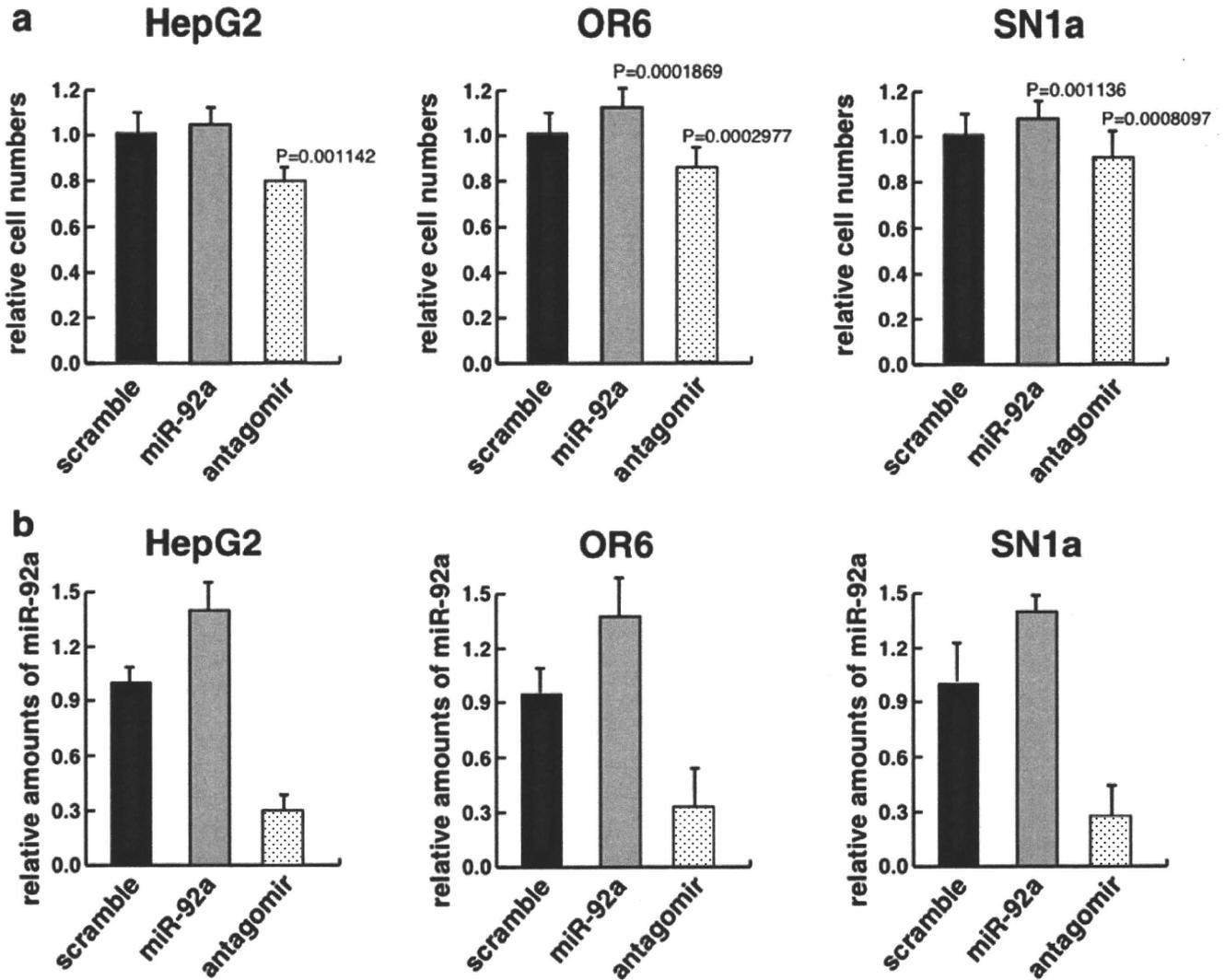


Figure 3 miR-92a modulates proliferation of HepG2, OR6 and SN1a cells. (a) Cell numbers of the HepG2, OR6 and SN1a cells transfected with synthetic miR-92a, anti-miR-92a antagomir, or scrambled control oligonucleotide were analyzed by MTT assays at 48 h for OR6 and SN1a and 72 h for HepG2 after transfection. Bars, s.d. (b) qRT-PCR analysis of miR-92a amounts in the cells transfected with miR-92a, anti-miR-92a antagomir or scrambled control at 48 h for OR6 and SN1a and 72 h for HepG2 after the transfection.

($n = 10$) by TaqMan qRT-PCR. Because miR-638 is stably present in human plasmas,¹³ we used miR-638 as the standard to improve the precision of the data. The ratio of miR-92a to miR-638 in the plasma samples from the HCC patients were decreased compared with that from the normal donors (Fig. 4a). Then, we further examined the ratio from the patients after surgical resection. Interestingly, the miR-92a/miR-638 levels were significantly higher than that in the plasmas from the patients before surgical resection (Fig. 4b).

DISCUSSION

In this study, we found that miR-92a was highly expressed in HCC (Figs 1,2). In addition, we demonstrated that the

expression level of miR-92a affects the proliferation of hepatoma cell lines, HepG2, OR6 and SN1a (Fig. 3). These results suggest that miR-92a may play an important role in tumor progression of hepatocyte. We do not know why, but addition of miR-92a did not significantly increase the proliferation of HepG2 cells. It may be possible that HepG2 cells themselves already contain enough miR-92a to promote cancer cell proliferation. In addition, miR-92a is a part of the miR-17-92 cluster, which is actively involved in the development and progression of various cancers.⁴⁻¹⁰ However, the molecular function of miR-92a is still unknown, and its mRNA targets have not been identified. Recently, it has been shown that one of the molecular mechanisms through which miR-92a increases cell proliferation is by negative regulation of an isoform of the cell-cycle regulator p63.¹¹ Thus, we examined