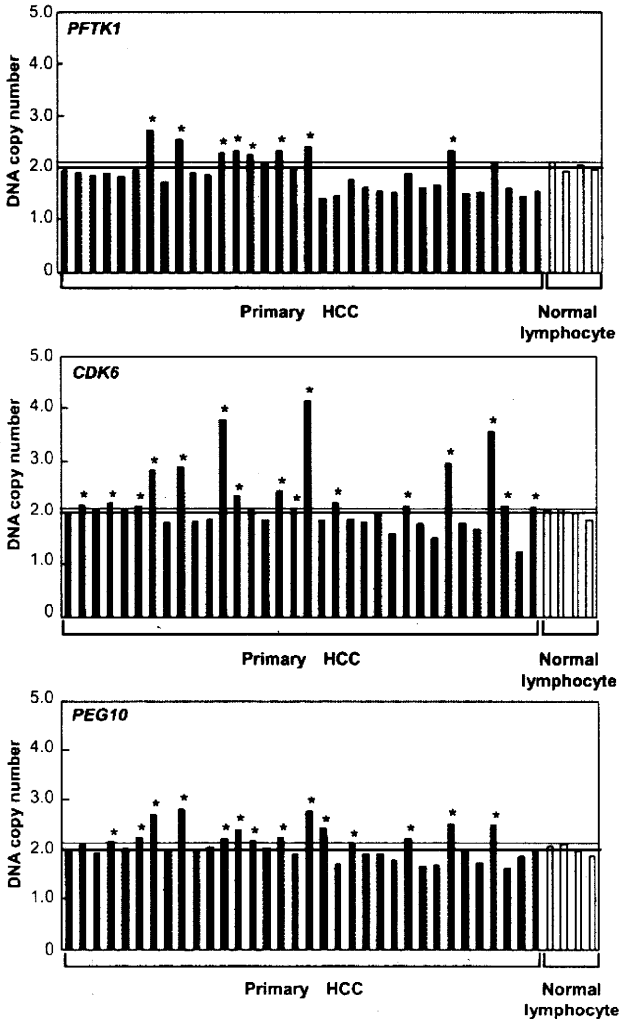


Fig. 3. DNA copy number of *CDK14* (previously *PFTK1*), *CDK6*, and *PEG10* in primary HCC tumors. The DNA copy number of each gene in 34 primary HCC tumors and four normal peripheral blood lymphocytes was determined as already described (for Fig. 2A). Asterisks indicate primary tumors showing copy number gain.

Table 2
Patient and tumor characteristics

| Characteristics | Value[a] |
|---|---|
| Sample size[b] | $n$ = 41 |
| Sex | |
| Male | 33 |
| Female | 8 |
| Median age, yr (range) | 67 (35–79) |
| Etiology of liver disease | |
| Hepatitis B virus | 9 |
| Hepatitis C virus | 21 |
| Other | 11 |
| Median tumor size, cm (range) | 5.0 (1.9–26) |
| Tumors, single or multiple | |
| Single | 26 |
| Multiple | 15 |
| Tumor differentiation | |
| Well | 7 |
| Moderate | 20 |
| Poor | 14 |
| Stage[c] | |
| I | 1 |
| II | 15 |
| III | 14 |
| IV | 11 |
| Background liver tissue | |
| Normal | 4 |
| Chronic hepatitis | 18 |
| Liver cirrhosis | 19 |
| Child–Pugh classification | |
| A | 40 |
| B | 1 |
| C | 0 |
| Median α-fetoprotein, ng/mL (range) | 14.9 (0.9–114,859) |

[a] Where no other unit is specified, values refer to number of patients.
[b] All patients were of Japanese ethnicity.
[c] International Union Against Cancer tumor–node–metastasis (UICC TNM) classification of malignant tumor.

adjacent nontumorous liver tissues and that upregulation of the gene correlated with both advanced metastatic HCCs and microvascular invasion [26]. Further studies are required to clarify the potential role of *CDK14* in HCC.

*PEG10* was first identified as an imprinted gene that is paternally expressed and maternally silenced [14]. It has been suggested that *PEG10* is derived from a retrotransposon that was previously integrated into the mammalian genome [14]. The overexpression of *PEG10* in HCC observed in this study is consistent with the previously reported overexpression of *PEG10* in tumors, including HCC [15–19], and in B-cell leukemia [27,28]. Furthermore, several lines of evidence suggest that *PEG10* may be important for the regulation of cell proliferation and cell death: *PEG10* is overexpressed in regenerating mouse liver [16], knockdown of *PEG10* inhibits the proliferation of cancer cells [29], and the PEG10 protein inhibits cell death mediated by SIAH1, a mediator of apoptosis [15]. The importance of *PEG10* for cell regulation is further suggested by the fact that targeted disruption of the mouse *Peg10* gene results in early embryonic lethality due to defects in the placenta [30].

The exact mechanism by which *PEG10* signals is unclear, but it is known to interact with members of the TGF-β receptor family [31]. Further evidence of a potential role for *PEG10* in cell growth and carcinogenesis is that its expression can be regulated by the protooncogene *MYC* [29], by E2F transcription factors that modulate the cell cycle [32], and by the sex hormone androgen [33].

Although the exact mechanism of *PEG10* function in tumors remains to be elucidated, and the findings in this study must be verified in future studies using a larger sample number, the data presented in this work suggest a role for amplification and overexpression of *PEG10* in hepatocarcinogenesis.

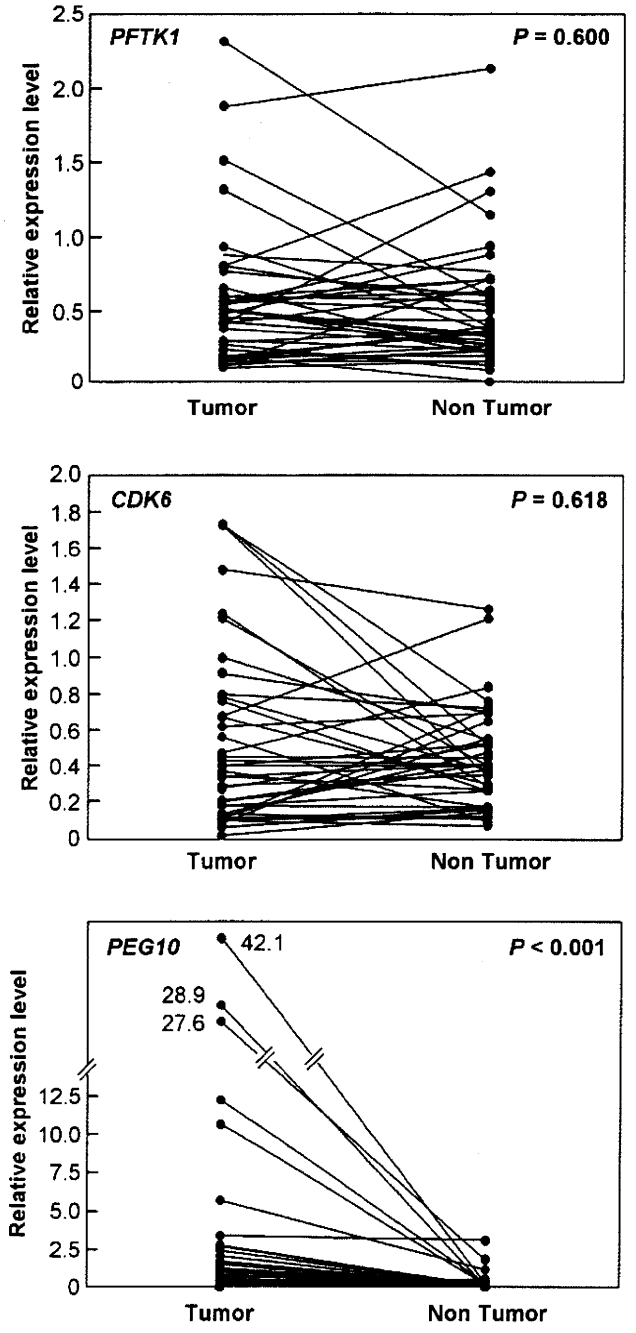


Fig. 4. Expression levels of *CDK14* (previously *PFTK1*), *CDK6*, and *PEG10* in paired tumor (T) and nontumor tissues (NT) from 41 patients with primary HCC. The expression level of each gene was determined as already described (for Fig. 2B). *CDK14*, *CDK6*, and *PEG10* were overexpressed in 22 (54%), 21 (51%), and 30 (73%) of the 41 tumors, respectively, compared with their nontumorous counterparts.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at 10.1016/j.cancergencyto.2010.01.004.

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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<br>≥5.0 log IU/mL<br>≥300 fmol/L<br>≥1 Meq/mL | • Peg-IFN-α2b (Peg-Intron) + ribavirin (Rebetol) for 48–72 weeks<br>• Peg-IFN-α2a (Pegasys) + ribavirin (Copegus) for 48–72 weeks | • Peg-IFN-α2b (Peg-Intron) + ribavirin (Rebetol) for 24 weeks |
| Low viral load<br><5.0 log IU/mL<br><300 fmol/L<br><1 Meq/mL | • Standard IFN for 24 weeks<br>• Peg-IFN-α2a (Pegasys) for 24–48 weeks | • Standard IFN for 8–24 weeks<br>• 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.<br>• 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.<br>• 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.<br>• 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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***Case Report***

# Relapse of hepatitis C in a pegylated-interferon-α-2b plus ribavirin-treated sustained virological responder

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A 41-year-old woman with chronic hepatitis C was treated with pegylated-interferon (PEG-IFN)-α-2b plus ribavirin for 24 weeks. She had hepatitis C virus (HCV) genotype 2a (1600 KIU/mL), and her liver histology showed mild inflammation and fibrosis. Four weeks after the start of the therapy, she achieved a rapid virological response (RVR) and then a sustained virological response (SVR). Serum alanine aminotransferase (ALT) levels remained within normal ranges and HCV RNA continued to be negative. However, ALT levels flared with the re-emergence of HCV RNA in the serum 1.5 years after discontinuation of therapy. HCV RNA obtained from sera before therapy and after relapse shared a 98.6% homology with the E2 region, and phylogenetic analyses indicated that they were the same HCV strain. These results eliminated the possibility of a re-infection and strongly indicated a late relapse of the disease. Therefore, follow-up is necessary for chronic hepatitis C patients after SVR, even if they respond well to therapy, including RVR.

**Key words:** chronic hepatitis C, genotype 2a, sustained virological response, relapse, phylogenetic analyses.

## INTRODUCTION

HEPATITIS C VIRUS (HCV) is an important cause of chronic liver disease, and more than 170 million people are infected worldwide, including 1.5–2 million people in Japan.[1] Approximately 70% of Japanese chronic hepatitis C patients are infected with genotype 1b, whereas the rest are infected with genotypes 2a or 2b.[2] At present, pegylated-interferon (PEG-IFN)-α plus ribavirin is the optimal therapy for chronic hepatitis C. Sustained virological response (SVR), defined as undetectable serum HCV RNA 24 weeks after therapy completion, is the primary goal of this therapy. Approximately 80% of patients infected with genotypes 2 or 3 achieve SVR after 24 weeks of treatment, whereas approximately 50% patients with genotype 1 achieve SVR after 48 weeks of treatment.

Late relapse, defined as a HCV RNA reappearance in serum after achieving SVR, is rare in SVR patients. Furthermore, distinguishing relapse from re-infection is difficult without comparing the HCV nucleotide sequence before the start of the therapy and after relapse. Here we describe the clinical course of an HCV genotype 2a-infected woman treated with PEG-IFN-α plus ribavirin for 24 weeks. She achieved a rapid virological response (RVR) because HCV RNA was undetectable by a qualitative polymerase chain reaction (PCR) assay 4 weeks after initiating therapy. However, she achieved SVR and suffered a relapse of chronic hepatitis C 1.5 years after therapy discontinuation. We analyzed nucleotide sequences within the E2 region of HCV RNA containing the hypervariable region (HVR)1 and the IFN sensitivity-determining region (ISDR) of nonstructural protein 5A (NS5A), using sera before treatment and after relapse and confirmed that they were the same HCV strain.

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## CASE REPORT

A 41-YEAR-OLD WOMAN had elevated serum alanine aminotransferase (ALT; 138 IU/L) and aspartate aminotransferase (AST; 248 IU/L) levels on a routine medical check-up in mid-September 2005. Because of liver dysfunction in October 2005, she visited Aiseikai Yamashina General Hospital for further examination. She had no family history of liver diseases. Her height was 145 cm and weight was 42 kg. No abnormalities were detected on physical examination; her average alcohol intake was less than 20 g/week. She had no history of i.v. drug abuse.

Table 1 shows the laboratory data. On 25 October 2005, transaminase and biliary enzyme levels were elevated. Serum anti-HCV antibody was positive. The HCV RNA load was 2400 KIU/mL (Amplicor Monitor ver. 2.0; Roche Diagnostic Systems, Tokyo, Japan), and she had the 2a HCV genotype. She hesitated to undergo IFN treatment in the beginning. We strictly prohibited her from alcohol and started treating her with 600 mg/day oral ursodeoxycholic acid (UDCA) and 40 mL i.v. glycyrrhizin twice a week (Stronger Neo-Minophagen C, SNMC). Her liver functions improved significantly, but did not normalize following treatment with UDCA and SNMC.

She was admitted to Aiseikai Yamashina General Hospital on 3 February 2006, and PEG-IFN plus ribavirin treatment was initiated for chronic hepatitis C. Abdominal ultrasonography revealed that the liver was almost normal in size, the edge was sharp and the internal echo was slightly coarse. Other tests including hepatitis B virus PCR, HBc antibody, HIV 1/2 antibodies, antinuclear antibody, anti-mitochondrial antibody, serum ceruloplasmin, copper and ferritin were normal. The laboratory test results obtained on 3 February 2006 are presented in Table 1. The liver biopsy specimen before treatment revealed mild fibrosis with mild inflammation, which was graded as A1F1 according to the classification of Ichida *et al.* or Bedossa and Poynard.[3,4] She received combination therapy consisting of PEG-IFN-α-2b (1.5 μg/kg; 60 μg) once a week plus 600 mg ribavirin daily.

After therapy initiation, ALT levels declined rapidly and remained within the normal range after completion of the treatment. Serum HCV RNA levels were measured by a quantitative PCR assay (Amplicor HCV Monitor ver. 2.0) before therapy initiation and after relapse and by a qualitative PCR assay (Amplicor HCV Test ver. 2.0) at 4, 8, 12, 16, 20 and 24 weeks (all during the treatment period) as well as at 4, 8, 12, 16, 20 and 24 weeks after therapy completion. Serum HCV RNA was qualitatively

Table 1 Laboratory findings

| | Normal | Initial visit (10/20/2005) | Before PEG-IFN + Rib (2/3/2006) | After relapse (1/25/2008) |
|---|---|---|---|---|
| White blood cell (μL) | (3900–9300) | 9070 | 8110 | 8800 |
| Red blood cell (×10⁴/μl) | (425–571) | 458 | 433 | 412 |
| Platelet (×10⁴/μL) | (12.7–35.6) | 29.2 | 29.7 | 27.3 |
| PT (%) | | 85% | 82% | 92% |
| Albumin (g/dL) | (4.0–5.0) | 4.2 | 4.2 | 4.1 |
| T. Bil (mg/dL) | (0.3–1.2) | 0.6 | 0.4 | 0.4 |
| AST (IU/l) | (<33) | 87 | 35 | 61 |
| ALT (IU/l) | (<35) | 195 | 38 | 96 |
| ALP (IU/l) | (115–360) | 278 | 240 | 247 |
| γ-GTP (IU/l) | (<47) | 256 | 48 | 88 |
| RPR | (–) | NA | (–) | (–) |
| HBsAg | (–) | (–) | (–) | (–) |
| ANA | (<40) | NA | <40 | <40 |
| Type IV collagen 7S (ng/mL) | (<5) | NA | 3.8 | 3.2 |
| Serum ferritin (ng/mL) | (5.3–179.7) | NA | 50.7 | NA |
| HCV RNA (Amplicor Monitor ver. 2.0) (KIU/mL) | (–) | 2400 | 1600 | 2600 |
| HCV genotype | | 2a | 2a | 2a |

ALP, alkaline phosphatase; ALT, alanine aminotransferase; ANA, antinuclear antibody; AST, aspirate aminotransferase; γ-GTP, γ-glutamyltranspeptidase; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; NA, not available; PT, prothrombin time; RPR, Rapid Plasma Reagin; T. Bil., total bilirubin.

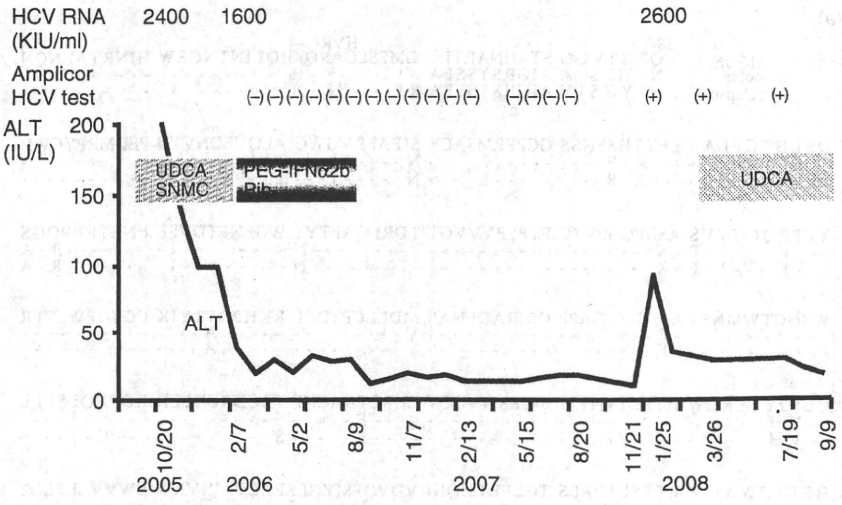


**Figure 1** Levels of alanine aminotransferase (ALT) and hepatitis C virus (HCV) RNA load during the clinical course. Pegylated-interferon (PEG-IFN)-α plus ribavirin combination therapy was started in February 2006 and continued for 24 weeks until August 2006. HCV RNA was undetectable within 4 weeks. ALT levels remained within normal ranges until November 2007. Relapse occurred in January 2008. SNMC, Stronger Neo-Minophagen C; UDCA, ursodeoxycholic acid.

undetectable 4 weeks after therapy initiation and remained undetectable 6 months after therapy completion. The patient had few side-effects, and the treatment was completed without reducing either of the drugs.

After achieving SVR, she underwent monthly liver function tests, and a qualitative PCR assay was performed occasionally. In December 2007, her liver function tests deteriorated. AST/ALT levels were elevated, and she tested positive for HCV RNA on 25 January 2008 (Fig. 1, Table 1). A quantitative PCR assay indicated that the HCV RNA titer was 2600 IU/L, and the HCV genotype was 2a. The patient again started taking 600 mg UDCA daily, and ALT returned to low levels (~30–40 IU/L). Repeated tests showed that HCV RNA was persistently positive.

To determine if HCV RNA that appeared 1.5 years after treatment completion was identical to that before therapy, we compared the nucleotide sequences of the two coding regions, namely, the E2 region containing HVR1 and ISDR of NS5A. Informed consent was obtained from the patient before analysis, and the serum samples obtained before treatment and after relapse were stored at −80°C until use.

Virological analyses proceeded as follows. To reconfirm HCV genotyping, direct sequencing of the 5′-untranslated region was performed, as described previously.[5,6] The genotypes were classified according to the nomenclature proposed in a previous report and were determined to be 2a in both the samples. HCV RNA was amplified by reverse transcription (RT)–PCR to directly sequence the E2 and ISDR regions.

In brief, RNA was extracted from 140 μL sera using a commercially available kit (QIAamp viral RNA kit; QIAGEN, Valencia, CA, USA) and dissolved in 50 μL diethylpyrocarbonate-treated water. This sample was used for RT with random hexamer primers (SuperScript III First-Strand Synthesis System for RT–PCR cDNA synthesis kit; Invitrogen, Carlsbad, CA, USA). The E2 region was amplified by nested PCR, and ISDR regions were amplified by hemi-nested PCR. Each 50-μL PCR reaction contained 100 nM of each primer, 1 ng template cDNA, 5 μL 10× Ex Taq buffer, 4 μL deoxyribonucleotide triphosphate mixture, and 1.25 U of Takara Ex Taq HS (Takara Ex Taq, Otsu, Japan).

The PCR primers were set based on a reference HCV sequence (accession no. AF177036).

The first PCR primer sequences for E2 were: sense (1422, 1441) 5′-ACTTCTCTATGCAGGGAGCG-3′ and antisense (2437, 2418) 5′-GTTTTGGTGGAGGTGGAG AA-3′; and sense (2171, 2190) 5′-TGCCTGATCGACTA CCCCTA-3′ and antisense (2730, 2711) 5′-AGGCC AGTGAGGGAATAGGT-3′. The second PCR primer sequences for E2 were: sense (1453, 1472) 5′-CGTT GTCATCCTTCTGTTGG-3′ and antisense (2261, 2242) 5′-CAACCCCTCCCACATACATC-3′; and sense (2189, 2208) 5′-TACAGGCTCTGGCATTACCC-3′ antisense (2698, 2679) 5′-TACCCGACCCTTGATGTACC-3′.

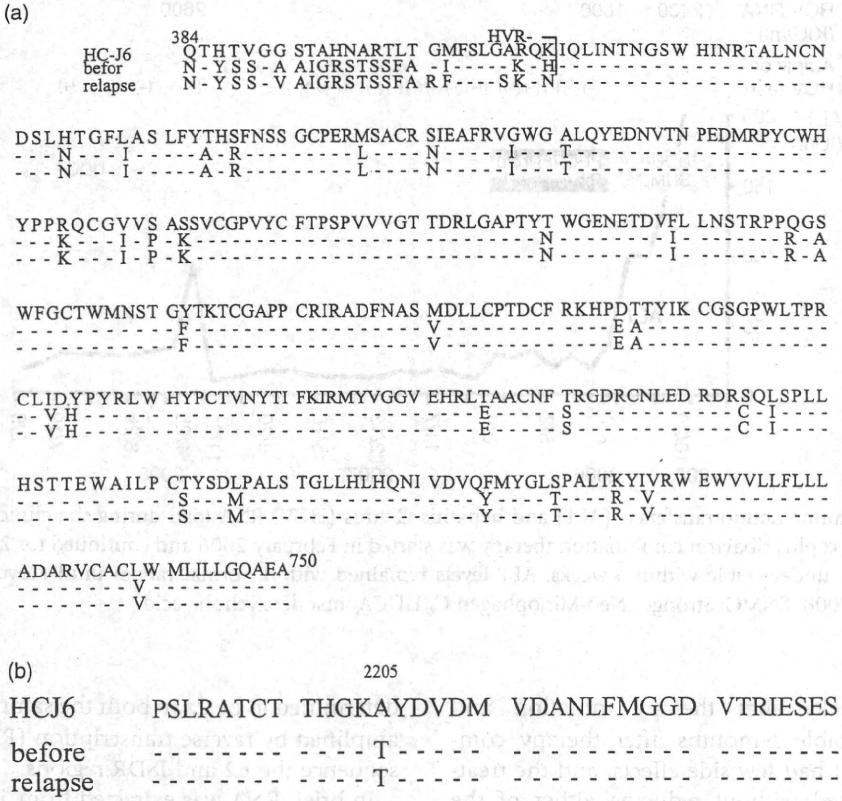


**Figure 2** (a) Comparison of the hepatitis C virus (HCV) E2 (top) region amino acid sequences. Sequencing was performed for serum samples obtained before therapy on 3 February 2006 and after relapse on 25 January 2008. Sequences were aligned against the HCJ6 HCV genotype 2a reference sequence (GenBank accession no. D00944). Hypervariable region (HVR)1 positions of E2 are indicated by numbers corresponding to the amino acid positions within the HCV genotype 2a polyprotein of the reference sequence. There were five amino acid mutations in these regions between the two samples. (b) IFN sensitivity-determining region (ISDR) sequences before therapy and after relapse showed the same single mutation at codon 2205. Sequences were aligned against the HCJ6 HCV genotype 2a reference sequence (GenBank accession no. D00944). ISDR positions are indicated as numbers corresponding to the amino acid positions within the HCV genotype 2a polyprotein of the reference sequence.

The first PCR primer sequences for ISDR were: sense (6866, 6885) 5′-ACGTCCATGCTAACAGACCC-3′ and antisense (7185, 7166) 5′-GGGAATCTCTTCTTGGG GAG-3′. The second PCR primer sequences for ISDR were the sense primer from the first-round PCR and a new antisense primer (7109, 7090) 5′-CGAGAG AGTCCAGAACGACC-3′.[7]

Polymerase chain reaction products were separated by electrophoresis on 1% or 2% agarose gels, stained with ethidium bromide, and visualized under ultraviolet light. The products were purified and sequenced with second-round PCR primers, using a dye terminator sequencing kit (BigDye Terminator ver. 1.1 cycle sequencing kit; Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 310 genetic analyzer (Applied Biosystems). Sequence alignments and phylogenetic analyses were performed with MEGA4 software.[8] Nucleotide sequences obtained from the two samples were compared to 23 published HCV genotype 2a sequences. A phylogenetic tree was constructed by the neighbor-joining method[9] based on the nucleotide sequence of the E2 region, with pairwise distances being estimated using the Kimura two-parameter method. Bootstrap values were determined on 1000 re-samplings of data sets.[10]

The E2 nucleotide sequences before treatment and after relapse were 98.6% similar. Except HVR1, two of the samples were 99.0% similar. The amino acid sequences in the E2 region, except HVR1, were identical between the two samples. There was a difference of five amino acids in HVR1 (Fig. 2a).

When compared to known HCV isolates of various genotypes whose entire coding region sequence has

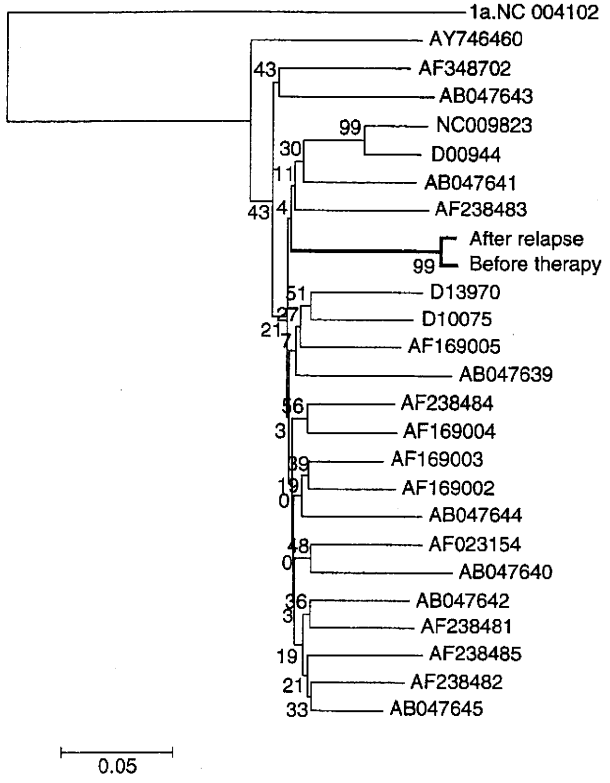


**Figure 3** A phylogenetic tree was constructed by the neighbor-joining method based on the nucleotide sequence of the E2 region (1101 nt) of 23 genotype 2a strains using the genotype 1a hepatitis C virus (HCV) isolate (NC004102) as an outgroup. The isolates obtained in the present study before therapy and after relapse are indicated in bold letters for clarity. Twenty-three reported genotype 2a HCV isolates, whose entire coding region sequence is known, are included for comparison, and their accession numbers are shown. Bootstrap values were determined on 1000 re-samplings of datasets.

been determined, the two isolates were the closest to a particular genotype 2a HCV isolate (accession no. AB047645) with 87.3% nucleotide sequence similarity; however, the isolates were only 64.4–72.7% similar to known genotype non 2a (1a NC004102, 1b D90208, 2b D01221, 3a D17763, 4a Y11604, 5a Y13184 and 6a Y12083). The phylogenetic tree of 23 genotype 2a HCV isolates, constructed based on the E2 1101-nucleotide sequence, indicated that the sample obtained after relapse bifurcated from a common trunk with the sample before treatment, and we confirmed that these two samples were the closest to each other among all known genotype 2a HCV isolates (Fig. 3). The results of sequencing analysis before therapy and after re-emergence of viremia ruled out the possibility of a re-infection and strongly suggested a late relapse of chronic hepatitis C.

Interferon sensitivity-determining region sequences before treatment and after relapse showed 98.9% similarity. The amino acid sequences of the two ISDR regions were completely identical. The sequences of the HCJ6 (accession no. D00944) strain were defined as wild-type ISDR, and those that deviated from this strain were defined as the mutant type. ISDR sequences before treatment and after relapse were different in only one codon (2205) when compared with the reference HCJ6 sequence (Fig. 2b).

## DISCUSSION

IN THIS STUDY, we clarified that E2 1101-nucleotide sequences of HCV isolated from sera before treatment and after relapse shared a 98.6% homology. Furthermore, phylogenetic analyses classified these two samples as the same strain. These results ruled out the possibility of a re-infection and strongly suggested a late relapse of chronic hepatitis C.

Hepatitis C virus is an RNA virus belonging to the genus *Hepacivirus* in the Flaviviridae family. Similar to other RNA viruses, HCV circulates as a genetically distinct population, demonstrating a quasispecies.[11] HCV HVR1, which is composed of 27 amino acids and is located at the 5′ terminus of the E2 gene, is highly variable among and within infected patients,[12–14] so it can be used to identify individual HCV isolates.[15,16] HCV HVR1 changes rapidly over time in the same individual. Our pairwise sequences were not completely identical but shared a high homology, which was equal to the homology reported previously.[16] These results suggest that the patient achieved SVR but suffered a relapse of hepatitis C after 1.5 years.

Some reports have indicated that in a majority of patients with SVR, low-level HCV RNA can be detected in lymphocytes, monocytes/macrophages and liver, despite constantly undetectable HCV RNA in sera.[17–19] This "occult" persistence of HCV replication could potentially play a role in late recurrence after treatment. However, the significance/mechanism of HCV RNA persistence in the liver or peripheral blood mononuclear cells is still uncertain, and data regarding occult persistence are conflicting.[20] Moreover, it is unclear as to how many of these late relapse patients were "true" relapsers and how many were re-infected. The relapse rates after SVR in IFN monotherapy are approximately 5–10%.[21,22] Nakayama *et al.* recently reported a late relapse of

hepatitis C after IFN-α plus ribavirin therapy and summarized late relapsing cases in Japan.[23] They indicated that compared to reports from foreign countries, late relapses were very rare in Japan, particularly after IFN and ribavirin therapy and that the relapse interval was principally restricted to within 2 years after therapy completion. Four hundred and fifty-five chronic hepatitis C patients were cured by PEG-IFN plus ribavirin therapy in the study group of Kyoto Prefectural University of Medicine and related hospitals, and this is the only case of late relapse to date (Itoh Y. *et al.*, 2009 unpublished data). This may be the first reported case of relapse after SVR with PEG-IFN plus ribavirin therapy.

Several host and viral characteristics are associated with the likelihood of response to IFN-based therapy. The HCV genotype and viral load are the most important viral predictors, and the ISDR sequence variation[24] and substitutions of amino acids 70 and/or 91 in the core region[25] within the HCV genome have been recently advocated in patients with genotype 1. It is interesting to note that only one amino acid varied in ISDR compared to the reference sequence in our case. For patients with HCV genotype 2a, Hayashi *et al.* reported that ISDR amino acid variations compared to the reference sequence and RVR as well as negative HCV at 4 weeks are important predictors of SVR in PEG-IFN monotherapy.[7] ISDR interacts with interferon-inducible double-stranded RNA-activated protein kinase (PKR) and inactivates HCV replication *in vitro*.[26] According to the report by Hayashi *et al.*,[7] an A-to-T mutation at codon 2205 (Fig. 2b) can be interpreted as wild type, and hence ISDR in this case contained no mutations, which may have influenced HCV RNA re-emergence after achieving SVR.

Patients with RVR, defined as a negative HCV RNA at 4 weeks, are more likely to have SVR.[7,27] In our case, HCV RNA was negative at 4 weeks, which indicated that this case may be cured; however, relapse of hepatitis C occurred after 1.5 years. The data concerning the efficacy of re-treatment of genotype 2 chronic hepatitis C are limited. According to the report by Moucari *et al.*,[28] there is a higher rate of SVR in genotype non-1 relapsers. Therefore, our patient could be retreated with a second PEG-IFN plus ribavirin combination therapy. However, because the patient is 41 years old and has stage F1 hepatic fibrosis, we will recommend that she wait for a new drug such as a protease inhibitor. Further research for unknown factors to predict late relapse after achieving SVR might be necessary.

In conclusion, SVR patients may have a potential risk of HCV reactivation. Annual surveillance including HCV RNA testing seems clinically reasonable for detecting spontaneous relapse and recurrence of hepatitis C in SVR patients.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

# Steatosis and hepatic expression of genes regulating lipid metabolism in Japanese patients infected with hepatitis C virus

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**Abstract**
*Purpose* Steatosis is a histological finding associated with the progression of chronic hepatitis C. The aims of this study were to elucidate risk factors associated with steatosis and to evaluate the association between steatosis and hepatic expression of genes regulating lipid metabolism.
*Methods* We analyzed 297 Japanese patients infected with hepatitis C virus and a subgroup of 100 patients who lack metabolic factors for steatosis. We determined intrahepatic mRNA levels of 18 genes regulating lipid metabolism in these 100 patients using real-time reverse transcription-polymerase chain reaction. Levels of peroxisome proliferator-activated receptor α and sterol regulatory element-binding protein 1 proteins were assessed by immunohistochemistry.
*Results* Steatosis was present in 171 (57%) of 297 patients. The presence of steatosis was independently associated with a higher body mass index, higher levels of γ-glutamyl transpeptidase and triglyceride, and a higher fibrosis stage. Steatosis was present in 43 (43%) of 100 patients lacking metabolic factors. Levels of mRNA and protein of peroxisome proliferator-activated receptor α, which regulates β-oxidation of fatty acid, were lower in patients with steatosis than in patients without steatosis.
*Conclusions* These findings indicate that impaired degradation of lipid may contribute to the development of hepatitis C virus-related steatosis.

**Keywords** Steatosis · Hepatitis C virus · Fibrosis · Gene expression · Peroxisome proliferator-activated receptor α

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## Introduction

The prevalence of hepatic steatosis ranges from 40 to 86% (mean ~55%) in patients infected with hepatitis C virus (HCV) [1]. This range is higher than in the general population of adults in the Western world (20–30%) [2]. Steatosis appears to be associated with a more rapid progression of liver fibrosis and a lower response to interferon-α-based therapy [3–5].

Patients with HCV infection may have metabolic cofactors, such as obesity, diabetes, and alcohol abuse that contribute to the development of fatty liver. It is likely that two types of steatosis, viral and metabolic, coexist in patients with chronic hepatitis C [1, 3]. Known risk factors associated with steatosis include HCV genotype 3, a higher body mass index (BMI), diabetes, hyperlipidemia, ongoing alcohol abuse, older age, the presence of fibrosis, and

hepatic inflammation [1, 5]. However, different populations may have different risk factors for steatosis, and the distribution of HCV genotype differs from region to region. For example, HCV genotype 3, which is thought to be directly responsible for steatosis [6–8], is far less frequent in Japan than in Europe [7] or the United States [9].

Although the mechanisms of HCV-related steatosis are not well known, several viral and host factors appear to be involved [3]. In vitro studies [10] and a transgenic mouse models [11] have shown that HCV core protein can induce steatosis. HCV core protein, in turn, inhibits the activity of microsomal triglyceride transfer protein, which is essential for the assembly and secretion of very low density lipoproteins [12]. The intrahepatic levels of microsomal triglyceride transfer protein mRNA show an inverse correlation with the degree of steatosis in patients with chronic hepatitis C [13]. HCV infection and HCV core protein upregulates the expression of sterol regulatory element-binding protein 1 (SREBP1), a key transcriptional factor that activates the expression of genes involved in lipid synthesis [14, 15]. In addition, HCV core protein binds to retinoid X receptor $\alpha$, a transcriptional regulator that controls many cellular functions including lipid metabolism [16]. HCV core protein also down-regulates the expression of peroxisome proliferator-activated receptor $\alpha$ (PPAR$\alpha$) and carnitine palmitoyl transferase 1 (CPT1) [17, 18], and the mRNA levels of PPAR$\alpha$ and CPT1 are found to be reduced in patients with chronic HCV infection [19].

In the present study, we investigated the risk factors associated with steatosis in Japanese patients with chronic HCV infection. To elucidate the molecular mechanisms underlying HCV-related (i.e., viral) steatosis, we also systematically measured the intrahepatic expression levels of genes that regulate lipid degradation, secretion, synthesis, and uptake in patients who lack metabolic factors for steatosis.

## Methods

### Patients

The study included a total of 297 Japanese patients with chronic HCV infection who underwent liver biopsy between April 2004 and June 2006 at the Hospital of Kyoto Prefectural University of Medicine, Kyoto, Japan. To eliminate selection biases, the patients were recruited consecutively. Inclusion criteria were as follows: patients older than 18 years, positive for anti-HCV (third-generation enzyme immunoassay; Chiron, Emeryville, CA), and positive for serum HCV-RNA (Amplicor HCV assay; Roche Diagnostic Systems, Tokyo, Japan). Exclusion criteria were as follows: positive for hepatitis B virus surface antigen (radioimmunoassay; Dainabot, Tokyo, Japan); other types of liver diseases, including primary biliary cirrhosis, autoimmune hepatitis, alcoholic liver disease, Wilson's disease, or hemochromatosis; coinfection with human immunodeficiency virus; treated with antiviral or immunosuppressive agents within 6 months of enrollment; treated with drugs known to produce hepatic steatosis, including corticosteroids, high dose estrogen, methotrexate, or amiodarone within 6 months of enrollment; a history of gastrointestinal bypass surgery.

BMI was calculated using the following formula: weight in kilograms/(height in meters)$^2$. Obesity was defined as a BMI $\geq$25, according to the criteria of the Japan Society for the Study of Obesity [20]. Diabetes was defined as a fasting glucose level $\geq$126 mg/dl or by the use of insulin or oral hypoglycemic agents to control blood glucose. The ongoing alcohol intake per week recorded and converted to average grams per day. Significant alcohol intake was defined as consumption of >20 g/day.

The Ethics Committee of the Kyoto Prefectural University of Medicine approved this study. Informed consent was obtained from each patient in accordance with the Helsinki declaration.

### Laboratory tests

Venous blood samples were taken in the morning after a 12-h overnight fast. The laboratory evaluation included a blood cell count and the measurement of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), $\gamma$-glutamyl transpeptidase ($\gamma$-GTP), total cholesterol, triglyceride, and fasting plasma glucose. These parameters were measured using the standard clinical chemistry techniques. The HCV genotype was determined according to the classification of Simmonds et al. [21]. The serum HCV-RNA level was quantified by Amplicor HCV monitor assay (version 2.0; Roche). These clinical and laboratory data were collected at the time of liver biopsy.

### Histopathological examination

Liver biopsy specimens were obtained percutaneously from all patients for diagnostic purposes and divided into two parts. One part was fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin, Masson's trichrome, and silver impregnation. The sections were analyzed by an experienced hepatologist (T.O.) who was blinded to the laboratory parameters and clinical data. The degrees of inflammation and fibrosis were evaluated according to the criteria proposed by Desmet et al. [22]. Steatosis was graded based on percent of hepatocytes in the biopsy involved: none (0%), mild (<33%), moderate (33–66%), or severe (>66%) [23, 24]. The other part of the liver

biopsy was frozen immediately in liquid nitrogen and stored at –80°C for mRNA analysis.

### Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR)

We quantified mRNA by real-time fluorescence detection. Total RNA was obtained using an RNeasy Kit (Qiagen, Tokyo, Japan). Residual genomic DNA was removed and single-stranded complementary DNA was generated using a Quantitect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol. Real-time quantitative RT-PCR experiments were performed with the LightCycler system using Faststart DNA Master Plus SYBR Green I (Roche Diagnostics, Penzberg, Germany) according to the manufacturer's protocol. The 18 genes chosen for the current study, their protein products, and the primer sequences for amplifying them are listed in Table 1. The primers were designed using Primer3 version 0.4 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) on the basis of sequence data obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/). *ACTB* ($\beta$-actin gene) was used as an endogenous control.

### Immunohistochemistry

Immunohistochemical staining for PPARα and SREBP1 was performed on formalin-fixed, paraffin-embedded sections from 100 liver biopsy specimens using rabbit polyclonal antibodies against human PPARα (clone H-98; Santa Cruz Biotechnology, Santa Cruz, CA) and SREBP1 (clone K-10; Santa Cruz Biotechnology), respectively. Deparaffinized sections were microwaved in a citrate buffer (pH 6.0) for 20 min. After blocking the endogenous peroxidase, the sections were incubated for 90 min at room temperature with 1:100 anti-PPARα or anti-SREBP1 antibodies. The sections were then incubated for 30 min at room temperature with peroxidase-labeled polymer-conjugated goat anti-rabbit immunoglobulin (Histofine Simple Stain Max-Po (Multi); Nichirei, Tokyo, Japan), followed by 3,3′-diaminobenzidine tetrahydrochloride as the chromogen. The sections were then lightly counterstained with hematoxylin. Negative controls were evaluated by substituting the primary antibody with nonimmunized rabbit serum. Immunoreactivity was scored according to the intensity of staining as follows: 1+, weak or absent; 2+, moderate; 3+, strong.

**Table 1** Genes and primer sequences used for reverse transcription-polymerase chain reaction assays

| Function/gene symbol | Alternate symbol | Protein product | Forward primer (5′ → 3′) | Reverse primer (5′ → 3′) |
|---|---|---|---|---|
| Nuclear receptor | | | | |
| *PPARA* | PPARα | Peroxisome proliferator-activative receptor α | ggaaagcccactctgcccccct | agtcaccgaggaggggctcga |
| *PPARG* | PPARγ | Peroxisome proliferator-activative receptor γ | cattctggcccaccaactttgg | tggagatgcaggctccactttg |
| *NR1H3* | LXRα | Liver X receptor α | cgggcttccactacaatgtt | tcaggcggatctgttcttct |
| *RXRA* | RXRα | Retinoid X receptor α | tccttctcccaccgctccatc | cagctccgtcttgtccatctg |
| Fatty acid oxidation | | | | |
| *CPT1A* | CPT1 | Carnitine palmitoyltransferase 1 | catcatcactggcgtgtacc | ttggcgtacatcgttgtcat |
| *ACADS* | SCAD | Short chain acyl-CoA dehydrogenase | ctcacgttggggaagaaaga | tgcgacagtcctcaaagatg |
| *ACADM* | MCAD | Medium chain acyl-CoA dehydrogenase | ttgagttcaccgaacagcag | agggggactggatattcacc |
| *ACADL* | LCAD | Long-chain acyl-CoA dehydrogenase | ttggcaaaacagttgctcac | ctcccacatgtatccccaac |
| *ACADVL* | VLCAD | Very long-chain acyl-CoA dehydrogenase | agccgtgaaggagaagatca | tgtgtttgaagccttgatgc |
| *EHHADH* | LBP | Enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase | cttcagccctggatgttgat | aaaagaagtgggtgccaatg |
| *HADHA* | LCHAD | Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase, alpha subunit | cacctctctgcctgttcctc | ggcaaagatgctgacacaga |
| *ACOX1* | AOX | Acly-CoA oxidase | tgatgcgaatgagtttctgc | agtgccacagctgagaggtt |
| *CYP2E1* | CYP2E | Cytochrome P450 CYP2E | cccaaaggatatcgacctca | agggtgtcctccacacactc |
| Intake of fatty acid | | | | |
| *SLC27A5* | FATP5 | Fatty acid transporor protein 5 | acacactcggtgtccctttc | ctacagggcccactgtcatt |
| Transfer of trigyceride | | | | |
| *MTP* | MTP | Microsomal triglyceride transfer protein | catctggcgaccctatcagt | ggccagctttcacaaaagag |
| Biosynthesis of fatty acid | | | | |
| *SREBF1* | SREBP1 | Sterol regulatory element-binding protein 1 | tgcattttctgacacgcttc | ccaagctgtacaggctctcc |
| *ACACA* | ACC | Acetyl CoA carboxylase | gagaactgccctttctgcac | ccaagctccaggcttcatag |
| *FASN* | FAS | Fatty acid synthase | ttccgagattccatcctacg | tgtcatcaaaggtgctctcg |

**Table 2** Patient characteristics

| Characteristic | |
|---|---|
| *n* | 297 |
| Age[a] | 58 (20–78) |
| Male gender (%) | 131 (44.9%) |
| BMI[a] | 22.7 (15.6–35.1) |
| Obesity (%) | 76 (25.6%) |
| Alcohol intake (%) | 67 (22.6%) |
| Diabetes (%) | 9 (3.0%) |
| HCV genotype (%) | |
| 1 | 212 (71.4%) |
| 2 | 76 (25.6%) |
| 3 | 2 (0.7%) |
| Unknown | 7 (2.3%) |
| HCV-RNA level (KIU/ml)[a] | 1100 (5–9400) |
| Platelet count ($\times 10^4/\mu L$)[a] | 17.6 (5.3–37.4) |
| AST (IU/L)[a] | 47 (14–413) |
| ALT (IU/L)[a] | 59 (9–537) |
| γ-GTP (IU/L)[a] | 39 (10–490) |
| Fasting glucose (mg/dL)[a] | 96 (68–223) |
| Total cholesterol (mg/dL)[a] | 173 (19–318) |
| Triglyceride (mg/dL)[a] | 91 (26–930) |
| Histological activity (%) | |
| 0 | 3 (1.0%) |
| 1 | 127 (42.8%) |
| 2 | 120 (40.4%) |
| 3 | 47 (15.8%) |
| Fibrosis (%) | |
| 0 | 4 (1.3%) |
| 1 | 100 (33.7%) |
| 2 | 120 (40.4%) |
| 3 | 62 (20.9%) |
| 4 | 11 (3.7%) |
| Steatosis (%) | |
| None | 126 (42.4%) |
| Mild (<33%) | 163 (54.9%) |
| Moderate (33–66%) | 7 (2.4%) |
| Severe (>66%) | 1 (0.3%) |

[a] Median (range)

**Table 3** Univariate analysis of factors associated with steatosis

| Factors | No steatosis ($n = 126$) | Steatosis ($n = 171$) | *P* |
|---|---|---|---|
| Age[a] | 56 (20–78) | 59 (27–75) | 0.019 |
| Male gender (%) | 44 (34.9%) | 87 (50.9%) | 0.007 |
| BMI[a] | 21.8 (16.5–30.7) | 23.9 (15.6–35.1) | <0.0001 |
| Alcohol intake (%) | 29 (23.0%) | 38 (22.2%) | 0.89 |
| Diabetes (%) | 4 (3.2%) | 5 (2.9%) | 1.00 |
| HCV genotype (%) | | | |
| 1 | 91 (72.2%) | 121 (70.8%) | |
| 2 | 31 (24.6%) | 45 (26.3%) | |
| 3 | 1 (0.8%) | 1 (0.9%) | |
| Unknown | 3 (2.4%) | 4 (2.4%) | 0.78 |
| HCV-RNA level (KIU/ml)[a] | 1257 (5–7030) | 1063 (5–9400) | 0.14 |
| Platelet count ($\times 10^4/\mu L$)[a] | 18.4 (5.9–32.7) | 17.4 (5.3–37.4) | 0.19 |
| AST (IU/L)[a] | 36 (15–413) | 58 (14–339) | <0.0001 |
| ALT (IU/L)[a] | 40 (9–537) | 73 (12–509) | <0.0001 |
| γ-GTP (IU/L)[a] | 25 (10–298) | 56 (12–490) | <0.0001 |
| Fasting glucose (mg/dL)[a] | 95 (68–207) | 97 (77–223) | 0.002 |
| Total cholesterol (mg/dL)[a] | 179 (109–285) | 171 (104–318) | 0.13 |
| Triglyceride (mg/dL)[a] | 83 (26–214) | 96 (32–930) | <0.0001 |
| Histological activity (%) | | | |
| 0 | 2 (1.6%) | 1 (0.6%) | |
| 1 | 72 (57.1%) | 55 (32.2%) | |
| 2 | 42 (33.3%) | 78 (45.6%) | |
| 3 | 10 (7.9%) | 37 (21.6%) | <0.0001 |
| Fibrosis (%) | | | |
| 0 | 3 (2.4%) | 1 (0.6%) | |
| 1 | 62 (49.2%) | 38 (22.2%) | |
| 2 | 47 (37.3%) | 73 (42.7%) | |
| 3 | 11 (8.7%) | 51 (29.8%) | |
| 4 | 3 (2.4%) | 8 (4.7%) | 0.001 |

[a] Median (range)

### Statistical analysis

Results are presented as numbers with percentages in parenthesis for qualitative data or as the medians and ranges for quantitative data. Univariate comparisons were made using a chi-square test for qualitative factors or a Mann–Whitney *U* test on ranks for quantitative factors with non-equal variance. Logistic regression analysis was used for multivariate analysis. *P* values below 0.05 by two-sided test were considered to be significant. Variables that achieved statistical significance on univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors for steatosis. All statistical analyses were performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA).

## Results

The characteristics of the 297 patients are summarized in Table 2. Steatosis was present in 171 (57.6%) patients. The grade of steatosis was mild in 163 (54.9%) patients, moderate in 7 (2.4%), and severe in 1 (0.3%).