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-alpha to treat chronic hepatitis C.

**Acknowledgments** The authors thank Kazue Yoshihara and Yoshiko Kubushiro for their technical assistance. This work was supported by a grant for the 2008 Strategic Research Project (No. W20012) provided by Yokohama City University, Japan.

## References

1. Adams RL, Lindsay JG (1967) Hydroxyurea reversal of inhibition and use as a cell-synchronizing agent. J Biol Chem 242:1314–1317
2. Bader T, Fazili J, Madhoun M, Aston C, Hughes D, Rizvi S, Seres K, Hasan M (2008) Fluvastatin inhibits hepatitis C replication in humans. Am J Gastroenterol 103:1383–1389
3. Belt RJ, Haas CD, Kennedy J, Taylor S (1980) Studies of hydroxyurea administered by continuous infusion: toxicity, pharmacokinetics, and cell synchronization. Cancer 46:455–462
4. Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M (1989) Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244:359–362
5. Foli A, Maiocchi MA, Lisziewicz J, Lori F (2007) A checkpoint in the cell cycle progression as a therapeutic target to inhibit HIV replication. J Infect Dis 196:1409–1415
6. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Gonçales FLJ, Häussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J (2002) Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 347:975–982
7. Garcia M, Yu XF, Griffin DE, Moss WJ (2008) Measles virus inhibits human immunodeficiency virus type 1 reverse transcription and replication by blocking cell-cycle progression of $CD4^+$ T lymphocytes. J Gen Virol 89:984–993
8. Hasford J, Baccarani M, Hehlmann R, Anseri H, Tura S, Zuffa E (1996) Interferon-alpha and hydroxyurea in early chronic myeloid leukemia: a comparative analysis of the Italian and German chronic myeloid leukemia trials with interferon-alpha. Blood 87:5384–5391
9. Ikeda M, Abe K, Dansako H, Nakamura T, Naka K, Kato N (2005) Efficient replication of a full-length hepatitis C virus genome, strain O, in cell culture, and development of a luciferase reporter system. Biochem Biophys Res Commun 329:1350–1359
10. Ikeda M, Abe K, Yamada M, Dansako H, Naka K, Kato N (2006) Different anti-HCV profiles of statins and their potential for combination therapy with interferon. Hepatology 44:117–125
11. Ikeda M, Kato N (2007) Modulation of host metabolism as a target of new antivirals. Adv Drug Deliv Rev 59:1277–1289
12. Kuo G, Choo QL, Alter HJ, Gitnick GL, Redeker AG, Purcell RH, Miyamura T, Dienstag JL, Alter MJ, Stevens CE, Tegtmeier GE, Bonino F, Colombo WS, Lee WS, Kuo C, Berger K, Shuster JR, Overby LR, Bradley DW, Houghton M (1989) An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science 244:362–364
13. Lori F, Malykh A, Cara A, Sun D, Weinstein JN, Lisziewicz J, Gallo RC (1994) Hydroxyurea as an inhibitor of human immunodeficiency virus-type 1 replication. Science 266:801–805
14. Lori F, Jessen H, Lieberman J, Finzi D, Rosenberg E, Tinelli C, Walker B, Siliciano RF, Lisziewicz J (1999) Treatment of human immunodeficiency virus infection with hydroxyurea, didanosine, and a protease inhibitor before seroconversion is associated with normalized immune parameters and limited viral reservoir. J Infect Dis 180:1827–1832
15. Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK (2001) Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet 358:958–965
16. Mori K, Abe K, Dansako H, Ariumi Y, Ikeda M, Kato N (2008) New efficient replication system with hepatitis C virus genome derived from a patient with acute hepatitis C. Biochem Biophys Res Commun 371:104–109
17. Naka K, Ikeda M, Abe K, Dansako H, Kato N (2005) Mizoribine inhibits hepatitis C virus RNA replication: effect of combination with interferon-$\alpha$. Biochem Biophys Res Commun 330:871–879
18. Pietschmann T, Lohmann V, Rutter G, Kurpanek K, Bartenschlager R (2001) Characterization of cell lines carrying self-replicating hepatitis C virus RNAs. J Virol 75:1252–1264
19. Saito I, Miyamura T, Ohbayashi A, Harada H, Katayama T, Kikuchi S, Watanabe Y, Koi S, Onji M, Ohta Y, Choo QL, Houghton M, Kuo G (1990) Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proc Natl Acad Sci USA 87:6547–6549
20. Sergerie Y, Boivin G (2008) Hydroxyurea enhances the activity of acyclovir and cidofovir against herpes simplex virus type 1 resistant strains harboring mutations in the thymidine kinase and/or the DNA polymerase genes. Antiviral Res 77:77–80
21. Tamura T, Matsuzaki M, Harada H, Ogawa K, Mohri H, Okubo T (1997) Upregulation of interferon-alpha receptor expression in hydroxyurea-treated leukemia cell lines. J Investig Med 45:160–167
22. Tanabe Y, Sakamoto N, Enomoto N, Kurosaki M, Ueda E, Maekawa S, Yamashiro T, Nakagawa M, Chen CH, Kanazawa N, Kakinuma S, Watanabe M (2004) Synergistic inhibition of intracellular hepatitis C virus replication by combination of ribavirin and interferon-alpha. J Infect Dis 189:1129–1139
23. The Benelux CML, Group Study (1998) Randomized study on hydroxyurea alone versus hydroxyurea combined with low-dose interferon-alpha 2b for chronic myeloid leukemia. Blood 91:2713–2721
24. Thomas DL (2000) Hepatitis C epidemiology. Curr Top Microbiol Immunol 242:25–41
25. Watashi K, Hijikata M, Hosaka M, Yamaji M, Shimotohno K (2003) Cyclosporin A suppresses replication of hepatitis C virus genome in cultured hepatocytes. Hepatology 38:1282–1288
26. Watashi K, Ishii N, Hijikata M, Inoue D, Murata T, Miyanari Y, Shimotohno K (2005) Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. Mol Cell 19:111–122

Antiviral Therapy 2010; 15:1179–1183 (doi: 10.3851/IMP1668)

# Short communication

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

*Akito Nozaki[1,2]*, Kazushi Numata[1], Manabu Morimoto[1], Masaaki Kondo[1], Kazuya Sugimori[1], Satoshi Morita[3], Eiji Miyajima[4], Masanori Ikeda[5], Nobuyuki Kato[5], Shin Maeda[6], Katsuaki Tanaka[1]*

[1]Gastroenterological Center, Yokohama City University Medical Center, Yokohama, Japan
[2]Department of Transfusion Medicine, Yokohama City University Medical Center, Yokohama, Japan
[3]Department of Biostatistics and Epidemiology, Yokohama City University Medical Center, Yokohama, Japan
[4]Department of Laboratory Medicine and Clinical Investigation, Yokohama City University Medical Center, Yokohama, Japan
[5]Department of Tumor Virology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan
[6]Department of Gastroenterology, Yokohama City University Graduate School of Medicine, Yokohama, Japan

*Corresponding author e-mail: akino@yokohama-cu.ac.jp

**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/m$^2$ 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 |

[a]Metavir score. [b]Presented as the maximal HCV RNA decrease in $\log_{10}$ IU/ml (HCV RNA range) per number of treatment weeks (W) shown. [c]HCV RNA decrease range. F, female; HU, hydroxyurea; IFN, interferon; M, male; PEG-IFN, pegylated interferon; RBV, ribavirin.

50% effective concentration ($EC_{50}$), 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/μl, absolute platelet count >70,000 cells/μl 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-α2b 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/μl, absolute leukocyte count <1,500 cells/μl and absolute platelet count <50,000 cells/μl 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).

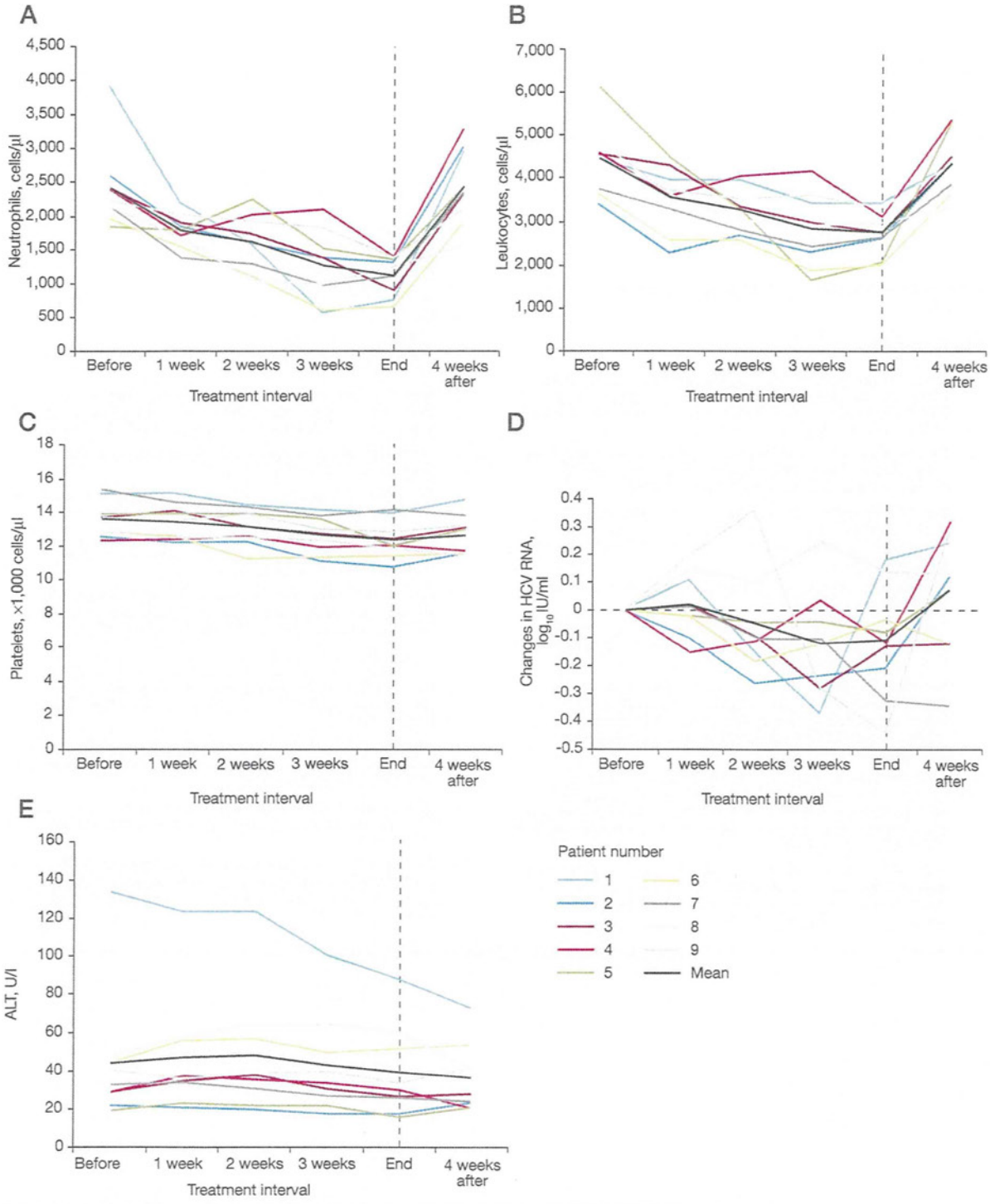
## Discussion

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 μmol/l with a 50% cytotoxic dose of 150–200 μmol/l) was considerably lower than the clinically achievable concentration (approximately 2,480 μmol/l) [9]. Furthermore, in this trial, the mean plasma concentration of HU was estimated as 272–990 μmol/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.

## Acknowledgements

The authors thank Yuko Ishida for her assistance. This work was supported by a grant from the 2008 Strategic Research Project (number W20012; Yokohama City University, Yokohama, Japan).

## Disclosure statement

The authors declare no competing interests.

## References

1. Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 1989; 244:359–362.
2. Kuo G, Choo QL, Alter HJ, *et al.* An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. *Science* 1989; 244:362–364.
3. Saito I, Miyamura T, Ohbayashi A, *et al.* Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc Natl Acad Sci U S A* 1990; 87:6547–6549.
4. Thomas DL. Hepatitis C epidemiology. *Curr Top Microbiol Immunol* 2000; 242:25–41.
5. Fried MW, Shiffman ML, Reddy KR, *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; 347:975–982.
6. Manns MP, McHutchison JG, Gordon SC, *et al.* Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; 358:958–965.
7. Ikeda M, Abe K, Dansako H, Nakamura T, Naka K, Kato N. Efficient replication of a full-length hepatitis C virus genome, strain O, in cell culture, and development of a luciferase reporter system. *Biochem Biophys Res Commun* 2005; 329:1350–1359.
8. Naka K, Ikeda M, Abe K, Dansako H, Kato N. Mizoribine inhibits hepatitis C virus RNA replication: effect of combination with interferon-α. *Biochem Biophys Res Commun* 2005; 330:871–879.
9. Nozaki A, Morimoto M, Kondo M, *et al.* Hydroxyurea as an inhibitor of hepatitis C virus RNA replication. *Arch Virol* 2010; 155:601–605.
10. Zala C, Rouleau D, Montaner JS. Role of hydroxyurea in treatment of disease due to human immunodeficiency virus infection. *Clin Infect Dis* 2000; 30 Suppl 2:S143–S150.
11. Lori F, Malykh A, Cara A, *et al.* Hydroxyurea as an inhibitor of human immunodeficiency virus-type 1 replication. *Science* 1994; 266:801–805.
12. Sergerie Y, Boivin G. Hydroxyurea enhances the activity of acyclovir and cidofovir against herpes simplex virus type 1 resistant strains harboring mutations in the thymidine kinase and/or the DNA polymerase genes. *Antiviral Res* 2008; 77:77–80.
13. Lori F, Jessen H, Lieberman J, *et al.* Treatment of human immunodeficiency virus infection with hydroxyurea, didanosine, and a protease inhibitor before seroconversion is associated with normalized immune parameters and limited viral reservoir. *J Infect Dis* 1999; 180:1827–1832.
14. Belt RJ, Haas CD, Kennedy J, Taylor S. Studies of hydroxyurea administered by continuous infusion: toxicity, pharmacokinetics, and cell synchronization. *Cancer* 1980; 46:455–462.
15. Bachir D, Hulin A, Huet E, *et al.* Plasma and urine hydroxyurea levels might be useful in the management of adult sickle cell disease. *Hemoglobin* 2007; 31:417–425.
16. Berry DA. Monitoring accumulating data in a clinical trial. *Biometrics* 1989; 45:1197–1211.
17. Berry DA. A case for Bayesianism in clinical trials. *Stat Med* 1993; 12:1377–1393.
18. Saban N, Bujak M. Hydroxyurea and hydroxamic acid derivatives as antitumor drugs. *Cancer Chemother Pharmacol* 2009; 64:213–221.
19. Foli A, Maiocchi MA, Lisziewicz J, Lori F. A checkpoint in the cell cycle progression as a therapeutic target to inhibit HIV replication. *J Infect Dis* 2007; 196:1409–1415.
20. Garcia M, Yu XF, Griffin DE, Moss WJ. Measles virus inhibits human immunodeficiency virus type 1 reverse transcription and replication by blocking cell-cycle progression of $CD4^+$ T lymphocytes. *J Gen Virol* 2008; 89:984–993.
21. Watashi K, Ishii N, Hijikata M, *et al.* Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. *Mol Cell* 2005; 19:111–122.
22. Di Bisceglie AM, Shindo M, Fong TL, *et al.* A pilot study of ribavirin therapy for chronic hepatitis C. *Hepatology* 1992; 16:649–654.
23. Reichard O, Yun ZB, Sönnerborg A, Weiland O. Hepatitis C viral RNA titers in serum prior to, during, and after oral treatment with ribavirin for chronic hepatitis C. *J Med Virol* 1993; 41:99–102.

Accepted 18 May 2010; published online 6 October 2010

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/1007-9327office
wjg@wjgnet.com
doi:10.3748/wjg.v16.i2.184

*World J Gastroenterol* 2010 January 14; 16(2): 184-192
ISSN 1007-9327 (print)


ORIGINAL ARTICLE

# An antioxidant resveratrol significantly enhanced replication of hepatitis C virus

Mitsuyasu Nakamura, Hidetsugu Saito, Masanori Ikeda, Ryota Hokari, Nobuyuki Kato, Toshifumi Hibi, Soichiro Miura

Mitsuyasu Nakamura, Ryota Hokari, Soichiro Miura, Department of Internal Medicine 2, National Defense Medical Collage, 3-2 Namiki, Tokorozawa, Saitama 3598513, Japan
Mitsuyasu Nakamura, Hidetsugu Saito, Toshifumi Hibi, Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 1608582, Japan
Masanori Ikeda, Nobuyuki Kato, Department of Molecular Biology, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikada-cho, Okayama 7008558, Japan
Author contributions: Nakamura M performed the majority of experiments; Ikeda M and Kato N provided reagents and were involved in editing the manuscript; Hokari R, Hibi T and Miura S provided financial support for this work; Saito H designed the study, provided financial support and wrote the manuscript.
Supported by The grant from the Japanese Ministry of Education and Science
Correspondence to: Hidetsugu Saito, MD, PhD, Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 1608582, Japan. hsaito@sc.itc.keio.ac.jp
Telephone: +81-3-33531211 Fax: +81-3-33518705
Received: September 25, 2009 Revised: November 5, 2009
Accepted: November 12, 2009
Published online: January 14, 2010

## 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 didnot 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 didnot affect antiviral effects of IFN or RBV.

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



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

**Peer reviewer:** Yutaka Yata, MD, PhD, Director, Department of Gastroenterology, Saiseikai Maebashi Hospital, 564-1 Kamishinden-machi, Maebashi-city, Gunma 371-0821, Japan

Nakamura M, Saito H, Ikeda M, Hokari R, Kato N, Hibi T, Miura S. An antioxidant resveratrol significantly enhanced replication of hepatitis C virus. *World J Gastroenterol* 2010; 16(2): 184-192 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i2/184.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i2.184

## 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 didnot 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 lead 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, antiatherosclerosis 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-b-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℃ until used. This stock solution was diluted with culture medium. The final

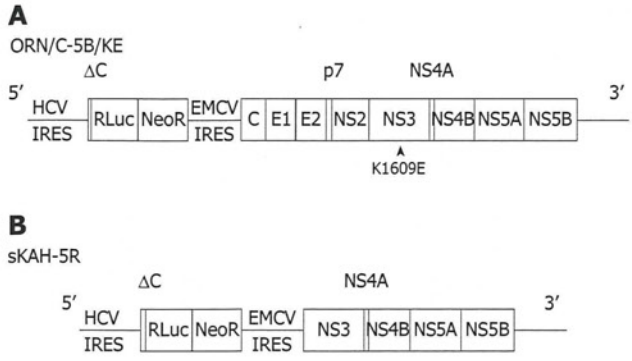



**Figure 1 Organization of hepatitis C virus (HCV) RNA used in the replicon systems.** A: Genome-length HCV RNA generated in OR6 cells; B: Subgenomic HCV RNA used in sKAH-5R and s1B-4R cells. Structural region of HCV RNA was deficient in the replicated RNA of sKAH-5R and s1B-4R cells.

concentration of DMSO was 0.2%, which didnot interfere with viral replication, the highest concentration of RVT used in this study was 100 μmol/L, that of AXN was 50 μmol/L, and that of VE was 15 μmol/L. To monitor the anti-HCV effects of IFN and RBV on replication, OR6 cells ($1.5 \times 10^4$ /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 μmol/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 RVB, RVT and AXN on the antiviral effect of IFN (1 U) was studied and compared using luciferase activity.

### *Luciferase reporter assay*

Approximately 1 to $4.5 \times 10^4$ 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 μmol/L) and AXN (1-50 μmol/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 $2 \times 10^5$ cells per dish onto 6-well plates. After 24 h culture, the cells were treated with RVT at final concentrations of 25 and 50 μmol/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 mol/L (Figure 2A). The activity gradually decreased at higher concentrations, but at 100 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 didnot increase OR6 cells until 15 μmol/L, and the cell viability decreased at higher concentrations than 20 μmol/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 μmol/L when estimated by the number of viable cells. Different from the effect of RVT, AXN didnot enhance luciferase activity (Figure 2C). The luciferase activity decreased at a concentration more than 10 μmol/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 μmol/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 μmol/L (Figure 4A). This concentration is not toxic to the sKAH-5R cells, but more than 20 μmol/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

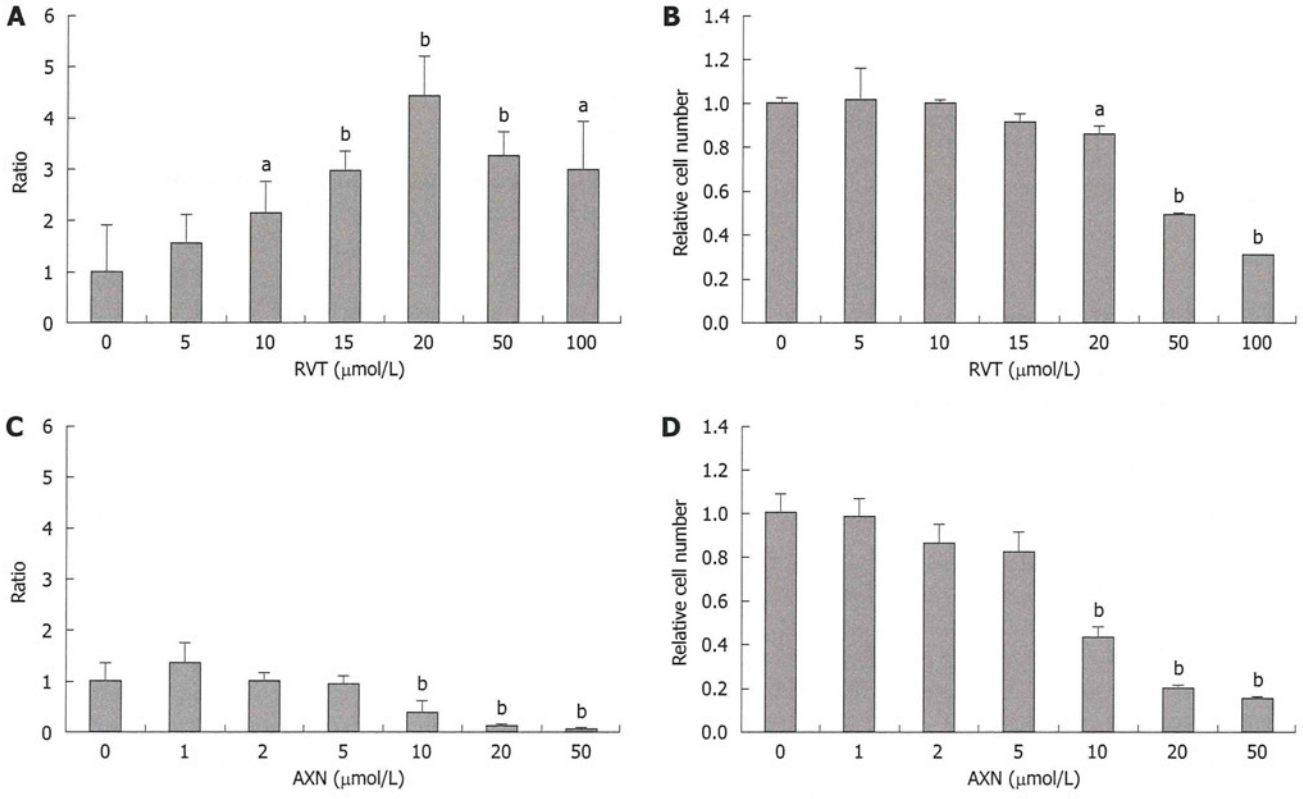



**Figure 2 Luciferase activity and cell viability of OR6 cells after addition of resveratrol (RVT) and astaxanthin (AXN) for 72 h.** The indicated concentrations of RVT or AXN were added to the culture medium of OR6 cells, and after 72 h of culture, cells were harvested with Runilla lysis buffer and 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. $^{a}P < 0.05$, $^{b}P < 0.01$.

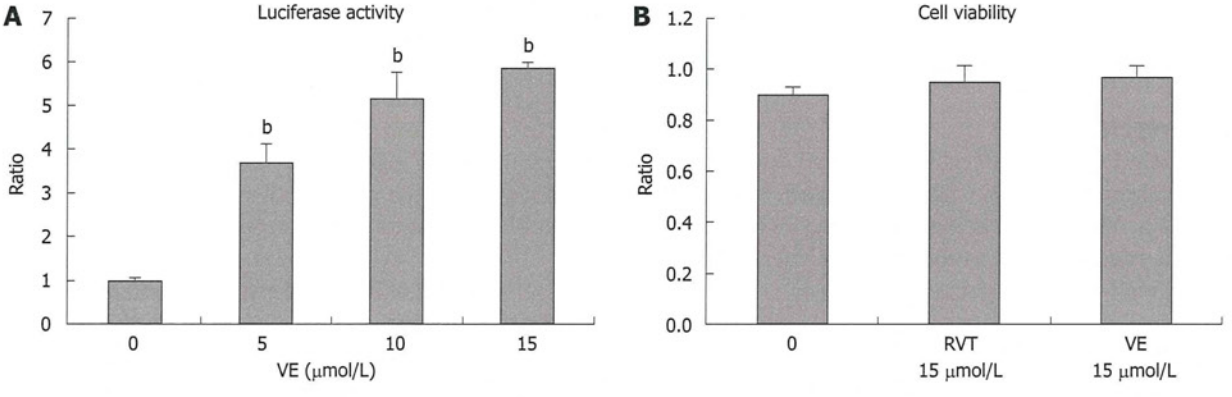



**Figure 3 The effect of vitamin E (VE) on luciferase activity and cell viability of OR6 cells (72 h).** Cells were treated with the indicated concentrations of VE for 72 h. A: Luciferase activity after addition of VE; B: Cell viability after addition of RVT and VE. $^{b}P < 0.01$.

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 didnot 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

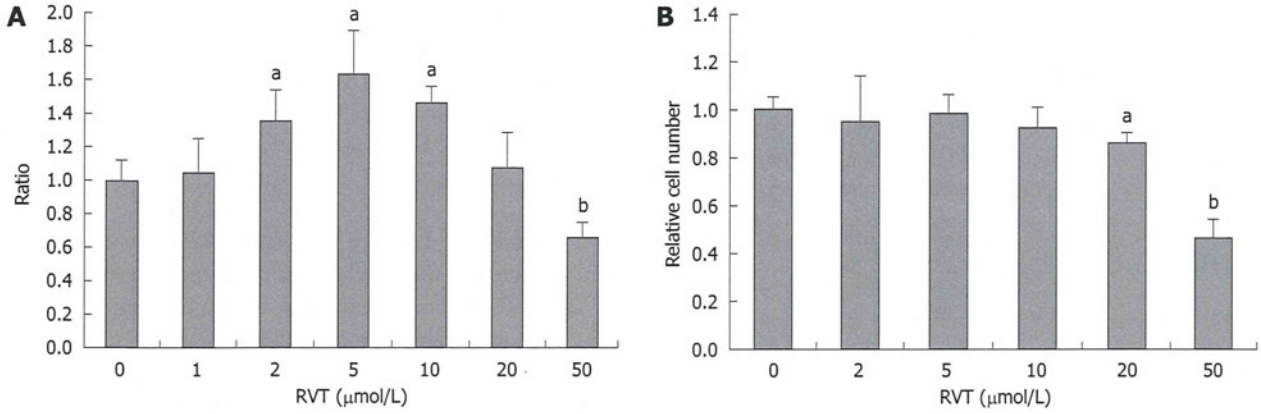



**Figure 4 The effect of resveratrol on subgenomic replicon cells, sKAH-5R.** RVT was added to the cells for 72 h and luciferase activity was assayed as was indicated in Figure 2. A: Luciferase activity after addition of RVT; B: Cell viability after addition of RVT. $^{a}P < 0.05$, $^{b}P < 0.01$.

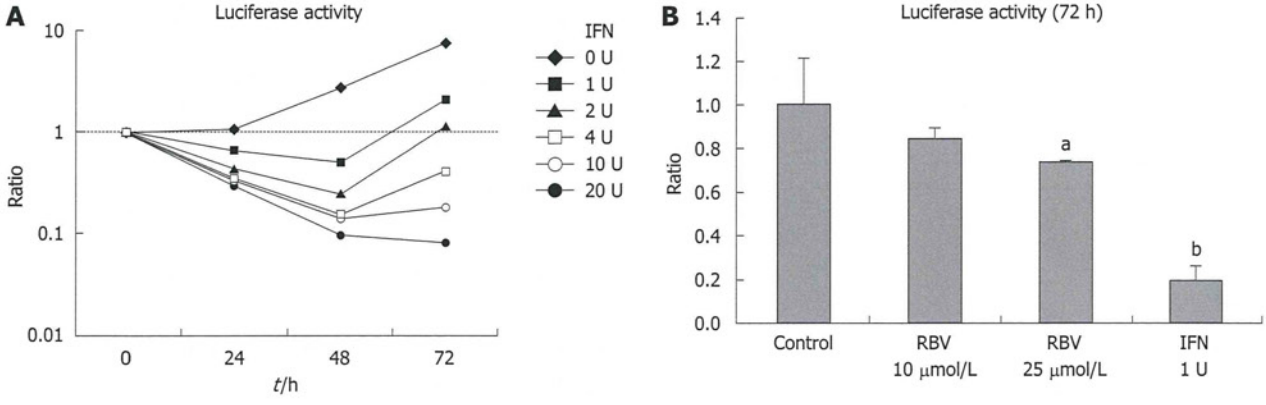



**Figure 5 Sequential change of luciferase activity of OS6 cells after addition of interferon (IFN).** A: IFN was added for the indicated duration and the ratio to the luciferase activity at time 0 was shown. Statistical significance ($^{a}P < 0.05$, $^{b}P < 0.01$) is shown below: at 24 h 0 *vs* $1^{a}$,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

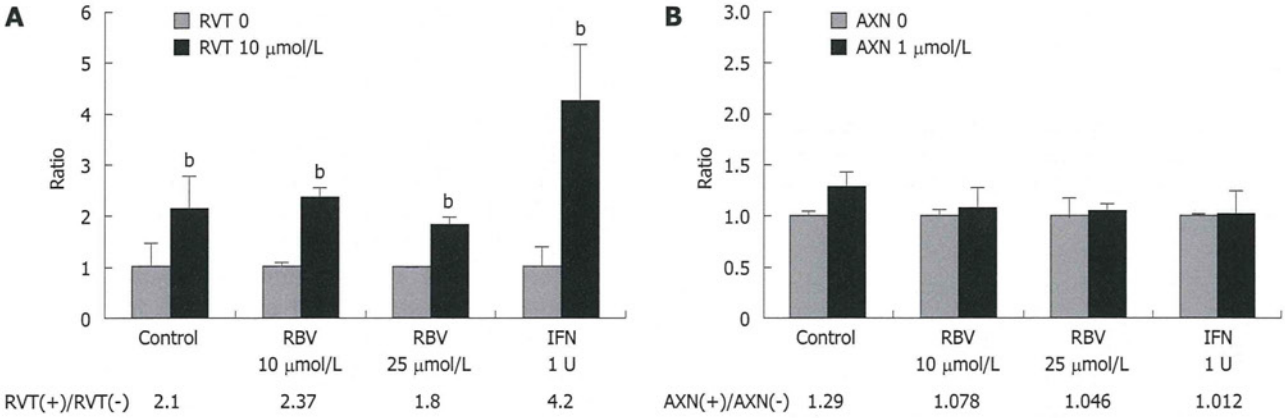



**Figure 6 The enhancing effect of RVT on replication of HCV RNA, even when treated with ribavirin (RBV) and IFN.** RVT was simultaneously added to RBV or IFN. A: The ratio was calculated as the ratio of luciferase activity with RVT to that without RVT. $^{b}P < 0.01$ *vs* RVT 0; B: The same procedure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

and 4.2-fold, respectively (Figure 6A). On the other hand, AXN didnot 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

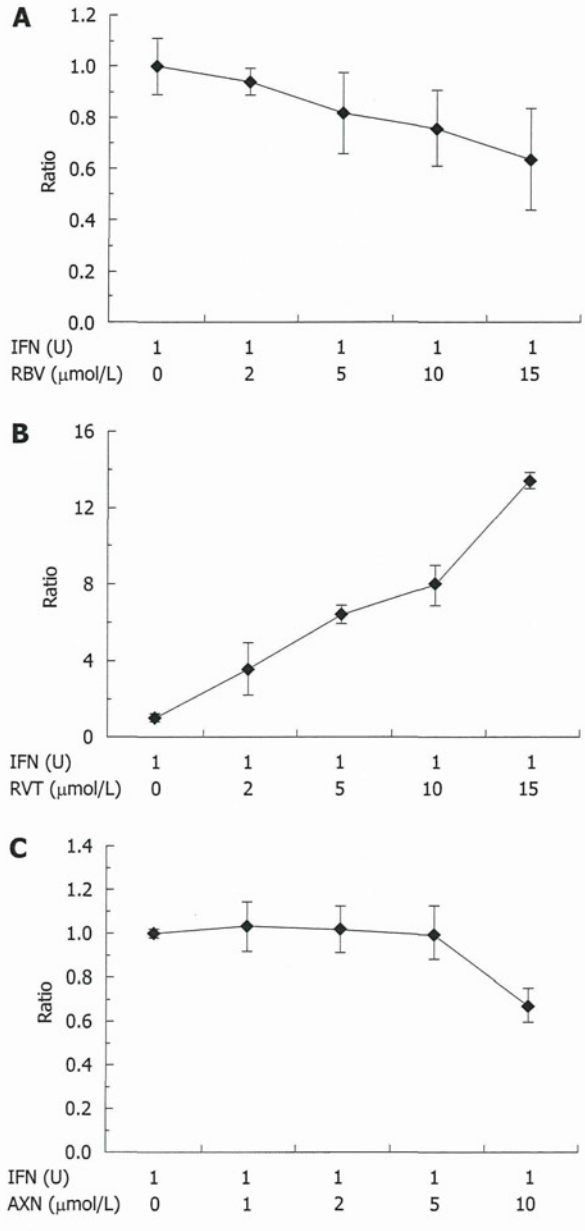



**Figure 7 The comparative effect of RBV (A), RVT (B) and AXN (C) on the luciferase activity of IFN-treated cells.** Cells were simultaneously treated with 1 U/mL of IFN and the indicated concentrations of RBV, RVT and AXN were added with IFN.

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 didnot 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 didnot 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 didnot 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 didnot 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 medical 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 $D_2$ 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 didnot 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-hydroxyl-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.

## REFERENCES


1 **Jain SK**, Pemberton PW, Smith A, McMahon RF, Burrows PC, Aboutwerat A, Warnes TW. Oxidative stress in chronic hepatitis C: not just a feature of late stage disease. *J Hepatol* 2002; **36**: 805-811

2 **Koike K**, Miyoshi H. Oxidative stress and hepatitis C viral infection. *Hepatol Res* 2006; **34**: 65-73

3 **Melhem A**, Stern M, Shibolet O, Israeli E, Ackerman Z, Pappo O, Hemed N, Rowe M, Ohana H, Zabrecky G, Cohen R, Ilan Y. Treatment of chronic hepatitis C virus infection via antioxidants: results of a phase I clinical trial. *J Clin Gastroenterol* 2005; **39**: 737-742

4 **Gabbay E**, Zigmond E, Pappo O, Hemed N, Rowe M, Zabrecky G, Cohen R, Ilan Y. Antioxidant therapy for chronic hepatitis C after failure of interferon: results of phase II randomized, double-blind placebo controlled clinical trial. *World J Gastroenterol* 2007; **13**: 5317-5323

5 **Manns MP**, Foster GR, Rockstroh JK, Zeuzem S, Zoulim F, Houghton M. The way forward in HCV treatment--finding the right path. *Nat Rev Drug Discov* 2007; **6**: 991-1000

6 **Kieffer TL**, Sarrazin C, Miller JS, Welker MW, Forestier N, Reesink HW, Kwong AD, Zeuzem S. Telaprevir and pegylated interferon-alpha-2a inhibit wild-type and resistant genotype 1 hepatitis C virus replication in patients. *Hepatology* 2007; **46**: 631-639

7 **Coon JT**, Ernst E. Complementary and alternative therapies in the treatment of chronic hepatitis C: a systematic review. *J Hepatol* 2004; **40**: 491-500

8 **Seeff LB**, Lindsay KL, Bacon BR, Kresina TF, Hoofnagle JH. Complementary and alternative medicine in chronic liver disease. *Hepatology* 2001; **34**: 595-603

9 **Brooks CL**, Gu W. How does SIRT1 affect metabolism, senescence and cancer? *Nat Rev Cancer* 2009; **9**: 123-128

10 **Sun AY**, Simonyi A, Sun GY. The "French Paradox" and beyond: neuroprotective effects of polyphenols. *Free Radic Biol Med* 2002; **32**: 314-318

11 **Baur JA**, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A, Prabhu VV, Allard JS, Lopez-Lluch G, Lewis K, Pistell PJ, Poosala S, Becker KG, Boss O, Gwinn D, Wang M, Ramaswamy S, Fishbein KW, Spencer RG, Lakatta EG, Le Couteur D, Shaw RJ, Navas P, Puigserver P, Ingram DK, de Cabo R, Sinclair DA. Resveratrol improves health and survival of mice on a high-calorie diet. *Nature* 2006; **444**: 337-342

12 **Docherty JJ**, Sweet TJ, Bailey E, Faith SA, Booth T. Resveratrol inhibition of varicella-zoster virus replication in vitro. *Antiviral Res* 2006; **72**: 171-177

13 **Evers DL**, Wang X, Huong SM, Huang DY, Huang ES. 3,4',5-Trihydroxy-trans-stilbene (resveratrol) inhibits human cytomegalovirus replication and virus-induced cellular signaling. *Antiviral Res* 2004; **63**: 85-95

14 **Faith SA**, Sweet TJ, Bailey E, Booth T, Docherty JJ. Resveratrol suppresses nuclear factor-kappaB in herpes simplex virus infected cells. *Antiviral Res* 2006; **72**: 242-251

15 **Palamara AT**, Nencioni L, Aquilano K, De Chiara G, Hernandez L, Cozzolino F, Ciriolo MR, Garaci E. Inhibition of influenza A virus replication by resveratrol. *J Infect Dis* 2005; **191**: 1719-1729

16 **Barros MP**, Pinto E, Colepicolo P, Pedersén M. Astaxanthin and peridinin inhibit oxidative damage in Fe(2+)-loaded liposomes: scavenging oxyradicals or changing membrane permeability? *Biochem Biophys Res Commun* 2001; **288**: 225-232

17 **Shen H**, Kuo CC, Chou J, Delvolve A, Jackson SN, Post J, Woods AS, Hoffer BJ, Wang Y, Harvey BK. Astaxanthin reduces ischemic brain injury in adult rats. *FASEB J* 2009; **23**: 1958-1968

18 **Aoi W**, Naito Y, Takanami Y, Ishii T, Kawai Y, Akagiri S, Kato Y, Osawa T, Yoshikawa T. Astaxanthin improves muscle lipid metabolism in exercise via inhibitory effect of oxidative CPT I modification. *Biochem Biophys Res Commun* 2008; **366**: 892-897

19 **Hussein G**, Nakagawa T, Goto H, Shimada Y, Matsumoto K, Sankawa U, Watanabe H. Astaxanthin ameliorates features of metabolic syndrome in SHR/NDmcr-cp. *Life Sci* 2007; **80**: 522-529

20 **Nakamura M**, Saito H, Hibi T. Advances in genomic research on hepatitis C virus with a useful tool, replicon system. *Keio J Med* 2008; **57**: 75-83

21 **Ikeda M**, Abe K, Dansako H, Nakamura T, Naka K, Kato N. Efficient replication of a full-length hepatitis C virus genome, strain O, in cell culture, and development of a luciferase reporter system. *Biochem Biophys Res Commun* 2005; **329**: 1350-1359

22 **Nakamura M**, Saito H, Ikeda M, Tada S, Kumagai N, Kato N, Shimotohno K, Hibi T. Possible molecular mechanism of the relationship between NS5B polymorphisms and early clearance of hepatitis C virus during interferon plus ribavirin treatment. *J Med Virol* 2008; **80**: 632-639

23 **Ikeda M**, Kato N, Mizutani T, Sugiyama K, Tanaka K, Shimotohno K. Analysis of the cell tropism of HCV by using in vitro HCV-infected human lymphocytes and hepatocytes. *J Hepatol* 1997; **27**: 445-454

24 **Yano M**, Ikeda M, Abe K, Dansako H, Ohkoshi S, Aoyagi Y, Kato N. Comprehensive analysis of the effects of ordinary nutrients on hepatitis C virus RNA replication in cell culture. *Antimicrob Agents Chemother* 2007; **51**: 2016-2027

25 **Docherty JJ**, Fu MM, Hah JM, Sweet TJ, Faith SA, Booth T. Effect of resveratrol on herpes simplex virus vaginal infection in the mouse. *Antiviral Res* 2005; **67**: 155-162

26 **Docherty JJ**, Fu MM, Stiffler BS, Limperos RJ, Pokabla CM, DeLucia AL. Resveratrol inhibition of herpes simplex virus replication. *Antiviral Res* 1999; **43**: 145-155

27 **Docherty JJ**, Smith JS, Fu MM, Stoner T, Booth T. Effect of topically applied resveratrol on cutaneous herpes simplex virus infections in hairless mice. *Antiviral Res* 2004; **61**: 19-26

28 **Heredia A**, Davis C, Redfield R. Synergistic inhibition of HIV-1 in activated and resting peripheral blood mononuclear cells, monocyte-derived macrophages, and selected drug-resistant isolates with nucleoside analogues combined with a natural product, resveratrol. *J Acquir Immune Defic Syndr* 2000; **25**: 246-255

29 **Guarente L**. Sirtuins as potential targets for metabolic syndrome. *Nature* 2006; **444**: 868-874

30 **Muriel P**, Rivera-Espinoza Y. Beneficial drugs for liver diseases. *J Appl Toxicol* 2008; **28**: 93-103

31 **Bujanda L**, García-Barcina M, Gutiérrez-de Juan V, Bidaurrazaga J, de Luco MF, Gutiérrez-Stampa M, Larzabal M, Hijona E, Sarasqueta C, Echenique-Elizondo M, Arenas JI. Effect of resveratrol on alcohol-induced mortality and liver lesions in mice. *BMC Gastroenterol* 2006; **6**: 35

32 **Sener G**, Toklu HZ, Sehirli AO, Velioğlu-Oğünç A, Cetinel S, Gedik N. Protective effects of resveratrol against acetaminophen-induced toxicity in mice. *Hepatol Res* 2006; **35**: 62-68

33 **Hassan-Khabbar S**, Cottart CH, Wendum D, Vibert F, Clot JP, Savouret JF, Conti M, Nivet-Antoine V. Postischemic treatment by trans-resveratrol in rat liver ischemia-reperfusion: a possible strategy in liver surgery. *Liver Transpl* 2008; **14**: 451-459

34 **Ahn J**, Cho I, Kim S, Kwon D, Ha T. Dietary resveratrol

alters lipid metabolism-related gene expression of mice on an atherogenic diet. *J Hepatol* 2008; **49**: 1019-1028

35 **Kawada N**, Seki S, Inoue M, Kuroki T. Effect of antioxidants, resveratrol, quercetin, and N-acetylcysteine, on the functions of cultured rat hepatic stellate cells and Kupffer cells. *Hepatology* 1998; **27**: 1265-1274

36 **Souza IC**, Martins LA, Coelho BP, Grivicich I, Guaragna RM, Gottfried C, Borojevic R, Guma FC. Resveratrol inhibits cell growth by inducing cell cycle arrest in activated hepatic stellate cells. *Mol Cell Biochem* 2008; **315**: 1-7

37 **Svegliati Baroni G**, D'Ambrosio L, Ferretti G, Casini A, Di Sario A, Salzano R, Ridolfi F, Saccomanno S, Jezequel AM, Benedetti A. Fibrogenic effect of oxidative stress on rat hepatic stellate cells. *Hepatology* 1998; **27**: 720-726

38 **Okuda M**, Li K, Beard MR, Showalter LA, Scholle F, Lemon SM, Weinman SA. Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. *Gastroenterology* 2002; **122**: 366-375

39 **Hézode C**, Cazeneuve C, Coué O, Roudot-Thoraval F, Lonjon I, Bastie A, Duvoux C, Pawlotsky JM, Zafrani ES, Amselem S, Dhumeaux D. Liver iron accumulation in patients with chronic active hepatitis C: prevalence and role of hemochromatosis gene mutations and relationship with hepatic histological lesions. *J Hepatol* 1999; **31**: 979-984

40 **Liu JP**, Manheimer E, Tsutani K, Gluud C. Medicinal herbs for hepatitis C virus infection. *Cochrane Database Syst Rev* 2001; CD003183

41 **Yano M**, Ikeda M, Abe K, Kawai Y, Kuroki M, Mori K, Dansako H, Ariumi Y, Ohkoshi S, Aoyagi Y, Kato N. Oxidative stress induces anti-hepatitis C virus status via the activation of extracellular signal-regulated kinase. *Hepatology* 2009; **50**: 678-688

42 **Miyanari Y**, Atsuzawa K, Usuda N, Watashi K, Hishiki T, Zayas M, Bartenschlager R, Wakita T, Hijikata M, Shimotohno K. The lipid droplet is an important organelle for hepatitis C virus production. *Nat Cell Biol* 2007; **9**: 1089-1097

43 **Delang L**, Paeshuyse J, Vliegen I, Leyssen P, Obeid S, Durantel D, Zoulim F, Op de Beeck A, Neyts J. Statins potentiate the in vitro anti-hepatitis C virus activity of selective hepatitis C virus inhibitors and delay or prevent resistance development. *Hepatology* 2009; **50**: 6-16

44 **Tada S**, Saito H, Ebinuma H, Ojiro K, Yamagishi Y, Kumagai N, Inagaki Y, Masuda T, Nishida J, Takahashi M, Nagata H, Hibi T. Treatment of hepatitis C virus with peg-interferon and ribavirin combination therapy significantly affects lipid metabolism. *Hepatol Res* 2009; **39**: 195-199

45 **Bechmann LP**, Zahn D, Gieseler RK, Fingas CD, Marquitan G, Jochum C, Gerken G, Friedman SL, Canbay A. Resveratrol amplifies profibrogenic effects of free fatty acids on human hepatic stellate cells. *Hepatol Res* 2009; **39**: 601-608

**S- Editor** Wang YR **L- Editor** O'Neill M **E- Editor** Zheng XM

Pathology International

*Pathology International* 2010; **60**: 351–357 doi:10.1111/j.1440-1827.2010.02526.x

Original Article

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

**Masatoshi Shigoka,[1] Akihiko Tsuchida,[1] Takaaki Matsudo,[1] Yuichi Nagakawa,[1] Hitoshi Saito,[1] Yoshiaki Suzuki,[1] Tatsuya Aoki,[1] Yoshiki Murakami,[2] Hidenori Toyoda,[3] Takashi Kumada,[3] Ralf Bartenschlager,[4] Nobuyuki Kato,[5] Masanori Ikeda,[5] Tomoki Takashina,[6] Masami Tanaka,[6] Rieko Suzuki,[6] Kosuke Oikawa,[7] Masakatsu Takanashi[6] and Masahiko Kuroda[6]**

*[1]Third Department of Surgery and [6]Department of Molecular Pathology, Tokyo Medical University, Tokyo, Japan, [2]Center for Genomic Medicine, Kyoto University, Kyoto, Japan, [3]Department of Gastroenterology, Ogaki Municipal Hospital, Ogaki, Japan, [4]Department of Infectious Diseases, Molecular Virology, University of Heidelbergand, Heidelberg, Germany, [5]Department of Tumor Virology, Okayama University Graduate School of Medicine, Okayama, Japan, and [7]First Department of Pathology, Wakayama Medical University, Wakayama, Japan*

**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

Correspondence: Masahiko Kuroda, MD, PhD, Department of Molecular Pathology, Tokyo Medical University, 6-1-1 Shinjuku, Shinjuku-ku, Tokyo 160-8402, Japan. Email: kuroda@tokyo-med.ac.jp

Received 8 November 2009. Accepted for publication 23 December 2009.



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 is 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 cancer[8] 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* hybridaization 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′-ACAGGCCGGGACAAGTGCAATA-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 hybridaization 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.

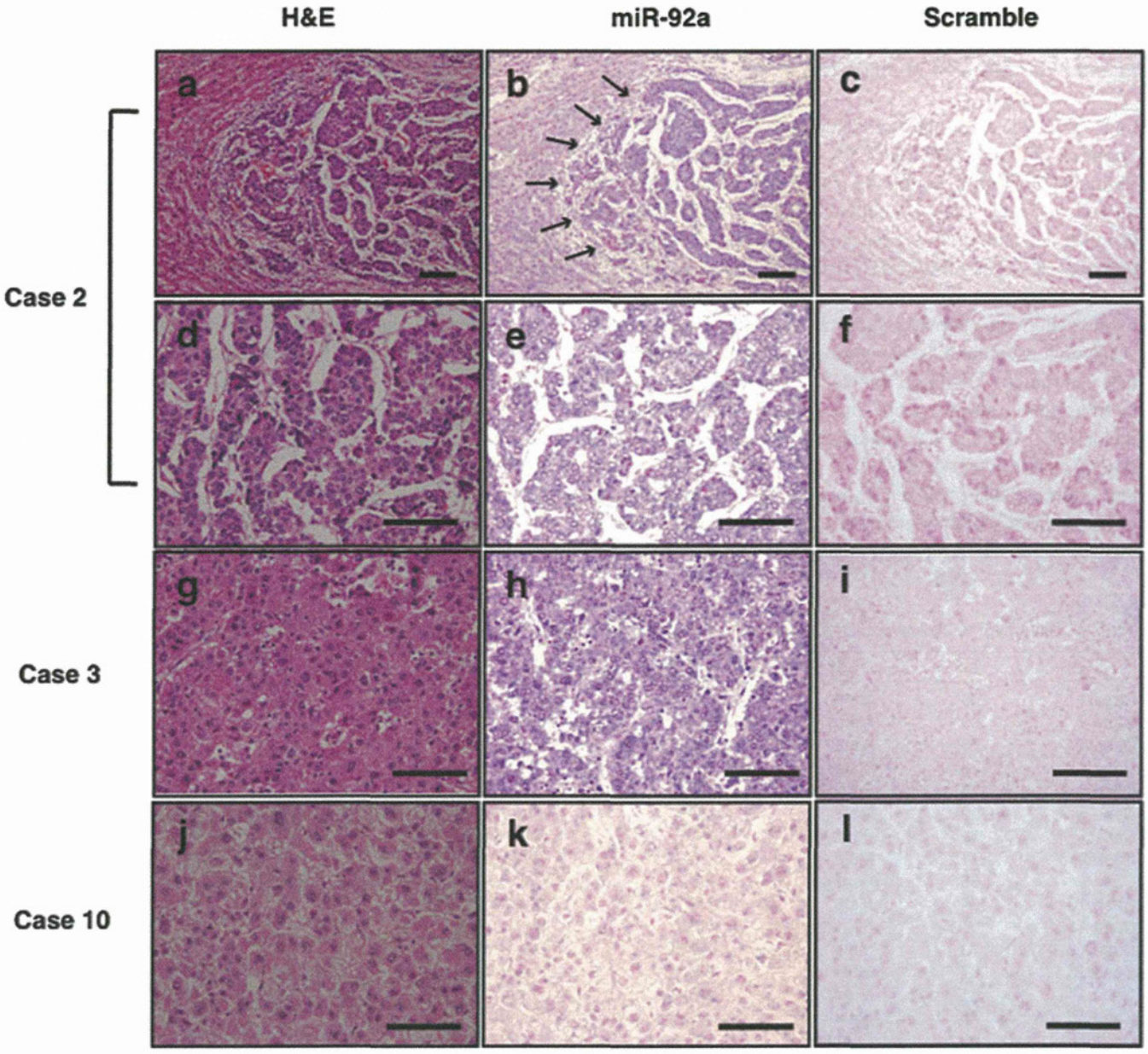



**Figure 1** MiRNA expression in hepatocellular carcinoma (HCC). *In situ* hybridization was performed using Locked nucleic acid (LNA)-modified probes for miR-92a and negative control. Case 2 and Case 3 were positive cases for miR-92a. Case 10 was a negative case for miR-92a. (**a–c**) Low power field 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 μm.

plasma was isolated using Isogen-LS (NIPPON GENE, Tokyo, Japan) according to the manufacturer's instructions. The RNA sample was suspended in 20 μL 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 ACUUGUCCCGGCCUGUCCCGGCC-3′ and 5′-AUUGCAC UUGUCCCGGCCUTT-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 Hiper-Fect (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 *Taq*Man 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).

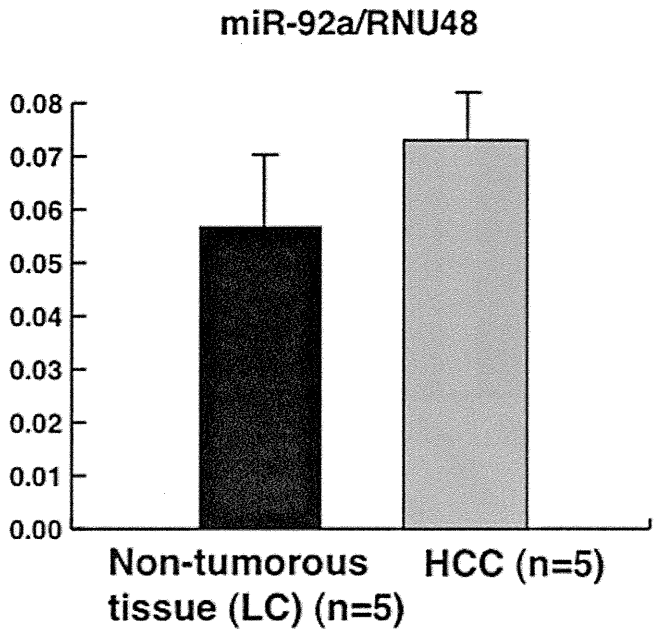



**Figure 2** Quantification of miR-92a expression in hepatocellular carcinoma (HCC) tissue samples. The ratios of miR-92a to RNU48 in HCC tissues and their adjacent non-tumorous liver cirrhosis (LC) tissues were analyzed by *Taq*Man qRT-PCR. *Bars*, s.d.

### 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

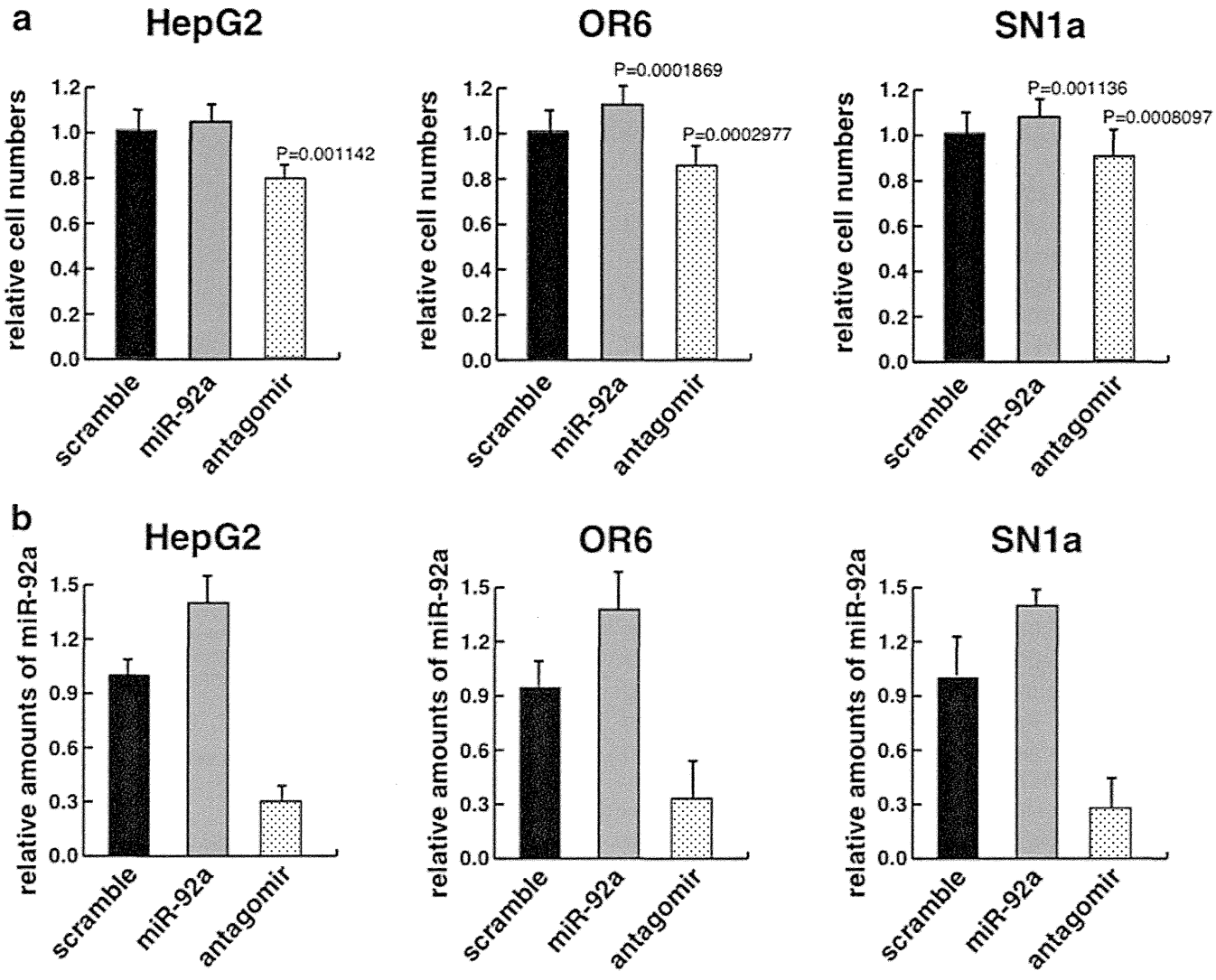



**Figure 3** miR-92a modulates proliferation of HepG2, OR6 and SN1a cells. (**a**) Cell numbers of the HepG2, OR6 and SN1a cells transfected with synthetic miR-92a, anti-miR-92a antagomir, or scrambled control oligonucleotide were analyzed by MTT assays at 48 h for OR6 and SN1a and 72 h for HepG2 after 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 *Taq*Man 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