Table 5 Supplements to guidelines for the treatment of patients with chronic hepatitis B (part II)

- Self-injection of IFN at home is recommended to patients, who are eligible to do it, for improving their quality of life.
- Treatment with nucleos(t)ide analogs should be continued in patients in whom cirrhosis or HCC has been cured.
- Antiviral treatment is considered in patients with ALT levels of ≥31 IU/L. To patients aged 35 years or older in whom viral replication persists, even to those with normal ALT levels, antiviral treatments are indicated. It is possible, however, to follow for outcomes in patients who are elderly or HBeAg-negative and in whom antiviral treatments are difficult, while they receive liver supportive therapy (e.g. SNMC, UDCA).
- In patients co-infected with HBV and HIV, entecavir cannot be used due to the possibility for emergence of HIV variants resistant to antiretroviral therapies.
- Immunosuppressive and anticancer drugs should be used with utmost caution, even in patients with low HBV DNA titers and normal ALT levels, because they can induce severe liver damage along with elevation in HBV DNA titers.

ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; IFN, interferon; SNMC, stronger neo-minophagen C; UDCA, ursodeoxycholic acid.

SUPPLEMENTS TO GUIDELINES FOR THE TREATMENT OF CHRONIC HEPATITIS B (PART II)

FURTHER, THE FOLLOWING five supplements have been added to the 2008 guidelines (Table 5).

To patients who are eligible, self-injection of IFN at home is recommended, taking into consideration their QOL. Because IFN-based therapies are not recommended for patients in whom HBV has been transmitted by perinatal infection, sequential treatment with IFN plus entecavir serves as another option in their antiviral treatment.

Treatment with nucleos(t)ide analogs should be extended to patients in whom cirrhosis or hepatocellular carcinoma (HCC) has been cured after successful therapies.

Antiviral treatment has to be considered in patients with ALT levels of 31 IU/L or more. Patients aged 35 years or older with normal ALT levels but in whom HBV replication persists, need to be considered for antiviral treatments. Elderly and HBeAg-negative patients, as well as those to whom the administration of antiviral drugs is difficult, can be followed regularly while they receive liver supportive therapy (e.g. stronger neo-minophagen C,[9] ursodeoxycholic acid [UDCA][10]).

Patients co-infected with HBV and HIV type 1 cannot receive entecavir due to the possibility of emergence of HIV mutants resistant to antiretroviral drugs.

Even in patients with low HBV DNA titers and normal ALT levels, HBV DNA loads can increase massively to induce severe liver damages in them, while they receive immunosuppressive or anticancer drugs. Hence, utmost caution should be exercised if they are to undergo antiviral treatments.

GUIDELINES FOR THE TREATMENT OF PATIENTS WITH CIRRHOSIS DUE TO HBV

TABLE 6 SUMMARIZES guidelines for the treatment of patients with type B cirrhosis. Patients with compensated or decompensated cirrhosis, who are infected with HBV, receive entecavir for persistent clearance of HBV DNA detectable by the real-time polymerase chain reaction and normalization of aspartate aminotransferase as well as ALT levels. Combined lamivudine plus adefovir therapy are indicated for patients in whom HBV mutants resistant to lamivudine or entecavir have developed. Guidelines for maintaining liver function, for preventing the development of HCC, include liver supportive therapy with glycyrrhizin and UDCA, either alone or in combination. For treatment toward sup-

Table 6 Guidelines for treatment of type B cirrhosis

Principles
- Compensated: termination of HBV infection by antiviral treatment with entecavir as the mainstay.
- Decompensated: reversal to compensation and prevention of HCC.

Methods
(1) Eradication of HBV and normalization of ALT/AST (compensated and decompensated cirrhosis).
   a) Entecavir.
   b) Combined lamivudine and adefovir (for patients with HBV mutants resistant to lamivudine or entecavir).
(2) Maintenance of liver function (improvement of ALT/AST and albumin) for preventing HCC.
   a) Liver supportive therapy such as SNMC or UDCA.
   b) Branched chain amino acids (Livact).
(3) Supplementation with nutrients (for stabilizing liver function in decompensated cirrhosis).

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; SNMC, stronger neo-minophagen C; UDCA, ursodeoxycholic acid.

pressing the development of HCC, branched chain amino acids (BCAA)[11] are implemented. Also, nutrient supplements are utilized for stabilizing liver function.

DISCUSSION AND CONCLUSION

THE STUDY GROUP for the Standardization of Treatment of Viral Hepatitis Including Cirrhosis, organized by the Ministry of Health, Labor and Welfare of Japan, has compiled a series of guidelines for the treatment of liver disease due to HBV and HCV ranging from chronic hepatitis to cirrhosis of various severities annually, since the fiscal year 2002. The principal aim of these guidelines is to decrease the incidence of HCC due to hepatitis virus infections in Japan. In accordance with this principle, supplements have been added to previous guidelines for the standardization of treatment of chronic viral liver disease every fiscal year. This article summarizes guidelines for the treatment of liver disease due to HBV. Guidelines for the treatment of liver disease due to HCV for the fiscal year 2008 are reported in the accompanying paper. They are formulated on evidence-based data that have been accumulated by members and cooperators of the study group. It will be necessary to improve these guidelines in the next fiscal year and henceforth, in accordance with many pieces of new evidence that are expected to evolve through enduring efforts and keen insights of members and cooperators of the study group.

In the treatment of chronic hepatitis B, novel therapeutic strategies have continued to evolve in previous guidelines. In guidelines of the fiscal year 2008, diverse new treatment arms are introduced for gaining the eventual goal of the "drug-fee state".

The Study Group for the Standardization of Treatment of Viral Hepatitis Including Cirrhosis has been drafted and displayed on the web site (www.jsh.or.jp/medical/index.html [in Japanese]) as well, guidelines for the treatment of a spectrum of liver diseases due to HBV, ranging from chronic hepatitis to cirrhosis of various severities for the fiscal year 2008. In view of the eventual goal of decreasing the incidence of HCC due to HBV infection, supplementation and adjustment are appended to previous guidelines, and new guidelines have been introduced to the treatment of cirrhosis due to HBV infection. As a general rule, antiviral treatments are the mainstay in guidelines for the treatment of chronic hepatitis B. In addition to them, it is necessary to always keep in mind the fundamental concepts of these guidelines. It is our sincere hope that, for the treatment of each patient, readers will conduct their clinical practice on the basis of these concepts, and then refer to appropriate individual guidelines, when they make decisions regarding treatment strategy, on a case-by-case basis. With respect to guidelines for the treatment of patients with cirrhosis, above all, expected achievable outcomes have to be taken into account in making treatment choices.

We can foretell that there is no end to the treatment of patients with chronic hepatitis and cirrhosis due to HBV, as it will keep evolving and improving in future guidelines. The enduring efforts of doctors and scientists, in pursuit of this goal, will fill in wide social and economic gaps in medical practices being served to the nation, and produce substantial and efficient interest in the medical economy on a national basis. In conducting treatment of patients with liver disease due to HBV infection, according to these guidelines, many new and unforeseen facets may surface that will require further improvements. Hence, it will be necessary to evaluate the therapeutic efficacy of these guidelines, and revise or add necessary supplements to them as required in the future.

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Review Article

Guidelines for the treatment of chronic hepatitis and cirrhosis due to hepatitis C virus infection for the fiscal year 2008 in Japan

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In the 2008 guidelines for the treatment of patients with chronic hepatitis C, pegylated interferon (Peg-IFN) combined with ribavirin for 48 weeks are indicated for treatment-naive patients infected with hepatitis C virus (HCV) of genotype 1. Treatment is continued for an additional 24 weeks (72 weeks total) in the patients who have remained positive for HCV RNA detectable by the real-time polymerase chain reaction at 12 weeks after the start of treatment, but who turn negative for HCV RNA during 13–36 weeks on treatment. Re-treatment is aimed to either eradicate HCV or normalize transaminase levels for preventing the development of hepatocellular carcinoma (HCC). For patients with compensated cirrhosis, the clearance of HCV RNA is aimed toward improving histological damages and decreasing the development of HCC. The recommended therapeutic regimen is the initial daily dose of 6 million international units (MIU) IFN continued for 2–8 weeks that is extended to longer than 48 weeks, if possible. IFN dose is reduced to 3 MIU daily in patients who fail to clear HCV RNA by 12 weeks for preventing the development of HCC. Splenectomy or embolization of the splenic artery is recommended to patients with platelet counts of less than $50 \times 103/mm^3$ prior to the commencement of IFN treatment. When the prevention of HCC is at issue, not only IFN, but also liver supportive therapy such as stronger neo-minophagen C, ursodeoxycholic acid, phlebotomy, branched chain amino acids (BCAA), either alone or in combination, are given. In patients with decompensated cirrhosis, by contrast, reversal to compensation is attempted.

Key words: chronic hepatitis, cirrhosis, hepatocellular carcinoma, hepatitis C virus, interferon, liver supportive therapy, pegylated interferon, ribavirin

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INTRODUCTION

SINCE THE FISCAL year 2002, guidelines for the treatment of patients with viral hepatitis have been compiled annually by the Study Group for the Standardization of Treatment of Viral Hepatitis Including Cirrhosis, under the auspice of the Ministry of Health Labor and Welfare of Japan, recruiting many specialists from all over the nation. They have been improved every year with many supplementary issues that have evolved, as our understanding of various aspects of viral hepatitis deepens and treatment options widen with time. For the fiscal year 2008, guidelines have been worked out for a comprehensive standardization of the treatment of chronic hepatitis and cirrhosis due to infection with hepatitis C virus (HCV) in Japan. It is hoped that these guidelines will be accepted widely and implemented for helping as many patients as possible who suffer from sequelae of persistent HCV infection.

Here, we relate excerpts of the 2008 guidelines for the treatment of patients with HCV-induced liver disease covering a wide range from those with normal aminotransferase levels to those with decompensated cirrhosis.

GUIDELINES FOR THE PRIMARY TREATMENT OF PATIENTS WITH CHRONIC HEPATITIS C

TABLE 1 SUMMARIZES the antiviral therapy of treatment-naive patients with chronic hepatitis C. In comparison with previous guidelines, the duration of combined treatment with pegylated interferon (Peg-IFN) and ribavirin is extended to 48–72 weeks for patients infected with HCV of genotype 1 in high viral loads (HVL: ≥5 log IU/mL by the Japanese criteria).[1,2] For patients infected with HCV of genotype 2 in HVL, Peg-IFN-α2b and ribavirin for 24 weeks are indicated. To patients with HCV-1 in low viral loads (LVL: <5 log IU/mL), either the standard IFN (not conjugated with polyethylene glycol) for 24 weeks, or the weekly monotherapy with Peg-IFN-α2a for 24–48 weeks, is given.[3] Patients with HCV-2 in LVL receive either the standard IFN for 8–24 weeks, or the weekly monotherapy with Peg-IFN-α2a for 24–48 weeks.

GUIDELINES FOR THE RE-TREATMENT OF PATIENTS WITH CHRONIC HEPATITIS C

FOR PATIENTS WHO receive re-treatment, first, it is imperatively prerequisite to: (i) identify factors for non-response to previous treatments; and (ii) decide whether to aim for clearance of HCV or to prevent the progression of hepatitis that can accelerate the development of hepatocellular carcinoma (HCC), and this can be monitored by alanine aminotransferase (ALT) and α-fetoprotein (AFP) levels toward normalizing or stabilizing their levels (Table 2).[4] Second, IFN combined with ribavirin is the mainstay of re-treatment of patients with chronic hepatitis C. Third, long-term IFN monotherapy is recommended to patients who are not indicated to IFN/ribavirin or who have failed to respond to the combination therapy. However, some patients do not tolerate IFN due to side-effects or their complicating morbidities. In addition, IFN monotherapy does not always improve ALT levels. Such patients need to receive liver supportive therapy including stronger neominophagen C (SNMC)[5] and ursodeoxycholic acid (UDCA),[6] as well as phlebotomy, either alone or in combination. Therapeutic target ALT levels are: (i) within ×1.5 the upper limit of normal (ULN) for patients in fibrosis stage 1 (F1); and (ii) less than 30 IU/L in those in fibrosis stages 2 or 3 (F2/F3), as far as possible.

Table 1 Guidelines for the primary treatment of patients with chronic hepatitis C

Genotypes	Genotype 1	Genotype 2
Viral loads		
High viral load ≥5.0 log IU/mL ≥300 fmol/L ≥1 Meq/mL	• Peg-IFN-α2b (Peg-Intron) + ribavirin (Rebetol) for 48–72 weeks • Peg-IFN-α2a (Pegasys) + ribavirin (Copegus) for 48–72 weeks	• Peg-IFN-α2b (Peg-Intron) + ribavirin (Rebetol) for 24 weeks
Low viral load <5.0 log IU/mL <300 fmol/L <1 Meq/mL	• Standard IFN for 24 weeks • Peg-IFN-α2a (Pegasys) for 24–48 weeks	• Standard IFN for 8–24 weeks • Peg-IFN-α2a (Pegasys) for 24–48 weeks

Peg-IFN, pegylated interferon.

Table 2 Guidelines for re-treatment of chronic hepatitis C

Principles
Selection has to be made between termination of HCV infection and normalization/stabilization of ALT as well as AFP levels (toward preventing aggravation of liver disease and development of HCC), after evaluating factors for non-response in the primary IFN treatment.

1 "IFN plus ribavirin" is the mainstay of re-treatment of patients who have failed to respond to the primary IFN therapy.
2 Long-term IFN is recommended to patients in whom ribavirin is not indicated or who have failed to respond to IFN/ribavirin; self-injection at home is approved for IFN-α (not for Peg-IFN).
3 Patients who are not indicated to IFN or have failed to improve ALT and AFP levels, in response to IFN, receive liver supportive therapy (SNMC, UDCA) and phlebotomy, either alone or in combination.
4 For preventing aggravation of liver disease (and development of HCC), ALT levels need to be controlled within 1.5 × ULN in patients in stage 1 fibrosis (F1), and as far as possible, 30 IU/L or lower in those in fibrosis stages 2–3 (F2/F3).
5 In treatment combined with ribavirin, dose and mode need to be selected, taking into consideration factors contributing to the response, such as age, sex, progression of liver disease, mutations in the HCV genome (amino acid substitutions in the core protein [aa70/aa91] and ISDR) and HCV RNA titers determined by the real-time PCR.

AFP, α-fetoprotein; ALT, alanine aminotransferase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; ISDR, interferon sensitivity determining region; PCR, polymerase chain reaction; Peg-IFN, pegylated interferon; SNMC, stronger neo-minophagen C; UDCA, ursodeoxycholic acid; ULN, upper limit of normal.

SUPPLEMENTS TO GUIDELINES FOR THE TREATMENT OF CHRONIC HEPATITIS C

FOR THE FISCAL year 2008, the following items were supplemented to the treatment of chronic hepatitis C (Table 3).

1 The treatment of patients infected with HCV-1 in HVL with Peg-IFN/ribavirin for 72 weeks is modified by the early virological response (EVR) within 12 weeks after the start. Patients who have remained positive for HCV RNA detectable by the real-time polymerase chain reaction at 12 weeks after the start of treatment, but who turn negative for HCV RNA till 13–36 weeks on treatment.[1,2]
2 Patients with HCV-1 in HVL who fail to clear HCV RNA detectable by real-time PCR but in whom ALT levels normalize are continued on Peg-IFN/ribavirin until 48 weeks, so that normalized ALT levels endure longer after the completion of therapy.[7]
3 Patients who are not indicated to Peg-IFN/ribavirin, or who have failed to respond to previous treatments, receive long-term IFN monotherapy. During the first 2 weeks, IFN in the conventional dose is given daily or three times a week. Patients who do not clear HCV RNA during the maximal treatment period of 8 weeks receive half the conventional dose of IFN indefinitely.[8]

GUIDELINES FOR THE TREATMENT OF PATIENTS WITH CHRONIC HEPATITIS C IN NORMAL ALT LEVELS

AS IN PREVIOUS guidelines, patients with chronic hepatitis C having normal ALT levels are stratified into four groups by ALT levels and platelet counts (Table 4). Patients with chronic hepatitis C who have normal ALT levels are reported to gain the sustained virological response (SVR) to antiviral treatments comparably frequently as those having elevated ALT levels. Taking this into consideration, patients with ALT levels of 30 IU/L or less and platelet counts of $150 \times 10^3/mm^3$ or more are followed for ALT every

Table 3 Supplements to guidelines for chronic hepatitis C

1 Criteria for extending the duration of Peg-IFN/ribavirin (to 72 weeks) in patients infected with HCV-1b in HVL: patients who have remained positive for HCV RNA detectable by the real-time polymerase chain reaction at 12 weeks after the start of treatment, but who turn negative for HCV RNA till 13–36 weeks on treatment.[1,2]
2 Patients with HCV-1b in HVL who fail to lose HCV RNA detectable by real-time PCR, but in whom ALT levels normalize by 36 weeks, Peg-IFN/ribavirin is given till 48 weeks for maintaining normalized ALT levels long after the completion of treatment.
3 Long-term IFN monotherapy in patients who are not indicated to Peg-IFN/ribavirin, or have failed to respond to it: the usual dose of IFN daily or three times in week is given for the first 2 weeks, and when HCV RNA does not disappear within the maximal duration of 8 weeks, long-term treatment with half the usual dose of IFN is continued indefinitely.

ALT, alanine aminotransferase; HCV, hepatitis C virus; HVL, high viral loads; PCR, polymerase chain reaction; Peg-IFN, pegylated interferon.

Table 4 Guidelines for the treatment of patients with normal ALT levels toward preventing the development of HCC

Platelets	$\geq 150 \times 10^3/mm^3$	$<150 \times 10^3/mm^3$
ALT		
≤30 IU/L	• Follow for ALT every 2–4 months. • If ALT levels elevate, start antiviral treatments taking into consideration the possibility of SVR and risk for HCC.	• Liver biopsy, if possible, and consider antiviral treatments for patients in A2/F2. • Follow for ALT every 2–4 months, and consider antiviral treatments when ALT levels elevate, for patients without biopsy.
31–40 IU/L	• Consider antiviral treatments for patients younger than 65 years.	• Start treatments for chronic hepatitis C. • Select treatments according to genotypes, viral load, age of patients, etc.

ALT, alanine aminotransferase; HCC, hepatocellular carcinoma; SVR, sustained virological response.

2–4 months. If ALT levels increase in them, antiviral treatments are considered based on the possibility of resolving HCV infection and the risk for developing HCC. In view of significant fibrosis present in patients with platelet counts of less than $150 \times 10^3/mm^3$, they are recommended to receive liver biopsy, if this is possible. Patients in fibrosis stage F2 or higher are evaluated for the indication to antiviral treatments. Patients with ALT levels between 31 and 40 IU/L are classified by platelet counts. Antiviral treatments are considered in those aged younger than 65 years who have platelet counts of $150 \times 10^3/mm^3$ or more, while guidelines for patients with chronic hepatitis are applied to those with platelet counts of less than $150 \times 10^3/mm$.[9,10]

GUIDELINES FOR THE TREATMENT OF PATIENTS WITH CIRRHOSIS DUE TO HCV

PATIENTS WITH COMPENSATED cirrhosis who are not infected with HCV-1 in HVL receive either IFN-β or IFN-α (Table 5). Since the fiscal year 2008, IFN-α has been approved for the treatment of patients infected with HCV-1 in HVL, with the aim of resolving infection and normalizing ALT as well as AFP levels by long-term therapy. Treatment duration was set at 1 year or longer, and because the longer the treatment duration the higher the SVR rate, 36 weeks has been recommended as the optimal treatment duration. Because the normalization of ALT/AST is important, even in patients who fail to clear HCV infection by these therapeutic regimens, treatment is better conducted for maintaining normal ALT/AST levels. Guidelines for maintaining liver function for preventing the development of HCC include liver supportive therapy with glycyrrhizin[5] and UDCA,[6] either alone or in combination. For treatment toward suppressing the development of HCC, branched chain amino acids (BCAA)[11] or phlebotomy are adopted. Also, nutrient supplements are applied for stabilizing liver function.

SUPPLEMENTS TO GUIDELINES FOR THE TREATMENT OF CIRRHOSIS DUE TO HCV

THE FOLLOWING ITEMS have been appended to supplement guidelines for the treatment of type C cirrhosis (Table 6).

Table 5 Guidelines for treatment of type C cirrhosis

Principles
- Compensated: termination of HCV infection
- Decompensated: reversal to compensation and prevention of HCC

Methods
(1) Eradication of HCV and normalization of ALT/AST (for patients with compensated cirrhosis).
  a) HCV-1b in HVL (≥5 log IU/mL)
     IFN-α (Sumiferon)
  b) Others
     IFN-α (Sumiferon)
     IFN-β (Feron)
(2) Maintenance of liver function (improvement of ALT/AST and albumin) for preventing HCC.
  a) Liver supportive therapy
     Stronger neo-minophagen C (SNMC), ursodeoxycholic acid (UDCA), etc.
  b) Branched chain amino acids (BCAA [Livact])
  c) Phlebotomy
(3) Supplementation with nutrients (for stabilizing liver function in decompensated cirrhosis).

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HVL, high viral loads; IFN, interferon.

Table 6 Supplements to guidelines for type C cirrhosis

1 To start with, IFN for compensated cirrhosis is desired at 6 MIU daily for 2–8 weeks, as far as possible, and to continue for 48 weeks or longer, as for chronic hepatitis C.
2 In patients with compensated cirrhosis who fail to clear HCV RNA within 12 weeks on IFN, long-term therapy at 3 MIU should be considered for preventing HCC.
3 In patients with platelet counts $<50 \times 10^3/mm^3$, splenectomy or embolization of splenic artery is recommended before re-treatment, and after thorough evaluation has been made on the response to IFN to be expected.
4 For the prevention of HCC, not only IFN, but also liver supportive therapy (SNMC, UDCA, etc.), phlebotomy and branched chain amino acids, either alone or in combination, are recommended for improving ALT/AST and AFP levels.

AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; MIU, million international units; SNMC, stronger neo-minophagen C; UDCA, ursodeoxycholic acid.

1 For treatment of type C cirrhosis with IFN, the initial dose of 6 million international units (MIU) daily is continued as long as possible (2–8 weeks). Thereafter, long-term IFN for 48 weeks or longer is desired as in the treatment of chronic hepatitis C.
2 In the treatment of type C cirrhosis, patients who fail to achieve EVR with the clearance of HCV RNA from serum within 12 weeks should receive long-term IFN at a dose of 3 MIU.
3 For patients with type C cirrhosis who have platelet counts of less than $50 \times 10^3/mm^3$, splenectomy or embolization of the splenic artery is desirable before commencing IFN therapy, after the efficacy of IFN has been evaluated thoroughly.[12]
4 For preventing the development of HCC, improvement in ALT, AST and AFP levels are aimed. Toward this end, not only IFN, but also liver supportive therapy (SNMC and UDCA), phlebotomy and BCAA are used, either alone or in combination.

DISCUSSION AND CONCLUSION

THE STUDY GROUP for the Standardization of Treatment of Viral Hepatitis Including Cirrhosis, organized by the Ministry of Health, Labor and Welfare of Japan, has compiled a series of guidelines for the treatment of liver disease due to HCV ranging from chronic hepatitis to cirrhosis of various severities for the fiscal year 2008. The principal aim of these guidelines is to decrease the incidence of HCC due to HCV infection in Japan. In accord with this principle, supplements have been added to previous guidelines for the standardization of treatment of chronic hepatitis C. They are prepared on evidence-based data that have been accumulated by members and cooperators of the study group. It is necessary to improve these guidelines in the next fiscal year and thereafter, in accordance with many pieces of new evidence that are expected to emerge through enduring efforts of members and cooperators of the study group.

In the treatment of chronic hepatitis C, the duration of antiviral treatments is extended to 72 weeks, which has been approved as of the fiscal year 2008, and criteria for the eligibility of extended treatment duration are clearly defined. Long-term antiviral treatments, extended up to 72 weeks, are hoped to increase the SVR even further. In addition, comprehensive guidelines for the treatment of cirrhosis have been improved with substantial additions, and their criteria for the indication made explicit.

The Study Group for the Standardization of Treatment of Viral Hepatitis Including Cirrhosis has drafted, and also displayed online (www.jsh.or.jp/medical/index.html [in Japanese]), guidelines for a spectrum of liver diseases due to HCV, from chronic hepatitis to cirrhosis of various severities. In view of the eventual goal of decreasing the incidence of HCC due to HCV infection, supplementation and adjustment are appended to previous guidelines, and new guidelines have been constructed for the treatment of cirrhosis due to HCV infection. As a general rule, antiviral treatments constitute the main body of guidelines for the treatment of chronic hepatitis C. Furthermore, the fundamental concept of these guidelines would need to be kept in mind always. It is our sincere hope that, for the treatment of each patient, readers will base their clinical practice on these guidelines, and refer to appropriate individual guidelines, when they make a decision on the treatment strategy, on a case-by-case basis. With respect to guidelines for the treatment of patients with cirrhosis, above all, expected achievable outcomes have to be taken into account in treatment choice.

It is our sincere desire that treatment of patients with chronic hepatitis and cirrhosis due to HCV will proceed following these guidelines. Efforts along these lines will rectify a wide gap in medical treatment served to the nation and raise substantial and efficient interest in the medical economy on the national basis. In practicing treatment according to these guidelines, it will be nec-

essary to evaluate their therapeutic efficacy, and revise or add necessary supplements to them as required in the future.

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Insulin resistance raises the risk for recurrence of stage I hepatocellular carcinoma after curative radiofrequency ablation in hepatitis C virus-positive patients: A prospective, case series study

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Aim: Several studies have reported that insulin resistance raises the risk of primary hepatocellular carcinoma (HCC). We conducted a prospective, case series study to test the impact of insulin resistance on the recurrence after curative radiofrequency ablation (RFA) of stage I HCC in HCV-positive patients.

Methods: From January 2006 to December 2007, 226 consecutive patients underwent treatment for primary HCC at our institutions, including 37 stage I cases. Among them, 33 were HCV-positive, and three, six and 24 received curative surgery, transarterial chemoembolization or RFA, respectively. In the 24 patients treated with RFA, recurrence-free survival was analyzed using the Kaplan–Meier method. The factors contributing to recurrence of HCC were subjected to univariate and multivariate analyses using the Cox proportional hazards model. Insulin resistance was estimated by the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR).

Results: Kaplan–Meier analysis showed that the recurrence-free survival was lower in patients with higher HOMA-IR (>2.3, $P = 0.0252$) or with lower serum albumin level (<3.3 g/dL, $P = 0.0004$). In the univariate analysis, HOMA-IR ($P = 0.0420$) and albumin ($P = 0.0036$) were significantly associated with recurrence of HCC. Multivariate analysis revealed albumin (odds ratio = 0.01, 95% confidence interval = 0.0002–0.015, $P = 0.0001$) and HOMA-IR (odds ratio = 3.85, 95% confidence interval = 1.57–14.2, $P = 0.0015$) to be independent predictors for recurrence of HCC.

Conclusion: Serum albumin level and HOMA-IR were independent risk factors for recurrence of stage I HCC after curative RFA in HCV-positive patients. Patients with these factors require closer surveillance.

Key words: hepatitis C virus, hepatocellular carcinoma, Homeostatic Model Assessment of Insulin Resistance, insulin resistance, radiofrequency ablation, recurrence

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is prevalent worldwide, especially in Africa and the Western Pacific Region. HCC is the third most common cause of cancer death in men and the fifth most common in women; every year, more than 600 000 people die from this disease (www.who.int/whosis/).

Risk factors for the development of primary HCC include viral infection such as hepatitis B virus (HBV) and hepatitis C virus (HCV), alcohol consumption, aflatoxin and immune-related hepatitis.[1] Regarding risk factors for recurrence, several studies have suggested male sex, presence of cirrhosis, high α-fetoprotein (AFP), large tumor foci, multiplicity of tumors, pathologically high-grade atypia of tumor cells and presence of portal venous invasion of tumor.[2–6] Recently, several epidemiological studies have revealed a close association between diabetes mellitus (DM) and HCC. Wideroff *et al.*[7] described the standardized incidence ratios in Denmark for primary liver cancer in subjects with DM compared with the general population as 4.0 (95% confidence interval [CI] = 3.5–4.6) and 2.1 (95% CI = 1.6–2.7) for men and women, respectively. El-Serag *et al.*[8] reported that DM increased the risk of chronic non-alcoholic liver disease and HCC in male patients without concomitant

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liver disease in the USA. Furthermore, patients with chronic hepatitis and cirrhosis tend to experience complications with DM or to show insulin resistance.[9] This is particularly the case for patients with HCV infection and non-alcoholic fatty liver disease (NAFLD), including its most severe form, non-alcoholic steatohepatitis (NASH), which can lead directly to HCC.[10–12] The HCV core protein induced insulin resistance by increasing tumor necrosis factor-α which disrupts tyrosine phosphorylation of insulin receptor substrate-1.[13] Thus, DM including insulin resistance seems to be closely associated with various liver diseases that can lead to HCC, although the impact of insulin resistance on the recurrence of HCC has not been evaluated.

In this study, to identify the impact of insulin resistance on recurrence after initial curative treatment for HCC, we designed a prospective, case series analysis to examine recurrence-free survival in consecutive patients with stage I HCC, stratified by Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) level, which is commonly used for measuring insulin resistance.[14,15] In particular, we focused on HCV-positive patients who were treated with radiofrequency ablation (RFA).

METHODS

Patients

FROM JANUARY 2006 to December 2007, 226 primary HCC patients underwent initial treatment at our institutions, and 199 of them were followed to the end of this study (April 2008). Among them, we had 37 consecutive patients with stage I HCC that met all the criteria: a single tumor of 2 cm or less diameter, with no vascular invasion, no lymph-node invasion and no distant metastasis.[16]

Hepatocellular carcinoma nodules were detected by imaging modalities including abdominal ultrasonography, dynamic computed tomography (CT), dynamic magnetic resonance imaging (MRI) and abdominal arteriography. Diagnosis of HCC was made from a typical hypervascular tumor stain on angiography and typical dynamic-study findings of enhanced staining in the early phase and attenuation in the delayed phase. Etiologies for HCC were HCV in 33 patients, HBV in two and others in two.

Treatment, follow up and determination of recurrence

Three patients were treated with surgical resection, six with transarterial chemoembolization (TACE) and 28 with RFA. Among them, we only recruited those who were positive for HCV and treated with RFA ($n = 24$). Therapeutic effect was judged to be curative using dynamic CT or MRI with total disappearance of imaging characteristics of HCC as described above.

Patients were thereafter followed on an out-patient basis using serum tumor markers such as AFP and protein induced by vitamin K absence or antagonists II (PIVKA-II) every month, and by abdominal ultrasound, dynamic CT scan or dynamic MRI every 3 months. Recurrent HCC was diagnosed using the imaging modalities described earlier as the appearance of another lesion different from the primary one. The follow-up period was defined as the interval from the date of initial treatment until the date of diagnosis of recurrence, or until April 2008 if HCC did not recur. We defined the local tumor progression at the initial HCC site as censored.

Statistical analysis

Baseline characteristics were compared using the Student's *t*-test for continuous variables or χ^2-test for categorical variables. Recurrence-free survival was estimated using the Kaplan–Meier method, and differences between curves were examined by log–rank test. There were 13 possible predictors for recurrence of HCC after the initial curative treatment: sex, age, body mass index (BMI), Child–Pugh classification, serum albumin level, total bilirubin level, alanine aminotransferase (ALT) activity, platelet count, prothrombin time, HOMA-IR (defined as fasting plasma glucose [mg/dL] × fasting immunoreactive insulin [μU/mL] / 405), hemoglobin A_{1c} (HbA_{1c}), and serum tumor markers (AFP and PIVKA-II). The parameters, that proved to be significant by log–rank test, were then subjected to the univariate and multivariate analyses using the Cox proportional hazards model. Statistical significance was declared if the *P*-value was 0.05 or less. In addition, we employed the quantitative insulin sensitivity check index (QUICKI), which directly correlates with the glucose clamp method,[17] to supplement the evaluation of insulin resistance by HOMA-IR: QUICKI = 1 / (log [immunoreactive insulin] + log [fasting plasma glucose]).

RESULTS

Patients' baseline characteristics and laboratory data

THE BASELINE CHARACTERISTICS and laboratory data of 24 patients (15 men and nine women, median age 73 years) are shown in Table 1. Twenty

Table 1 Baseline demographic and clinical characteristics

		Normal range
Sex (male/female)	15/9	
Age (years)	73 (61–82)	
BMI	22.3 (19.5–33.5)	
Child-Pugh classification (A/B/C)	20/4/0	
Follow-up period (days)	365 (60–770)	
ALB (g/dL)	3.75 (2.4–4.4)	3.9–4.9
ALT (IU/L)	48.5 (21–98)	7–40
T-Bil (mg/dL)	0.96 (0.6–2.1)	0.2–1.2
PLT ($\times 10^4/\mu L$)	8.85 (4.1–21)	14.1–32.7
PT (%)	74 (56–118)	70–120
FPG (mg/dL)	107 (75–155)	70–110
FIRI (μg/dL)	10.8 (2.78–32.2)	2–10
HOMA-IR	2.96 (0.76–7.39)	<1.6
HbA1c (%)	5.2 (3.7–7.2)	<5.6
AFP (ng/dL)	26.7 (2.2–203)	<20
PIVKA-II (mAU/mL)	24 (9–127)	<40

Values are median (range).
AFP, α-fetoprotein; ALB, albumin; ALT, alanine aminotransferase; BMI, body mass index; FIRI, fasting immunoreactive insulin; FPG, fasting plasma glucose; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; PIVKA-II, protein induced by vitamin K absence or antagonists II; PLT, platelets; PT, prothrombin time; T-Bil, total bilirubin.

patients were classified into Child-Pugh class A, four patients into class B and none into class C. The median follow-up period was 365 days (range 60–770 days), and no patient died during the study.

Possible risk factors for recurrence of HCC

No local tumor progression was diagnosed in this study period. Seven patients experienced the defined recurrence in the liver, but no one showed distant metastasis. One-year recurrence-free survival in total patients was 64%; Figure 1(a,b) shows Kaplan–Meier curves for recurrence-free survival according to HOMA-IR level (≤2.3 and >2.3), which produced significant difference ($P = 0.0252$). Serum albumin level (≥3.3 and <3.3 g/dL; $P = 0.0004$) was also a significant variable (Fig. 1c).

The Cox proportional hazards model was used to analyze risk factors for recurrence of stage I HCC after the curative RFA, using the 13 variables described earlier (Table 2). HOMA-IR level (odds ratio [OR] = 1.66, 95% CI = 1.01–2.72, $P = 0.0420$), and serum albumin level (OR = 0.08, 95% CI = 0.01–0.45, $P = 0.0036$) were identified as significant risk factors by univariate analysis. Multivariate analysis identified albumin (OR = 0.01,

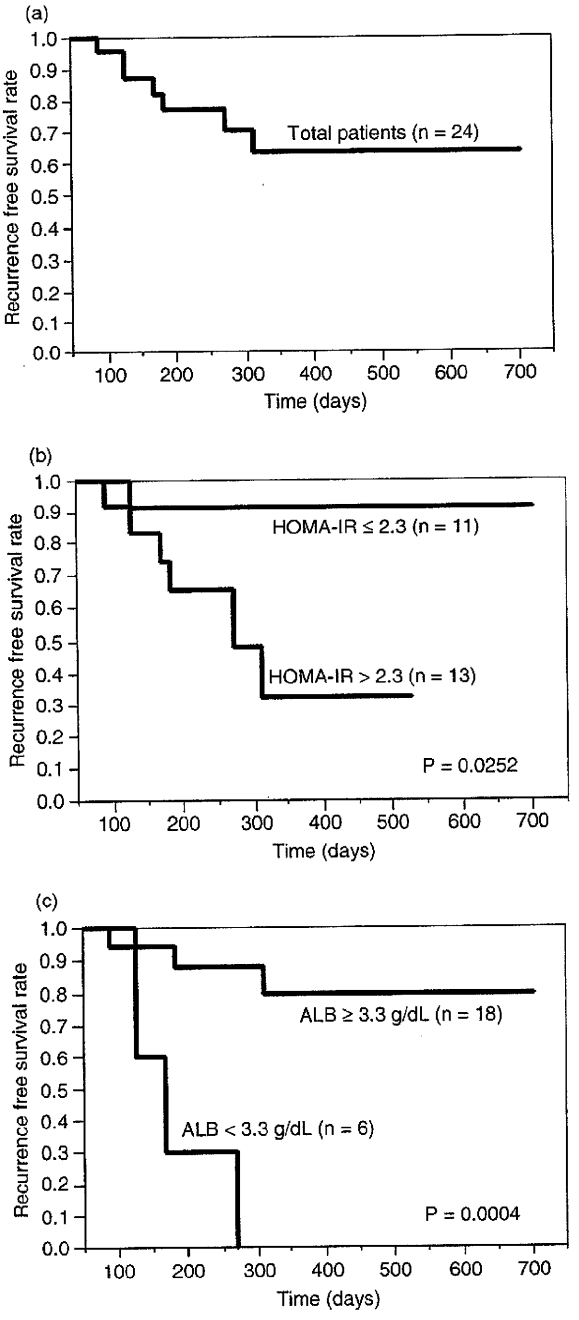

Figure 1 Kaplan–Meier curves for recurrence-free survival in (a) total patients and in subgroups divided according to (b) Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) level or (c) serum albumin level. ALB, albumin.

Table 2 Univariate analyses of possible risk factors for recurrence of hepatocellular carcinoma by Cox proportional hazards model

	95% CI			
	OR	Lower	Upper	*P*-value
Men (vs women)	1.20	0.25	8.41	0.8242
Age (years)	1.06	0.93	1.23	0.3451
BMI	0.90	0.59	1.22	0.6036
Child B (vs A)	4.81	0.60	31.3	0.1253
ALB (g/dL)	0.08	0.01	0.45	0.0036
T-Bil (mg/dL)	2.75	0.27	19.7	0.3603
ALT (IU/L)	0.99	0.95	1.02	0.6923
PLT ($\times 10^4/\mu L$)	0.86	0.65	1.05	0.1770
PT (%)	0.95	0.87	1.01	0.1617
HOMA-IR	1.66	1.01	2.72	0.0420
HbA1c (%)	0.69	0.27	1.53	0.3850
AFP (ng/dL)	1.00	0.99	1.02	0.1242
PIVKA-II (mAU/mL)	1.00	0.96	1.03	0.6172

OR is shown with a unit increase in continuous variables.
AFP, α-fetoprotein; ALB, albumin; ALT, alanine aminotransferase; BMI, body mass index; CI, confidence interval; FIRI, fasting immunoreactive insulin; FPG, fasting plasma glucose; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; OR, odds ratio; PIVKA-II, protein induced by vitamin K absence or antagonists II; PLT, platelets; PT, prothrombin time; T-Bil, total bilirubin.

95% CI = 0.0002–0.15, $P = 0.0001$) and HOMA-IR level (OR = 3.85, 95% CI = 1.57–14.2, $P = 0.0015$) as significant independent risk factors for recurrence.

Table 3 shows the patients' baseline characteristics and laboratory data divided according to HOMA-IR level (≤2.3 and >2.3). No significant differences were noted between the two subgroups except fasting plasma glucose and fasting immunoreactive insulin. Two patients in the HOMA-IR 2.3 or less subgroup took oral hypoglycemic drugs, sulfonylurea derivatives and voglibose. Three patients in the HOMA-IR more than 2.3 subgroup took oral hypoglycemic drugs; two took sulfonylurea derivatives and one took pioglitazone. No patient received insulin treatment.

We supplementally analyzed the data by excluding the patients under treatment with these oral hypoglycemics and also the patients with fasting plasma glucose above 140 mg/dL, in order to avoid possible unreliability in HOMA-IR evaluation. In nine patients, each remaining in HOMA-IR of 2.3 or less and HOMA-IR of more than 2.3, serum albumin (OR = 0.02, 95% CI = 0.0002–0.40, $P = 0.0060$) and HOMA-IR (OR = 3.49, 95% CI = 1.45–13.8, $P = 0.0033$) were still significant.

In a similar manner, evaluation of insulin sensitivity by QUICKI gave the results that lower QUICKI (≤0.33,

Table 3 Baseline demographic and clinical characteristics of patients classified according to HOMA-IR level

	HOMA-IR ≤2.3 ($n = 11$)	HOMA-IR >2.3 ($n = 13$)	*P*-value
Sex (male/female)	7/4	8/5	0.9157
Age (years)	70 (61–82)	74 (63–80)	0.1846
BMI	23.55 (19.5–33.5)	22.1 (19.5–25.1)	0.1219
Follow-up period (days)	393 (155–701)	337 (60–770)	0.2785
Child–Pugh classification (A/B)	10/1	10/3	0.3483
ALB (g/dL)	3.9 (2.4–4.4)	3.4 (2.7–4.4)	0.3304
ALT (IU/L)	40 (21–98)	53 (25–80)	0.6103
T-Bil (mg/dL)	0.8 (0.7–2.1)	1.0 (0.6–1.7)	0.6655
PLT ($\times 10^4/\mu L$)	8.5 (4.1–21)	8.9 (4.9–13.9)	0.5766
PT (%)	72 (56–95.5)	74 (58–118)	0.9953
FPG (mg/dL)	90 (75–119)	109 (86–155)	0.0151
FIRI (μg/dL)	7.98 (2.78–10.8)	14.1 (7.86–32.2)	0.0005
HOMA-IR	1.79 (0.76–2.27)	3.76 (2.91–7.39)	<0.0001
HbA1c (%)	5.05 (3.7–7.2)	5.3 (4.1–6.8)	0.6848
AFP (ng/dL)	23.2 (2.2–153.2)	28 (8–203)	0.7339
PIVKA-II (mAU/mL)	21 (9–127)	28 (9–67)	0.8071
Presence of oral hypoglycemic drugs (yes/no)	2/9	3/10	0.7678
Presence of insulin treatment (yes/no)	0/11	0/13	1.0000

Values are median (range).
AFP, α-fetoprotein; ALB, albumin; ALT, alanine aminotransferase; BMI, body mass index; FIRI, fasting immunoreactive insulin; FPG, fasting plasma glucose; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; PIVKA-II, protein induced by vitamin K absence or antagonists II; PLT, platelets; PT, prothrombin time; T-Bil, total bilirubin.

i.e. impaired insulin sensitivity) was associated significantly with the increased risk of HCC recurrence (OR = 7.97, 95% CI = 1.32–152, $P = 0.0213$).

DISCUSSION

SEVERAL EPIDEMIOLOGICAL STUDIES have revealed the association of DM with cancer incidence and cancer mortality for various organs such as the liver, biliary tract, pancreas, endometrium, kidney, colon, bladder and breast.[7,18–21] The mechanism by which insulin acts as a carcinogenic factor is currently a focus of interests. First, insulin functions as a growth factor by phosphorylating insulin receptor substrate 1 and activating the downstream mitogen-activated protein kinase cascade, which affects cellular proliferation.[22,23] Second, hyperinsulinemia increases peripheral lipolysis and hepatic accumulation of free fatty acids, and the excess β-oxidation in mitochondria and microsome leads to the production of reactive oxygen species[24,25] that play a significant role in carcinogenesis.[26,27] Adipocyte-secreted cytokines (adipokines) such as tumor necrosis factor-α and interleukin-6 also play a significant role in both insulin residence and carcinogenesis.[28,29] Thus, these factors could cooperatively induce the insulin resistance and carcinogenesis.

We demonstrated in the present study that a higher HOMA-IR level increases the risk of early recurrence after initial curative RFA of stage I HCC in HCV-positive patients. This finding basically agrees with previous studies[7,8,18] that suggested an association between insulin resistance and carcinogenesis as described above, but HbA_{1c} level did not predict recurrence (Table 2). This might be explained by the clinical relevance that HbA_{1c} level in patients with liver cirrhosis is often underestimated because of anemia. Therefore, the results of the present study suggest that the role of hyperinsulinemia is more important than that of hyperglycemia as reflected by HbA_{1c} in the recurrence of HCC. We therefore should pay attention to levels not only of glucose and HbA_{1c} but also of insulin when we follow patients who are at risk for HCC.

Interventional modalities to improve insulin-resistance could be a key to prevent the primary or recurrent HCC in patients complicated with such metabolic disorders. For instance, metformin and thiazolidine derivatives could be potential candidates for this purpose.[30,31] Oral branched-chain amino acid (BCAA) granules might be a candidate for preventing HCC recurrence in DM cases because, in addition to improving hypoalbuminemia,[32,33] this agent improves insulin resistance without stimulating insulin secretion.[34] Improvements of insulin resistance and glucose tolerance by BCAA have been reported in clinical trials.[35,36] Furthermore, Muto *et al.*[37] described that oral supplementation with BCAA granules inhibited liver carcinogenesis in HCV-positive liver cirrhosis with DM and obesity. Such effect of BCAA is also supported in experimental models.[38,39] These reports,[32–39] together with our present findings (Table 2 and Fig. 1b), suggest that insulin resistance is a significant risk factor for early recurrence of HCC and thus might be a critical target to prevent the recurrence and development of second primary HCC.

A limitation of this study is that the therapeutic effect of the primary HCC was judged as curative by imaging diagnosis but not by surgical pathology. Although the recurrent HCC developed apart from the primary tumor, we could not totally differentiate the recurrent lesion and a second primary HCC. A higher recurrence rate in this study (Fig. 1a) might be explained by this fact. Advanced medical imagings such as positron emission tomography would help solving such limitations in future study of this kind. Furthermore, the basic question, if de novo, namely, the first or second primary liver carcinogenesis is regulated by insulin resistance, should be addressed by recruiting HCC-free cirrhotics. However, such study requires a larger sample size and a longer observation period. Instead, we focused on the recurrent HCC, including possible second primary tumors, which develop at a 2–3-fold higher incidence than the first primary one.

Other study limitations are the short observation period, the small number of recruited patients and also the small number of detected events. Such a sample size essentially raises the possibility of β-error, including the absence of the statistical power of AFP and PIVKA-II for predicting the recurrence (Table 2). Previous reports agree that these tumor markers are risk factors of HCC recurrence,[2,6] although some criticisms remain.[4,5] In addition, we should state that the small number of events particularly restricts the reliability of multivariate analysis, while the calculation itself was possible in our study.

In conclusion, we presented for the first time that insulin resistance is significantly associated with the early recurrence of stage I HCC after curative RFA in HCV-positive patients. Increased HOMA-IR, which sensitively reflects insulin resistance, might be a useful biomarker for prediction of high-risk patients who cause early recurrence of HCC.

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Acyclic retinoid inhibits angiogenesis by suppressing the MAPK pathway

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Acyclic retinoid (ACR) is currently under clinical trial as an agent to suppress the recurrence of hepatocellular carcinoma (HCC) through its ability to induce apoptosis in premature HCC cells. ACR has an anticancer effect *in vivo* as well, although it shows weak apoptosis-inducing activity against mature HCC cells, suggesting the existence of an additional action mechanism. In this study, we investigated the antiangiogenic activity of ACR. ACR inhibited angiogenesis within chicken chorioallantoic membrane (CAM) in as similar a manner as all-*trans* retinoic acid (atRA). Although suppression of angiogenesis by atRA was partially rescued by the simultaneous addition of angiopoietin-1, suppression of angiogenesis by ACR was not rescued under the same condition at all. Conversely, although suppression of angiogenesis by ACR was partially inverted by the simultaneous addition of vascular endothelial growth factor (VEGF), suppression of angiogenesis by atRA was not affected under the same condition. These results suggested that mechanisms underlying the suppression of angiogenesis by ACR and atRA were different. ACR selectively inhibited the phosphorylation of VEGF receptor 2 (VEGFR2) and of extracellular signal-regulated kinase (ERK) without changing their protein expression levels, and inhibited endothelial cell growth, migration, and tube formation. The inhibition of the phosphorylation of ERK, endothelial growth, migration, tube formation, and angiogenesis by ACR was rescued by the overexpression of constitutively active mitogen-activated protein kinase (MAPK). Finally, ACR, but not atRA, inhibited HCC-induced angiogenesis in a xenografted CAM model. These results delineate the novel activity of ACR as an antiangiogenic through a strong inhibition of the VEGFR2 MAPK pathway.

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Angiogenesis has an important role in tumor growth by supplying nutrients and providing a route for metastasis.[1] Therefore, tumor angiogenesis is a good target for the treatment of solid cancers. Tumor cells induce angiogenesis by producing and releasing several angiogenic factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and angiopoietins (Angs).[1] The VEGF/VEGF receptor (VEGFR) signaling pathway is essential for drawing endothelial cells from preexisting blood vessels and in stimulating their growth,[2] whereas the Ang/Tie2 signaling pathway is important for sustaining the interaction between endothelial and mural cells and stabilizing the vasculature.

Retinoids (vitamin A and its derivatives) are natural fat-soluble hormones, the biological effects of which are believed to be mediated, all or in part, by the modulation of target gene expression through two families of nuclear receptors: retinoic acid receptors (RARs) and retinoid X receptors (RXRs).[3] Retinoids exert antitumor activity by modifying the transactivation of $p21^{CIP1}$, interferon receptor, and signal transduction and activator of transcription.[4,5] We previously reported that all-*trans* retinoic acid (atRA) inhibits angiogenesis on chorioallantoic membrane (CAM) through disruption of vascular remodeling by inducing Ang2 expression and suppressing Ang/Tie2 signaling.[6] Acyclic retinoid (ACR)

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is a synthetic retinoid and activates the RAR and RXR.[7] Oral administration of ACR for 12 months significantly reduced the incidence of post-therapeutic recurrence of hepatocellular carcinoma (HCC) compared with the placebo group.[8] In this study, ACR did not cause the typical toxic effects observed with conventional retinoids.[8] Now, ACR is under clinical trials as a chemopreventive drug against the recurrence of HCC. Nuclear receptor RXR in HCC is highly phosphorylated through the Ras-extracellular signal-regulated kinase (ERK) pathway, inactivated, and accumulates in the line as a dominant-negative receptor.[9,10] ACR inhibits the phosphorylation of RXR by inactivating the Ras-ERK pathway, recovering transactivation by retinoic acid, and induces apoptosis in human HCC cell lines.[9,10] ACR also has an anticancer effect *in vivo*.[11] However, it exerts only weak apoptosis-inducing activity against mature HCC cells *in vivo*. This result suggests an existence of an additional molecular mechanism underlying the anticancer effect of ACR. Therefore, we predicted that ACR might have antiangiogenic activity.

Herein, we found that in contrast to the antiangiogenic mechanism of atRA, ACR inhibited angiogenesis through the inhibition of the VEGF receptor mitogen-activated protein kinase (MAPK) pathway. Moreover, ACR suppressed HCC-induced angiogenesis in a xenografted CAM model. These results suggest that ACR will also be clinically useful as an antiangiogenic agent, in addition to its current usage as a chemopreventive agent.

MATERIALS AND METHODS

Reagents

Acyclic retinoid (2E,4E,6E,10E)-3,7,11,15-tetramethylhexadeca-2,4,6,10,14-pentaenoic acid) was provided by Kowa (Tokyo, Japan). AtRA was purchased from Sigma-Aldrich (St Louis, MO, USA). ACR was dissolved in ethanol and dimethyl sulfoxide (DMSO) to yield stock solutions of 10 mM and 1 M, respectively, whereas atRA was dissolved in ethanol to yield a stock solution of 17 mM.

Chicken CAM Assay

In vivo antiangiogenic activity of ACR and atRA was assessed by CAM assay as described previously.[12] In brief, fertilized Dekalb chicken eggs (Omiya Kakin, Saitama, Japan) were placed in a humidified egg incubator. After a 4.5-day incubation at 38°C, a 1% solution of methylcellulose containing ACR or atRA at various concentrations was loaded inside a silicon ring that was placed onto the surface of CAM. After a further incubation for 2 days, a fat emulsion was injected into the chorioallantois, so that the vascular networks stood out against the white background of the lipid. Antiangiogenic responses were evaluated under a stereomicroscope and photographed with a $\times 7.25$ objective. Quantitative analyses were carried out with angiogenesis-measuring software (ver.2.0; KURABO, Osaka, Japan).[12]

Matrigel Plug Assay

Matrigel (BD Biosciences, Bedford, MA, USA) was mixed with 200 units/ml heparin (Nacalai Tesque, Kyoto, Japan), with and without 50 ng/ml VEGF (Pepro Tech, Rocky Hill, NJ, USA) and 5 μM ACR in 0.1% DMSO. The matrigel mixture was injected subcutaneously into 5-week-old female C57BL/6 mice (Charles River, Yokohama, Japan). The mice were killed 7 days later. The matrigel plugs were removed and fixed in 4% paraformaldehyde for 4 h, dehydrated through a graded ethanol series, and embedded in paraffin (Nacalai Tesque). Vertical sections (5 μm) were mounted on slides and stained with hematoxylin and eosin, and observed under an inverted microscope (model DM IRB, Leica Microsystems, Wetzlar, Germany).

Cell Cultures

Human umbilical vein endothelial cells (HUVECs) and bovine aortic endothelial cells were cultured as described.[12] HepG2 cells, human HCC, were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal calf serum.

Transfection and Luciferase Assay

Transfection into HUVECs was carried out using a combination of LipofectAMINE 2000 Plus reagent (Invitrogen) and a constitutively active MAPK kinase vector (1.5 μg each per 35-mm dish).[13]

Western Blotting Analysis

After rinsing several times with TBS (20 mM Tris–HCl, 137 mM NaCl), cells were lysed in 1% Triton X-100 in 20 mM HEPES, pH 6.8, containing Complete protease inhibitor cocktail (1 tablet per 50 ml; Roche, Indianapolis, IN, USA), 1 mM EDTA, 1 mM PMSF, and 0.5 mM Na_3VO_5, and directly subjected to western analysis using phospho-VEGFR2-specific antibodies (1:1000 dilution; Cell Signaling Technology, Danvers, MA, USA), phospho-FGFR1-specific antibodies (1:1000 dilution; Cell Signaling Technology), or phospho-ERK-specific antibodies (1:2000 dilution, Cell Signaling Technology). Cell lysates were also subjected to western analysis using antibodies to VEGFR2, FGFR1, and ERK. Immunoreactive bands of proteins were detected with ECL-Plus chemiluminescence reagents (GE Healthcare, Buckinghamshire, UK).

In Vitro Tube Formation Assay

Tube formation by HUVECs on matrigel was assessed as described previously.[14] Unpolymerized matrigel (Becton Dickinson, Bedford, MA, USA) was diluted to a final concentration of 5 mg/ml with MCDB-131 medium, aliquoted 150 μl each into 24-well plates, and allowed to polymerize for 30 min at 37°C. HUVECs were transfected with a constitutively active MAPK kinase-expressing vector. Two days later, HUVECs were seeded onto the polymerized gel at 2×10^5 cells/well; thereafter, 100 ng/ml VEGF, 1 μM, 5 μM,

and 10 μM ACR and/or atRA were added, and incubated for 6 h. *In vitro* tube formation was examined under a phase-contrast microscope and photographed with a $\times 10$ objective.

HCC-Induced Angiogenesis in a Xenografted CAM Model

Hepatocellular carcinoma-induced angiogenesis in a xenografted CAM model was assessed as previously described.[15,16] HepG2 cell suspensions with or without 5 μM ACR or atRA were delivered at 4×10^5 cells per embryo onto the top of the CAM on day 8 using a gelatin sponge, called Gelform (Pfizer, New York, NY, USA) implant. After a further 4-day incubation, a fat emulsion was injected into the chorioallantois, so that the vascular networks stood out against the white background of the lipid. Antiangiogenic responses were evaluated under a stereomicroscope and photographed with a $\times 25$.

Statistical Analysis

Data are expressed as means $\pm$ s.d. Statistical significance was assessed by one-way analysis of variance, followed by Shaffer's *t*-test.

RESULTS

Comparison Between the Effects of ACR and atRA on Blood Vessel Formation in CAM

To determine whether ACR could inhibit *in vivo* angiogenesis, we carried out CAM assay (Figure 1). The formation of intricate vascular networks, developing within control CAM

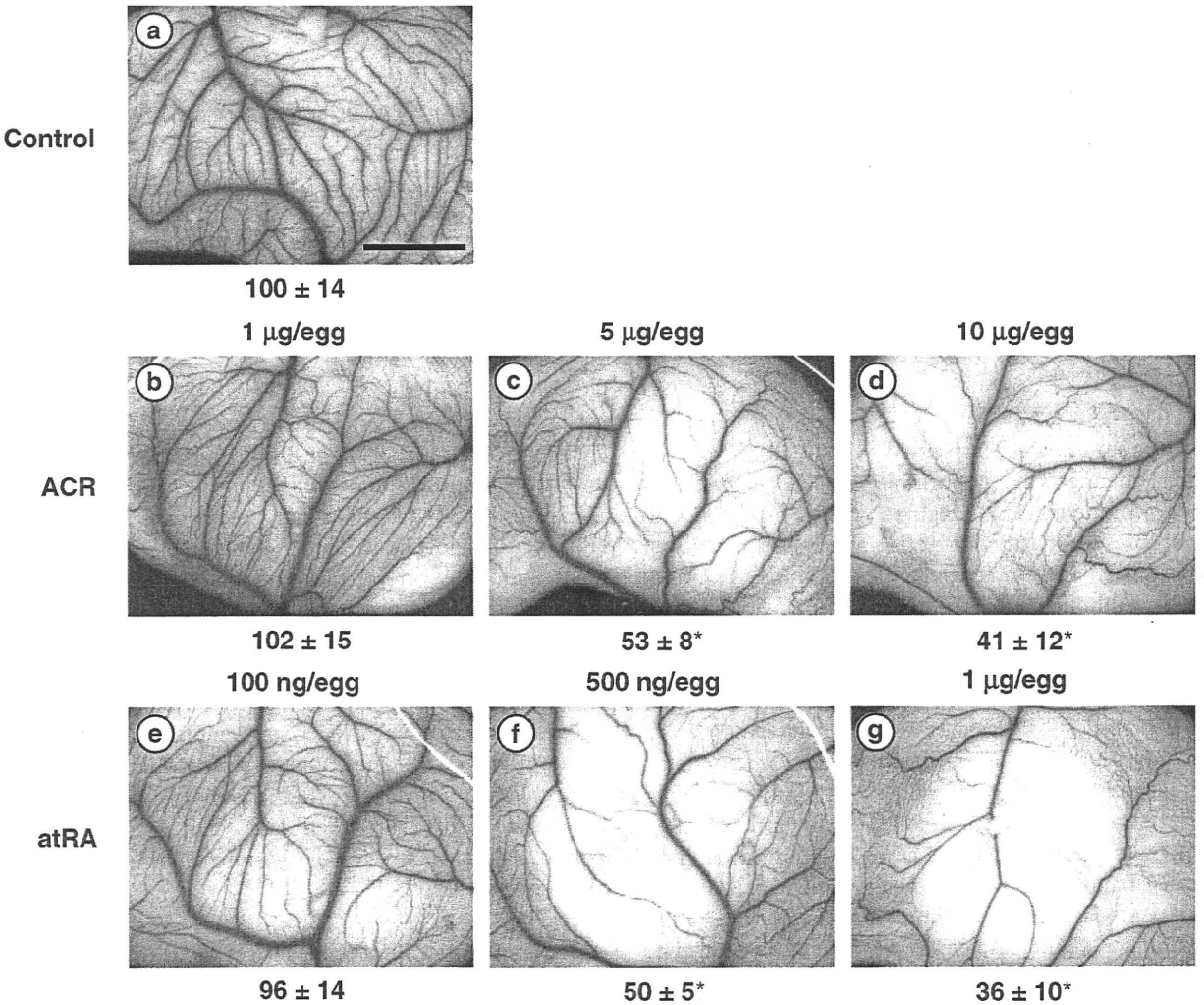

Figure 1 Suppression of *in vivo* angiogenesis in CAM by ACR and atRA. The 4.5-day-old CAMs were treated with ACR and atRA for 48 h, and then patterns of angiogenesis were photographed. Panel (**a**), vehicle (1% ethanol plus 1% DMSO); panel (**b**), 1 μg/egg ACR; panel (**c**), 5 μg/egg ACR; panel (**d**), 10 μg/egg ACR; panel (**e**), 100 ng/egg atRA; panel (**f**), 500 ng/egg atRA; panel (**g**), 1 μg/egg atRA. Scale bar, 5 mm. Total numbers of branches of blood vessels were analyzed with angiogenesis-measuring software and are shown under each panel. A total of 12 eggs (6 eggs per experiment $\times$ 2 experiments) were evaluated and representative results are shown. An asterisk indicates a significant difference ($P<0.05$) from the control.

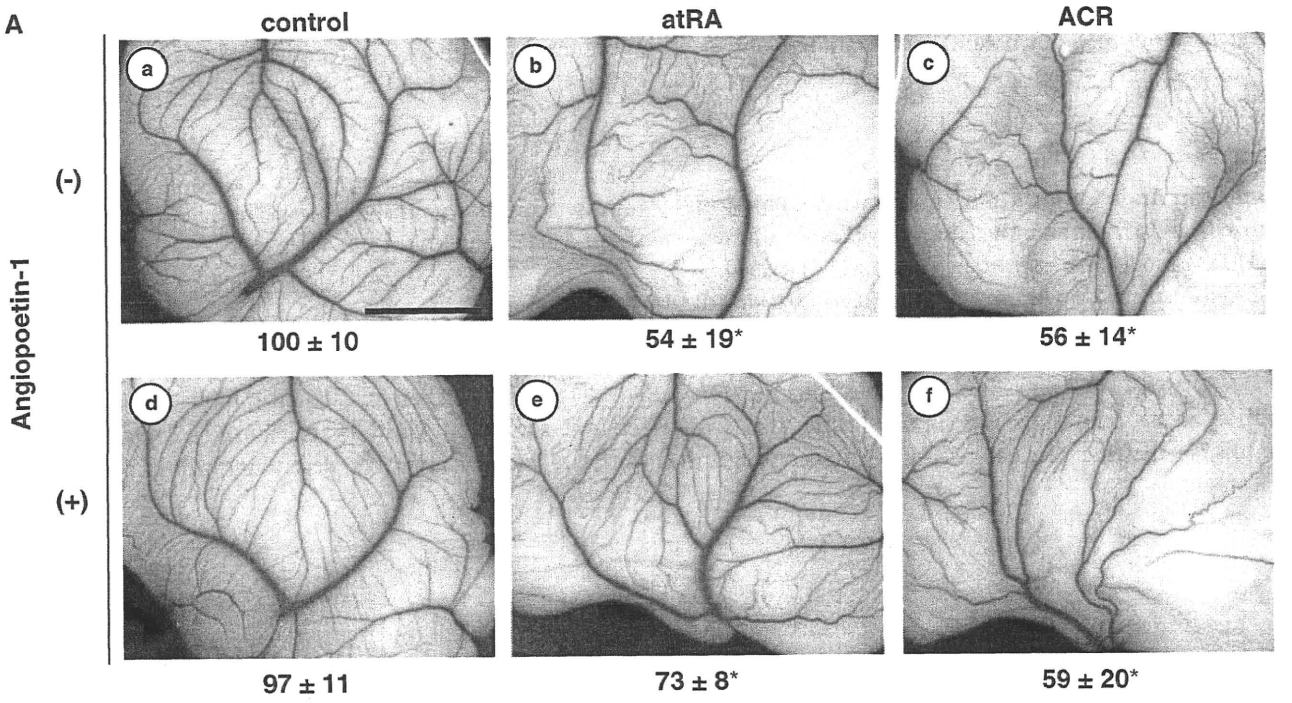

Figure 2 The antiangiogenic effect of atRA, but not ACR, was rescued by simultaneous treatment with Ang1 in CAM. (**A**) The 4.5-day-old CAMs were treated with ACR and atRA for 48 h and then patterns of angiogenesis were photographed. Panel (**a**), vehicle (1% ethanol plus 1% DMSO); panel (**b**), 500 ng/egg atRA; panel (**c**), 3 μg/egg ACR; panel (**d**), vehicle plus 300 ng/egg human recombinant Ang1; panel (**e**), 500 ng/egg atRA plus 300 ng/egg human recombinant Ang1; panel (**f**), 3 μg/egg ACR plus 300 ng/egg human recombinant Ang1. Scale bar, 5 mm. Total numbers of branches of blood vessels were analyzed with angiogenesis-measuring software and are shown under each panel. A total of 18 eggs (6 eggs per experiment × 3 experiments) were evaluated and representative results are shown. An asterisk indicates a significant difference ($P < 0.05$) from the control. This result shows the representative result from three independent experiments, all of which gave similar results.

(Figure 1, panel a), was suppressed with ACR in a dose-dependent manner at concentrations of 1–10 μg/egg (0.3–3.3 mM inside the ring) (Figure 1, panels b–d) and with atRA in a dose-dependent manner at about 10 times lower concentrations of 100–1000 ng/egg (33–333 μM) (Figure 1, panels e–g). Although the inhibition of angiogenesis with atRA was partially rescued by simultaneous treatment with Angl at a concentration of 300 ng/egg as consistently as we reported previously[6] (Figure 2A, panel e), inhibition of angiogenesis with ACR was not rescued with Angl at all (Figure 2A, panel f). Furthermore, although atRA stimulated the transactivation activity of the *Ang2* promoter twofold (Supplementary Figure 1, column 2), ACR hardly showed such an activity (Supplementary Figure 1, columns 3 and 4). On the other hand, inhibition of angiogenesis with ACR, but not with atRA, was rescued by simultaneous treatment with VEGF (compare Figure 3A, panels e and f). To determine whether ACR might inhibit VEGF-induced blood vessel formation *in vivo*, we examined the effect of ACR in the matrigel plug assay (Figure 3B). Invasion of cells into gels was observed in the control matrigel that contained VEGF without ACR (panel a). When ACR was included in the matrigel at a concentration of 5 μM, the VEGF-induced invasion of cells was inhibited by about 54% (panel b).

Effect of ACR and atRA on Endothelial Cell Growth, Migration, and Tube Formation

We investigated the molecular mechanism by which ACR inhibited angiogenesis. First, we compared the effect of ACR and atRA on vascular endothelial cells. ACR (5 μM) suppressed the growth, migration, and tube formation (Figure 4A, lane 2, closed column; Figure 4B, lane 2, closed column; Figure 4C, panel b, respectively). These suppressive effects by ACR were, all or in part, rescued by overexpressing a constitutive active *MEK* gene (Figure 4A, lane 2, open column; Figure 4B, lane 2, open column; Figure 4C, panel e, respectively). Conversely, atRA did not suppress, rather it enhanced all of them (Figure 4A, lane 3, closed column; Figure 4B, lane 3, closed column; Figure 4C, panel c, respectively).

ACR Suppressed Phosphorylation of VEGFR2 and ERK

Next, we examined the effect of ACR and atRA on the phosphorylation of angiogenic growth factor receptors expressed by endothelial cells. As seen in the upper panel of Figure 5a, induction of phosphorylated 230 kD VEGFR2 after VEGF treatment was blocked to about 20% by pretreatment with 5 μM ACR for 24 h (compare lanes 4 with 5). In contrast, pretreatment with 5 μM atRA for 24 h did not block the