through analysis of full-length core gene sequences identified seven polymorphisms significantly associated with increased HCC risk (36G/C [aaK12N], 209A [aaR70Q], 271U/C [aaL91M], 309A/C, 435A/C, 481A and 546A/C).[51] HCV core gene sequence data might provide useful information about HCC risk.

> Recommendation 5: Amino acid substitutions in the HCV core region (aa70 and aa91) should be determined before IFN treatment in order to predict the response to treatment. (Level 2b, Grade B.)

NS3 protein secondary structure

Recently, Ogata *et al.* reported that HCV-1b strains can be classified into different groups based on the secondary structure of an amino-terminal portion of the NS3 protein and that specific strains are more prevalent among patients with HCC.[52] Moreover, the cumulative incidence of HCC was highest among patients infected with specific group HCV-1b, in whom the risk of HCC significantly increased compared with that among patients infected with another group (hazard ratio = 4.95 [95% confidence interval = 1.43–17.11]) after adjustment for age and histological stage.[53]

> Informative statement: NS3 protein secondary structure may be related to hepatocarcinogenesis. (Grade B.)

NATURAL HISTORY OF CH-C

Progression to cirrhosis and HCC

PREVIOUS PUBLICATIONS REPORTED that approximately 60–80% of patients with acute hepatitis C develop chronic infection in the natural course.[54–57] Because it is difficult to ascertain precisely when the HCV infection occurred except for patients who had blood transfusions, and because chronic infection progresses slowly and asymptomatically, the natural entity of the disease has not been elucidated fully. Seeff *et al.* compared the long-term prognosis of HCV antibody-positive and -negative young men and reported that liver disease-related death was very rare in HCV antibody-positive patients.[58,59] Kenny-Walsh studied the liver histology of 363 young women 17 years after HCV infection and showed that 83% had no or mild hepatic fibrosis whilst 2% had liver cirrhosis.[60] These results demonstrate that progression to serious liver disease is a rare event two decades after infection of young people with HCV.

On the other hand, in blood transfusion-associated CH-C patients the mean interval to liver cirrhosis is estimated to be approximately 20–30 years and that to HCC approximately 30–40 years.[61,62] Because HCC is the most serious complication of HCV-infected people, it is desirable to predict the overall incidence of HCC in each patient. Up to now, many investigators have reported a close relationship between the stage of hepatic fibrosis and incidence of HCC. According to reports from Japan, the annual incidence of new HCC in liver cirrhosis is estimated to be approximately 5–8%.[63–65]

> Informative statement: The natural history of CH-C is highly variable. HCV infection does not have much impact on the overall mortality of all the infected people, whereas progression to liver cirrhosis is observed 20–30 years and to HCC 30–40 years after infection. In Japan, the annual incidence of HCC in liver cirrhosis is estimated to be 5–8%. (Level 2b, Grade B.)
>
> Recommendation 6: Treatment of HCV-infected people should be determined in consideration of the higher annual incidence of HCC in patients with liver cirrhosis in Japan as compared to Western countries. (Level 2b/3, Grade B.)

Progression of fibrosis

The rate of progression of fibrosis varies among patients with CH-C. Poynard *et al.*[66] calculated the average progression rate of hepatic fibrosis in CH-C to be 0.133 fibrosis units/year. In Japan, Shiratori *et al.*[67] reported this to be 0.10 fibrosis units/year. In HCV carriers with persistently normal aminotransferase levels (PNALT), progression of hepatic fibrosis is slower. Persico *et al.*[68] reported that median histological scores did not differ after 5 years of follow up in PNALT and Okanoue *et al.*[69] calculated the average progression rate of hepatic fibrosis in PNALT to be 0.05 fibrosis units/year.

> Informative statement: On average, progression of hepatic fibrosis in CH-C is 0.10–0.13 fibrosis score units/year. The hepatic stage/grade score of HCV carriers with PNALT are generally low and the progression of hepatic fibrosis is slow. Excessive alcohol intake, insulin resistance and hepatic steatosis are the major factors which induce the progression of hepatic fibrosis. (Level 2b, Grade B.)

Alanine aminotransferase (ALT) levels

Alanine aminotransferase is an easy tool to evaluate hepatocellular damage in liver diseases. In the past, a higher incidence of HCC was reported in liver cirrhotic patients with elevated ALT levels.[70] The normal range of serum ALT level varies according to the institutions or hospitals, but it is likely to be located between 30 IU/L

and 40 IU/L. Recently, Kumada et al.[71,72] demonstrated that the cumulative incidence of hepatocarcinogenesis increased in parallel with the increase in ALT average integration value in CH-C even in patients with normal ALT levels. In a community-based study, an elevated ALT level (>35 IU/l) was shown to be a significant risk factor of HCC development.[73]

> Recommendation 7: To prevent the occurrence of HCC, levels of serum ALT should be controlled at below 30 IU/l. (Level 3, Grade A.)

IFN administration

More than two decades have passed since IFN began to be used to treat CH-C patients. Nowadays, more than 70% of HCV-infected people can be cured by the combination therapy of PEG-IFN plus RBV. However, even in patients who were cured of HCV infection and attained an SVR, the occurrence of HCC may be reported long after completion of IFN therapy. The risk factor of HCC occurrence after IFN therapy is a combination of advanced hepatic fibrosis score before therapy, older age and male sex.[74–76] Bruno et al.[75] reported that annual incidence of HCC occurrence in liver cirrhosis after attaining SVR was 0.66%, which was one-third of the incidence of HCC in liver cirrhosis without a virological response (non-SVR).

> Recommendation 8: Surveillance is required for the occurrence of HCC in patients with CH-C and liver cirrhosis. Even if IFN-based therapy is successful in attaining SVR, screening for the detection of HCC by computed tomography (CT), magnetic resonance imaging or ultrasonography and measurement of the serum tumor markers should be carried out routinely, especially for patients with advanced hepatic fibrosis, older age and male sex, because they are at high risk for the occurrence of HCC. (Level 2b, Grade A.)

Indication of IFN therapy for CH-C

Interferon-based therapy is used to treat chronic HCV-infected patients worldwide and PEG-IFN plus RBV is the first choice indication for CH-C patients. Because IFN and RBV have a variety of adverse effects including depression and thyroid dysfunction, "who and how" to treat should be determined with caution. The AALSD practice guideline advocates that treatment decision should be individualized based on the severity of liver disease, the potential for serious side-effects, the likelihood of treatment response, the presence of comorbid condition and the patient's readiness for treatment.[1]

> Recommendation 9: Treatment decision of IFN therapy for CH-C should be individualized based on the body/

mental condition, probability of successful therapy and prolonged survival, and likelihood of provoking serious adverse effects. Scores of hepatic stage/grade should be considered as well. For aged patients, in whom HCV infection is regarded as the major determinant of survival, IFN-based therapy should be considered with caution. (Level 3, Grade A.)

PEG-IFN AND RBV COMBINATION THERAPY

Factors associated with virological response to PEG-IFN and RBV combination therapy

TREATMENT WITH PEG-IFN-α-2A or -2b together with RBV has been evaluated in two nationwide phase III registration trials in Japan.[77,78] In one trial, which determined efficacy of PEG-IFNα-2b and RBV,[79] the SVR rate to 48-week combination therapy was 48% (121/254) in patients with HCV genotype 1b and a high viral load (≥100 KIU/mL). Another trial using PEG-IFN-α-2a and RBV demonstrated an SVR rate to 48-week combination therapy of 59% (57/96) in patients with HCV genotype 1b and a high viral load (≥100 KIU/mL).[80] Based on these results, the currently recommended standard therapy for the patients with CH-C in Japan is the combination of a PEG-IFN together with RBV, except for the treatment naïve patients with a low viral load for whom a PEG-IFN monotherapy is recommended.

These clinical trials identified the following factors that are associated with non-SVR in patients with HCV genotype 1b and a high viral load: (i) older patients; (ii) non-responders to previous IFN therapy; (iii) advanced fibrosis; (iv) female sex; and (v) poor adherence below 80%. In marked contrast to the data from Europe and the USA, the SVR rate in Japanese female patients is lower than that in the male patients. Several community-based retrospective studies in Japan also demonstrated that female patients, especially older female patients, are more difficult to treat compared with other patients.[81,82] Other factors associated with virological response reported from Japan include the low-density lipoprotein cholesterol level,[83] α-fetoprotein (AFP) level,[83] whole-body insulin sensitivity index,[84] single nucleotide polymorphisms of MAP-KAPK3,[85] RIG-I/IPS-1 ratio,[86] Th1/Th2 ratio[45] and PKR response.[87] Association between viral mutations and treatment response is discussed in depth above.

> Recommendation 10: Predictors associated with a non-SVR to PEG-IFN and RBV include: (i) age older than 60 years, particularly older women; (ii) advanced fibro-
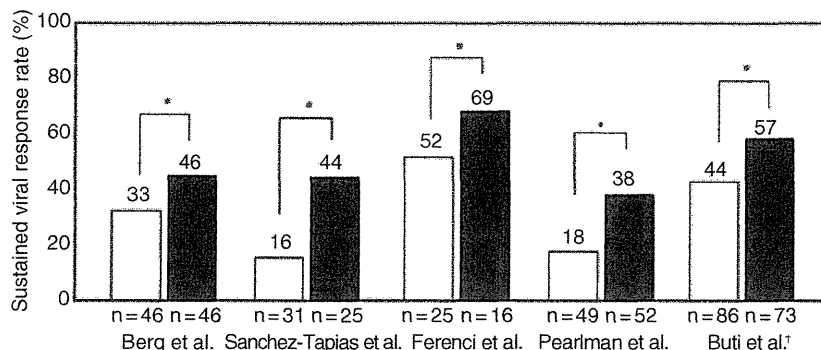
Figure 2 Comparison of sustained virological response rate between 48-week (open column) and 72-week (closed column) treatment with pegylated interferon and ribavirin in patients with partial early virological responder, which is defined as ≥2 log reduction in hepatitis C virus (HCV) RNA level compared to baseline HCV RNA level but detectable HCV RNA at treatment week 12. *Statistical significance between two treatment groups. †Comparison in patients with ≥80% adherence is shown.

sis; (iii) non-responder to previous IFN therapy; and (iv) poor adherence below 80%. (Level 2a, Grade B.)

Response-guided therapy for patients with HCV genotype 1

Measuring the rate of viral clearance from serum is helpful in predicting the likelihood of a response to PEG-IFN and RBV, and useful for determining the optimal duration of therapy. In two nationwide registration trials conducted in Japan,[77,78] the SVR rate was high, from 76–100% in patients whose HCV RNA was cleared rapidly from serum by week 4, and 71–73% in patients who achieved undetectable HCV RNA from week 5 to week 12. In contrast, the SVR rate in patients with late clearance of HCV RNA from week 13 to week 24 was low at 29–36%. No patients without clearance of HCV RNA by week 24 achieved SVR. It should be noted that time point of HCV clearance was determined by measurement of serum HCV RNA utilizing the Ampricor HCV method in these trials.

> Recommendation 11: Measuring the time of viral clearance from serum is helpful in predicting the likelihood of a response to PEG-IFN and RBV. Measurement of HCV RNA is recommended at weeks 4, 12 and 24. (Level 1, Grade A.)

As mentioned above, patients whose HCV RNA measured by Amplicor HCV had not cleared by week 24 were unable to achieve SVR with 48-week standard PEG-IFN and RBV therapy. However, in a retrospective study conducted in 52 patients without HCV RNA clearance from serum by week 24, the rate of ALT normalization 6 months after the completion of therapy (so-called biochemical response) was 56% (5/9) and 62% (8/13) of

patients achieved ALT normalization up to 2 years after the completion of therapy (sustained biochemical response).[88] Therefore, the proposal that recommends a continuation of PEG-IFN and RBV therapy for 48 weeks in biochemical responders at week 24 even without HCV clearance has been accepted widely in Japan. This proposal is in marked contrast to the AASLD practice guideline,[1] in which treatment discontinuation is strongly recommended in patients whose HCV RNA remains positive at week 24.

> Recommendation 12: It is impossible to achieve SVR in patients without HCV RNA clearance by week 24 measured by Amplicor HCV. (Level 1, Grade A.) However, it is recommended to continue the therapy for 48 weeks even in patients without HCV RNA clearance by week 24 if ALT normalizes at week 24, because a sustained biochemical response can be obtained in these patients. (Level 4, Grade C.)

The strategy of extending therapy in patients with delayed virological responses, defined as clearance of HCV RNA between weeks 12 and 24, was evaluated in five studies.[89-93] These results cannot be compared directly with each other because of the heterogeneous study populations, differences in the baseline characteristics and the different regimens utilized amongst them. Nevertheless, the results showed a trend toward a higher SVR rate by extending therapy from 48 to 72 weeks in patients with delayed virological response (Fig. 2).[89-93]

In Japan, a randomized controlled trial was conducted in 113 patients with HCV genotype 1b and a high viral load, comparing a 48-week treatment group and extended treatment group where patients were treated for an additional 44 weeks after clearance of

HCV RNA from serum. In this trial, the SVR rate was 36% in the 48-week treatment group and 53% in the extended treatment group, and the SVR rate was significantly higher in patients in the extended treatment group who became HCV RNA-negative during the period week 16–24 (9% vs 78%, $P = 0.005$).[54] In addition, in a case–control study matched for age, sex and the timing of HCV RNA clearance from serum, the SVR rate was high at 62% in the 72-week treatment group ($n = 65$) compared to 33% in the 48-week treatment group ($n = 130$), and the extended treatment was particularly effective in patients with HCV core mutations at aa70 and aa91 as well as patients a with wild type of ISDR sequence.[79] Accordingly, 72-week extended treatment is recommended for patients who are slow to clear of HCV RNA between weeks 12 and 24.

Currently, HCV RNA clearance from serum is determined by real-time PCR detection, although most of former studies utilized the Amplicor HCV method for this purpose. Because real-time PCR is highly sensitive, it should be reevaluated in terms of who gains benefit from extended therapy. Currently, there is no sufficient evidence to determine this. Nevertheless, substantial number of community-based Japanese study using real-time PCR detection suggested that SVR could be obtained by 72-week treatment if HCV RNA became undetectable by week 36. Accordingly, when determining the timing of HCV RNA clearance using real-time PCR detection, 72-week treatment could be recommended for patients who achieve HCV RNA clearance between weeks 12 and 36.

> Recommendation 13: 72-week extended therapy should be considered for patients with HCV genotype 1 who have delayed HCV RNA clearance from serum between weeks 12 and 24. (Level 2a, Grade B.)
>
> Recommendation 14: When using a real-time detection PCR method for measurement of HCV RNA, SVR can be obtained by 72-week extended treatment in patients who have achieved HCV RNA clearance by week 36. (Level 2b, Grade C.)

Response-guided therapy for patients with HCV genotype 2

Six trials have evaluated a shortening of the duration of therapy from 24 weeks to 12–16 weeks for patients with chronic HCV genotype 2 and 3.[80,95–99] Although the data from some of these trials suggest that patients with genotype 2 and 3 infection who achieve viral clearance from serum by week 4 can shorten their treatment duration to 12–16 week,[80,95,99] the benefit of a shortening the duration of therapy remains controversial.[96] In a recent

study by Mangia *et al.*, the factors associated with relapse after shorter duration of therapy are identified as age over 45 years, pre-treatment platelet count of less than 140×10^9/L, and body mass index over 30 kg/m²,[100] suggesting shortening the duration of therapy can be considered only in particular patients without predictors associated with relapse. Because most Japanese patients have risk factors for relapse such as older age and advanced fibrosis, shortening the duration of the therapy is not generally recommended for Japanese patients with genotype 2, even if they achieve viral clearance by week 4.

PEG-IFN and RBV combination therapy in patients with compensated cirrhosis

In the early Western registration trials, patients with HCV-related compensated cirrhosis did achieve SVR but at lower rates than did those without cirrhosis.[101–103] Subsequently, there was one treatment study that focused exclusively on patients with compensated cirrhosis.[104] In this study, 124 patients with compensated cirrhosis were assigned randomly to an RBV 1000/1200-mg (standard dose) group and 600/800-mg (low dose) group to determine the efficacy of PEG-IFN and RBV combination therapy. The SVR was achieved in 52% of patients who received the standard RBV dose and in 38% of those treated with the low dose. Serious adverse events developed in 14% and 18% of recipients of the standard and low RBV doses, respectively, while dose reduction was necessary in 78% and 57% of the two groups, respectively. HCV genotype 2/3 and platelet count over 150×10^9/L were identified as factors contributing to SVR. Thus, patients with HCV-related compensated cirrhosis can be treated successfully with PEG-IFN and RBV but careful observation is needed because of an anticipated higher rate of adverse effects. Although PEG-IFN and RBV for patients with compensated cirrhosis has not been approved yet in Japan, the following recommendation is reasonable.

> Recommendation 15: Patients with HCV-related compensated cirrhosis can be treated successfully with PEG-IFN and RBV but careful observation is needed because of an anticipated higher rate of adverse effects. (Level 3, Grade B.)

Retreatment with PEG-IFN and RBV combination therapy for patients who failed to respond to previous IFN treatment

Seven randomized controlled trials have been reported so far that examine the efficacy of PEG-IFN and RBV

combination therapy in patients who failed to respond to previous standard IFN therapy with or without RBV.[105-111] The SVR rate varies among these trials ranging 6–45%, and was lower among non-responders to previous IFN therapy compared with relapsers. In a study using PEG-IFNα-2b and RBV at two different doses (1.5 μg/kg per week of PEG-IFNα-2b together with 800 mg/day of RBV or 1.0 μg/kg per week of PEG-IFN together with 1000–1200 mg/day of RBV), the SVR rate was low at 10% and 6% in non-responders to previous treatment, but was high at 50% and 32% in relapsers, respectively.[109] In a phase III clinical trial in Japan, the SVR rate was also low in non-responders but sufficiently high in relapsers.[77] Accordingly, PEG-IFN and RBV combination therapy is well indicated for patients who relapse after standard IFN therapy with or without RBV.

Data on retreatment of patients who failed to respond to previous PEG-IFN plus RBV therapy have been evaluated in two trials.[112,113] In a randomized controlled trial that used two different doses of PEG-IFN-α-2a (360 or 180 μg/week) with two different durations of therapy (72- or 48-week),[112] an SVR was achieved in 7–14% of patients. It should be noted, however, that the SVR was favorable at 52% in patients who achieved HCV RNA clearance from serum by week 12 in the 72-week treatment arm.[112] In the other trial that used PEG-IFN-α-2b and RBV in 2333 patients who failed to respond to previous PEG or standard IFN together with RBV, an SVR was achieved in 56% of patients whose HCV RNA was cleared from serum by week 12 and in 48% of those with genotype 1.[113] Accordingly, it is reasonable to propose that SVR could be obtained by retreatment with PEG-IFN and RBV in patients who achieve HCV RNA clearance by week 12 of retreatment, even if they failed to respond to previous PEG-IFN and RBV combination therapy.[112,113] In contrast, in the AASLD practice guideline, retreatment with PEG-IFN and RBV is not recommended for patients who did not achieve an SVR after a prior full course of PEG-IFN and RBV. Because it is still unclear who is more likely to respond to retreatment with PEG-IFN and RBV, and new drugs such as protease inhibitors may be indicated in the near future for patients who failed to respond to previous PEG-IFN and RBV therapy, data with retreatment of PEG-IFN and RBV should be accumulated to enable a conclusive recommendation.

> *Recommendation 16: Retreatment with PEG-IFN and RBV can be considered for non-responders and relapsers who were treated previously with IFN-based therapy with or without RBV. An SVR could be obtained in these*

> *patients whose HCV RNA is cleared from serum by week 12 of retreatment with PEG-IFN and RBV. (Level 2b, Grade B.)*

MONOTHERAPY WITH IFN OR PEG-IFN

IN JAPAN, IFN monotherapy has been used to treat HCV infection since 1992. Today, IFN monotherapy is used only in patients with specific characteristics because combination therapy with PEG-IFN and RBV has achieved a high rate of SVR. Recently, a large randomized control trial (RCT) of maintenance therapy with a low dose of PEG-IFN was reported.[114] There were no differences in progression of liver disease between a PEG-IFN group and a control group. However, Japanese studies of elderly patients or patients who received maintenance therapy for longer periods showed that IFN can improve outcomes in advanced hepatic fibrosis.

Naïve patients with low viral loads

Previous studies showed that 3 MIU of IFN monotherapy achieved SVR rates of 15–45% in patients with fewer than 2×10^6 copies of HCV.[115-118] Monotherapy with 180 μg/week of PEG-IFN-α-2a or 1.5 μg/kg per week of PEG-IFN-α-2b produced SVR rates of 16–46% in patients with fewer than 2×10^6 copies.[119-121] In Japanese patients with fewer than 1×10^5 copies of HCV, 6 MIU of IFN treatment for 24 weeks achieved an SVR rate of 86% (127/148).[122] PEG-IFN monotherapy for 48 weeks similarly achieved an SVR rate of 86% (106/123). A recent RCT showed that PEG-IFN monotherapy for 24 weeks produced the same SVR rate as similar treatment for 48 weeks in patients with fewer than 1×10^5 copies of HCV. On the basis of these results, monotherapy with IFN or PEG-IFN is considered to be an effective treatment for naïve patients with fewer than 5.0 log copies/mL of HCV.[123]

> *Recommendation 17: Monotherapy with IFN or PEG-IFN can be considered for naïve patients with low viral loads (<5.0 log copies/mL). (Level 2a, Grade B.)*

Patients with chronic kidney disease

Patients with chronic kidney disease (CKD) who undergo hemodialysis have a high prevalence of HCV infection. In Japan, one study reported that HCV RNA was detected in 117 (22%) of 543 patients who underwent maintenance hemodialysis.[124] Hemodialysis patients infected with HCV have a higher mortality rate than uninfected hemodialysis patients.[125] This higher

mortality is attributed to the frequent progression to cirrhosis and/or HCC in HCV-infected patients who receive hemodialysis.

Because RBV is excreted renally, it is currently contraindicated in patients with CKD who have a creatinine clearance of less than 50 mL/min. In addition, pharmacokinetic studies have shown that the clearance of IFN is lower in patients who undergo hemodialysis than in patients who have normal renal function.[126]

Studies of antiviral therapy in patients who undergo hemodialysis suggest that IFN monotherapy is generally well tolerated and that SVR rates are higher than those in patients with normal renal function.[127] The overall SVR rate was reported to be 33–37% in hemodialysis patients.[128] However, the number of subjects in these trials was too low to support confident conclusions. Adverse events are common in this population, and many patients discontinue therapy prematurely because of such events. A recent RCT showed in EASL 2008 that 135 μg/week of PEG-IFN-α-2a for 48 weeks achieved an SVR rate of 39% (23/38), whereas a dose of 90 μg/week produced an SVR rate of 35% (16/43). In 74% of the patients, treatment was completed as scheduled.

Another important point is when to initiate antiviral therapy in hemodialysis patients. IFN might induce allograft rejection and renal failure.[129] Therefore, IFN therapy should be considered before renal transplantation. The next issue to be resolved is the efficacy and safety of low-dose RBV combination therapy in hemodialysis patients.

In 2008, KDIGO proposed guidelines for the treatment of patients with CKD.[130] In Japan, a committee including hepatologists and specialists for CKD is planning a clinical trial for HCV-infected patients with CKD.

> *Recommendation 18: 3 MIU of IFN thrice weekly or 90 or 135 μg of PEG-IFN-α-2a weekly is recommended for patients with CKD. (Level 2a, Grade B.)*

Patients with acute HCV infection

Acute HCV infection progresses to chronic infection in approximately 70% of patients.[131] Antiviral treatment should therefore be considered for this group of patients. On the other hand, it is difficult to identify patients with self-limited disease not requiring therapy. The results of previous studies indicate that anti-HCV treatment should be initiated if HCV RNA is detected continuously for more than 12–16 weeks. If treatment is initiated within this period, monotherapy with IFN or PEG-IFN achieves an SVR rate of more than 80% in patients with acute HCV infection.[132] Reliable evidence

showing that additional treatment with RBV improves the SVR rate in such patients is not available.

> *Recommendation 19: Patients with acute HCV infection should be considered as candidates for antiviral therapy. If HCV RNA is detected continuously for 12 or 16 weeks from the onset, treatment with 6 MIU of IFN or 180 μg of PEG-IFN monotherapy should be initiated. (Level 2a, Grade B.)*

Patients who receive curative treatment for HCC

Hepatocellular carcinoma frequently recurs in HCV-infected patients, even after curative therapy for HCC. Prevention of the recurrence of HCC is essential in such patients. Several RCT showed that the incidence of HCC was low in an IFN-treated group, compared to a control group (Table 4).[133,134] For example, Kubo *et al.* reported that 3 MIU IFN monotherapy thrice weekly for 96 weeks inhibited the recurrence of HCC in patients who had undergone a curative resection.[134] Furthermore, Shiratori *et al.* performed an RCT in 74 patients who had received curative percutaneous ethanol injection therapy for HCC. They reported that second and third recurrences of HCC were less frequent in patients who received IFN.[135] In an Italian study of 150 patients who had undergone curative resection, the recurrence rate of HCC 2 years after operation was significantly lower among patients who received IFN.[136]

Japanese studies showed that the survival rate was also improved by IFN treatment owing to the suppression of HCC and/or the progression of hepatic failure.[137,138]

> *Recommendation 20: IFN therapy should be considered for patients after curative treatment for HCC. (Level 1, Grade A.)*

Maintenance therapy for patients with advanced hepatic fibrosis

Previous studies of patients with advanced hepatic fibrosis, defined as a fibrosis score 3 or 4, showed that IFN monotherapy inhibited the occurrence of HCC, compared to patients who did not receive IFN.[64,139,140] In Japanese studies, IFN was effective not only in SVR patients, but also in non-SVR patients.[139,141] On the other hand, an Italian study showed that the incidence of HCC decreased only in cirrhotic patients in whom HCV was eradicated by IFN therapy.[75]

Case–control studies in patients older than 60 years showed that a low dose of IFN reduced ALT and AFP levels and decreased the incidence of HCC, compared to a control group.[142,143] RCT for IFN monotherapy non-

Table 4 Interferon monotherapy for patients after curative treatment for hepatocellular carcinoma

Author	Study design	No. of patients (IFN group vs non-IFN group)	Age (IFN group vs non-IFN group)	Interferon	Sustained virological response	Follow-up duration (months)	HCC recurrence (IFN group vs non-IFN group)	Survival (IFN group vs non-IFN group)
Ikeda *et al.*	RCT	10 vs 10	60 vs 65	beta	0	25	10% vs 70% $P = 0.0004$	
Kubo *et al.*	RCT	15 vs 15	62 vs 60	alpha	2 (13%)	54	60% vs 87% $P = 0.055$	80% vs 50% $P = 0.041$
Suou *et al.*	Pilot study	18 vs 22	61 vs 62	alpha	6 (33%)	60	28% vs 82% $P < 0.001$	100% vs 73% $P < 0.05$
Shiratori *et al.*	RCT	49 vs 25	61 vs 63	alpha	14 (29%)	60	80% vs 92%	68% vs 48%
Lin *et al.*	RCT	8 vs 6	61 vs 59	alpha	no data	27	63% vs 83% $P = 0.34$	
Jeong *et al.*	Prospective case–control study	16 vs 16	69 vs 68	alpha	2 (13%)	36	69% vs 80% $P = 0.157$	100% vs 88% $P = 0.45$
Sakaguchi *et al.*	Case–control study	24 vs 33	69 vs 67	alpha	1 (4%)	36	14% vs 73% $P = 0.011$	100% vs 94% $P = 0.25$
Mazzaferro *et al.*	RCT	76 vs 74	65 vs 67	alpha	2 (3%)	45	76% vs 94% $P = 0.49$	64% vs 52% $P = 0.47$
Akamatsu *et al.*	Retrospective study	53 vs 399	60 vs 68	no data	17 (32%)	72		88%, 71% vs 53.2% $P = 0.025$
Kudo *et al.*	Case–control study	43 vs 84	65 vs 66	alpha or pegylated IFN	2 (5%)	60	56% s 71% $P = 0.04$	86% vs 56% $P = 0.004$

IFN, interferon; HCC, hepatocellular carcinoma; RCT, randomized control study.

responders showed that histological fibrosis and activity was improved in the assigned IFN-treated group. In contrast, in the untreated group, the fibrosis score did not decline.[144] In Japan, several studies support the effectiveness of low-dose IFN maintenance therapy.[145–147] In the USA, an RCT of 53 patients in whom a histological response, but not a viral response was induced by 6 MIU of IFN showed that 3 MIU of IFN for 24 months improved the degree of hepatic fibrosis.

However, the Hepatitis C Antiviral Long-Term Treatment against Cirrhosis (HALT-C) trial found no difference in the progression of liver disease between a low-dose PEG-IFN group and a control group.[114] The large discrepancy in the effectiveness of IFN maintenance therapy between the HALT-C trial and Japanese trials might be attributed to several factors. First, the study designs differed. One of the most important differences was related to the patients' clinical characteristics. For example, patients enrolled in Japanese studies were older than those in the HALT-C trial. Elderly patients have a higher incidence of HCC than younger patients. It is suggested that the tumor-suppressive effect of IFN maintenance therapy might be more clearly demonstrated in a high-risk group, including elderly patients.[138]

Until more data become available, the decision to perform IFN maintenance therapy should be made on an individual basis.

> Recommendation 21: IFN maintenance therapy is a treatment option that can inhibit the progression of liver disease in patients with advanced hepatic fibrosis, especially in those who are elderly. However, the effect of monotherapy with IFN or PEG-IFN remains uncertain in non-responders to combination therapy with PEG-IFN plus RBV. (Level 2a, Grade C.)

CONSENSUS ON THERAPEUTIC STRATEGY FOR CH-C

Indication of antiviral therapy

IKEDA *ET AL.* elucidated the necessities of antiviral therapy for elderly patients with chronic HCV infection.[132] At 5 and 10 years, hepatocarcinogenesis rates in the intermediate ($100–140 \times 10^9$/L) and low platelet ($<100 \times 10^9$/L) groups were 10.9% and 21.6% in the IFN group ($n = 217$) and 19.5% and 43.0% in the untreated group ($n = 459$), respectively ($P = 0.0005$). IFN independently decreased the risk of carcinogenesis risk with a hazard ratio of 0.56 ($P = 0.035$). On the other hand, in the high platelet ($\geq 150 \times 10^9$/L) group,

no significant difference was found in 5- and 10-year carcinogenesis rates between the IFN-treated group ($n = 228$) and the untreated group ($n = 585$) ($P = 0.69$). Furthermore, IFN treatment significantly increased cumulative survival in the lower platelet subgroup ($P = 0.0001$) but did not affect the higher platelet subgroup ($P = 0.08$). Thus, the necessities of antiviral therapy are shown to be greater in elderly patients with advanced fibrosis, although adverse effects of IFN are reported to be more frequent and the efficacy of IFN to be lower in such patients.[148–150]

Therefore, the indication of antiviral therapy should be considered in the following order: the necessity of treatment, first; safety of treatment, second; and efficacy of treatment for a patient, last. Antiviral therapy should not be given up because the expected SVR rate is low.

> Recommendation 22: Antiviral therapy should be offered even to CH-C patients whose SVR rates are expected to be low if type C chronic liver disease is the prognostic determinant (prognosis is improved by HCV elimination) for the individual patient, and the expected adverse effects are tolerable to the patients. (Level 6, Grade B/C.)

Effect of drug adherence of PEG-IFN and RBV on virological response

The relationship between drug exposure and antiviral effect of PEG-IFN plus RBV combination therapy has been reported in several papers.[101,151–155] McHutchison *et al.* revealed that the SVR rate in patients who received 80% or more of their total planned doses of PEG-IFN-α-2b and RBV for 80% or more of the scheduled duration of therapy was significantly higher than that of patients who received less than 80% of one or both drugs (51% vs 34%) and also suggested that the impact of dose reduction was greatest in patients for whom the dose had to be decreased within the first 12 weeks of treatment.[152]

Recently, Oze *et al.* evaluated how reducing drug doses affects complete early virological response (c-EVR) defined as HCV RNA negativity at week 12, using 984 patients with CH-C genotype 1.[156] As a result, the mean dose of PEG-IFN-α-2b, and not RBV, during the first 12 weeks was the independent factor for c-EVR ($P = 0.02$), not RBV.

Hiramatsu *et al.* reported on whether dose reduction of RBV (or PEG-IFN) has an effect on virological relapse in PEG-IFN plus RBV treatment for patients with CH-C genotype 1.[157] In the analysis of 472 patients responding to PEG-IFN-α-2b plus RBV, stepwise reduction of the

RBV dose was associated with a stepwise increase in relapse rate from 11% to 60% (Fig. 3).

Improving the treatment tolerability for genotype 2 or 3 patients has focused on dose reduction of treatment drugs. Weiland et al. examined low-dose PEG-IFN-α-2a (135 µg/week) with a weight-based standard dose of RBV (11 mg/kg daily) for genotype 2 and 3 patients.[158] Recently, Inoue et al. reported neither PEG-IFN nor RBV drug exposure were critical in reaching rapid virological response and SVR.[159]

Recommendation 23: In genotype 1 patients, PEG-IFN is dose-dependently correlated with c-EVR, independent of RBV dose. The administration over 80% of the scheduled dose of PEG-IFN-α-2a or over 1.2 µg/kg per week of PEG-IFN-α-2b should be chosen as a starting dose: a marked dose reduction of PEG-IFN should not be risked at the start even for patients with disadvantage (e.g. aged patients). (Level 2b/3, Grade B.)

Recommendation 24: In genotype 1 patients, RBV shows a dose-dependent correlation with the relapse after treatment. Maintaining the RBV dose over 80% of the scheduled dose or over 10 mg/kg per day (12 mg/kg per day, if possible) during the complete treatment period can lead to suppression of the relapse in HCV genotype 1 patients responding to PEG-IFN-α-2b plus RBV, especially in c-EVR patients. (Level 2b/3, Grade B.)

Recommendation 25: In genotype 2/3 patients, reducing drug doses of PEG-IFN and RBV (down to 400 mg/day) has no significant effect on virological responses. (Level 2a, Grade B.)
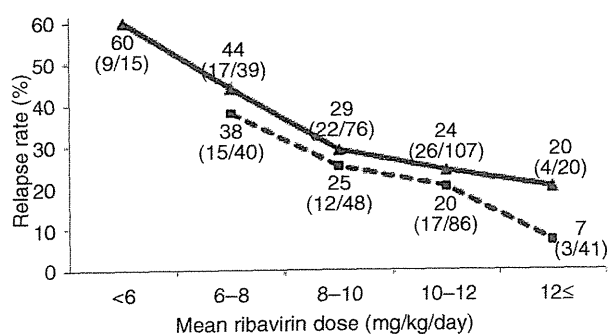

Figure 3 Relapse rate according to pegylated interferon (PEG-IFN)-α-2b and ribavirin doses during treatment of patients who completed treatment, which was stratified with the mean ribavirin doses (-▲-). Group with the mean PEG-IFN dose <1.4 µg kg/week (-■-). Group with the mean PEG-IFN dose ≥1.4 µg kg/week. There was no significant difference between the two PEG-IFN-α-2b-dose groups (P = 0.17).

Treatment for patients without elimination of HCV

Tarao et al. showed the rate of HCC appearance was significantly higher in HCV-related cirrhotic patients with a high ALT value (≥80 IU/mL) than in those with a lower ALT value (<80 IU/mL).[70] This suggested that suppression of inflammation in the liver with HCV infection is very important to prevent the hepatocarcinogenesis in patients with HCV-related cirrhosis.

Omata et al. assessed the effects of oral ursodeoxycholic acid (UDCA) on serum biomarkers. CH-C patients with elevated ALT were assigned randomly to 150 (n = 199), 600 (n = 200) or 900 mg/day (n = 197) UDCA intake for 24 weeks. As a result, the median changes in serum ALT at the end of treatment were shown to be −15.3, −29.2 and −36.2%, respectively, although serum HCV RNA did not change in any group.[160]

A glycyrrhizin product, Stronger Neo-Minophagen C (SNMC; Minophagen Pharmaceutical, Tokyo, Japan), is used widely in Japan and has been reported to improve ALT levels and liver inflammation.[161,162] Furthermore, Ikeda et al. reported liver carcinogenesis was suppressed by long-term administration of glycyrrhizin, using a cohort of 1249 patients, and its favorable effect on hepatocellular carcinogenesis in those patients with IFN-resistant CH-C.[163,164]

Repeated phlebotomy has been shown to be effective for the improvement of serum ALT as well as progression of fibrosis,[32] however, it remains controversial whether the effects of IFN improve with extensive phlebotomy.[165–169]

In Japan, Yano et al. showed the iron removal by repeated phlebotomy improved serum ALT levels in patients with CH-C.[170]

Recommendation 26: Patients whose HCV RNA was not eradicated by PEG-IFN plus RBV and whose ALT and/or AFP levels were not improved by IFN monotherapy or those without indication for IFN therapy should be treated with the liver-supporting therapy (SNMC, UDCA), and if the effect of this medication is inadequate, phlebotomy can be used in combination. (Level 3/6, Grade B/C.)

Treatment of patients with decompensated cirrhosis

The compensated patients who failed to eradicate HCV by antiviral therapy and decompensated patients should be referred for consideration of liver transplantation and liver supporting therapy should be performed. Long-

term nutritional supplementation with oral branched-chain amino acid (BCAA) has been shown to be useful to prevent progressive hepatic failure and to improve surrogate markers.[171,172] Early interventional with oral BCAA was shown to prolong the liver transplant waiting period by preserving hepatic reserve in cirrhosis.

> *Recommendation 27: Patients with compensated cirrhosis for the prevention of hepatocellular carcinogenesis, should be treated by not only IFN but also with liver supporting therapy (SNMC, UDCA) and/or phlebotomy and/or BCAA in order to improve the liver inflammation and AFP levels. (Level 3, Grade C.)*

Novel antiviral drugs

Telaprevir, a protease inhibitor specific to the HCV non-structural 3/4A serine protease, reduced HCV RNA levels rapidly in early studies. McHuthison *et al.* reported the improved SVR rate with triple therapy for 12 weeks followed by PEG-IFN-α-2a and RBV for 12 weeks.

Thus, the treatment for CH-C is progressing. Therefore, as a treatment strategy, PEG-IFN plus RBV combination therapy should be performed early for aged patients and the patients with the advanced fibrosis. However, the novel antiviral drugs, such as protease inhibitors and polymerase inhibitors, should be taken into account as a candidate of treatment for the patients who can wait for the oncoming drugs.

> *Recommendation 28: Novel antiviral drugs, such as a protease inhibitor or a polymerase inhibitor, in combination with PEG-IFN plus RBV, can improve the SVR rates in genotype 1 CH-C patients. (Level 2a, Grade A.)*

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Liver

BASIC STUDIES

Hepatitis C virus protein and iron overload induce hepatic steatosis through the unfolded protein response in mice

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Abstract

Background/Aim: Hepatic iron overload and steatosis play critical roles in the progression of hepatitis C virus (HCV)-associated chronic liver disease. However, how these two pathophysiological features affect each other remains unknown. The aim of this study was to investigate how hepatic iron overload contributes to the development of hepatic steatosis in the presence of HCV proteins. *Methods:* Male C57BL/6 transgenic mice expressing the HCV polyprotein and nontransgenic littermates were fed an excess-iron diet or a control diet. Mice in each group were assessed for the molecules responsible for fat accumulation in the liver. *Results:* Hepatic iron levels were positively correlated with triglyceride concentrations in the liver for all mice. As compared with the livers of nontransgenic mice fed the control diet, the livers of transgenic mice fed the excess-iron diet showed a lower expression of carnitine palmitoyl transferase I, a higher expression of sterol-regulatory element-binding protein 1 and fatty acid synthetase and an activated unfolded protein response indicated by a higher expression of unspliced and spliced X-box DNA-binding protein 1 (XBP-1), phosphorylated eukaryotic initiation factor-2α (p-eIF2α), CCAAT/enhancer-binding protein homology protein (CHOP) and abundant autophagosomes concomitant with increased production of reactive oxygen species. Six-month treatment with the anti-oxidant N-acetyl cysteine dramatically reduced hepatic steatosis in transgenic mice fed the excess-iron diet through decreased expression of unspliced and spliced XBP-1, p-eIF2α, and CHOP. *Conclusions:* The iron-induced unfolded protein response appears to be one of the mechanisms responsible for fat accumulation in the liver in transgenic mice expressing the HCV polyprotein.

Hepatic steatosis and iron overload are not only the pathophysiological features of hepatitis C virus (HCV)-associated chronic liver disease (1, 2) but also risk factors for the development of hepatocellular carcinoma (HCC) (3, 4). Thus, these pathophysiological features appear to play critical roles in the pathogenesis of HCV-associated chronic liver disease. The mechanisms underlying HCV-related steatosis are diverse. HCV core protein has been demonstrated to inhibit microsomal transfer protein activity and very low-density lipoprotein secretion (5) and to upregulate the promoter activity of sterol-regulatory

element-binding protein (SREBP) 1c, a transcription factor involved in lipid synthesis (6). Persistent activation of peroxisome proliferator-activated receptor α has also been reported to be essential for the development of hepatic steatosis in transgenic mice expressing the HCV core protein (7). As for hepatic iron overload, we have shown that HCV-induced reactive oxygen species (ROS) increase the hepatic iron concentration by reducing hepcidin transcription in transgenic mice expressing the HCV polyprotein (8), and that even modest iron supplementation results in the development of liver tumours,

including HCC, through mitochondrial injury in these transgenic mice (9). However, it remains unknown how these two pathophysiological features affect each other in the progression of HCV-associated chronic liver disease. In our previous study, marked hepatic steatosis was observed at 6 months after commencement of iron overloading in transgenic mice, which was followed by the development of liver tumours. These results clearly indicated that hepatic iron overload was involved in the development of hepatic steatosis in the presence of HCV proteins. The aim of this study was to investigate how hepatic iron overload contributes to the development of hepatic steatosis in transgenic mice expressing the HCV polyprotein. In the present study, we report that iron-induced ROS-activated unfolded protein response may be postulated as one of the possible mechanisms of HCV-related fat accumulation in the liver.

Materials and methods

Animals

The transgene pAlbSVPA-HCV, containing the full-length polyprotein-coding region under the control of the murine albumin promoter/enhancer, was described in detail (10, 11). HCV polyprotein has been demonstrated to be processed into individual proteins in the liver and to be expressed at a biologically relevant level in which transcripts of RNA encoding the complete viral polyprotein are detectable only by a reverse-transcription polymerase chain reaction (11). Of the four transgenic lineages with evidence of RNA transcription of the full-length HCV-N open reading frame (FL-N), the FL-N/35 lineage proved capable of breeding in large numbers. There is no inflammation in the transgenic liver (11). Male FL-N/35 transgenic mice and age-matched C57BL/6 mice (control mice) were fed a normal rodent diet including carbonyl iron (45 mg/kg diet, control diet) or an excess-iron diet (carbonyl iron 225 mg/ kg diet) at the age of 8 weeks, bred, maintained and killed by an intraperitoneal injection of 10% pentbarbital sodium preceded by 12-h fasting at 12 months after initiation of feeding according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals. As another experiment, six FL-N/35 transgenic mice were fed the control diet for 6 months, and then they were divided into two groups: three fed the excess-iron diet for 6 months with administration of N-acetyl cysteine (NAC) and those without NAC. NAC was contained in drinking water (1 g/L).

Hepatic iron and triglyceride content

Iron concentrations in the livers were measured by atomic absorption spectrometry (Hitachi Z-6100, Hitachi Ltd., Tokyo, Japan), as described previously (9), and expressed as μg Fe/g of tissue (wet weight). Lipids were extracted from the homogenized liver tissue by the method of Bligh and Dyer (12). The triglyceride levels were measured with a TGE-test Wako kit (Wako Pure Chemicals, Tokyo, Japan) according to the manufacturer's instructions. The protein concentrations in the liver were determined by the method

of Lowry *et al.* (13), using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

Immunoblotting

Lysates of liver were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA), blocked overnight at 4 °C with 5% skim milk and 0.1% Tween 20 in Tris-buffered saline and subsequently incubated for 1 h at room temperature with an anti-human ferritin antibody (Dako, Glostrup, Denmark), anti-rabbit carnitine palmitoyl transferase I (CPT I) antibody, anti-rabbit CPT II antibody (Alpha Diagnostic International, San Antonio, TX, USA), anti-rabbit SREBP1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-rabbit fatty acid synthetase (FAS) antibody (Cell Signaling Technology Inc., Boston, MA, USA), anti-mouse X-box DNA-binding protein 1 (XBP-1) antibody (Santa Cruz Biotechnology Inc.) or anti-bacterially expressed, mouse CCAAT/enhancer-binding protein homology protein (CHOP) fusion protein antibody (Abcam, Cambridge, England). Exceptionally, the proteins were blocked for 1 h at room temperature and subsequently incubated overnight at 4 °C with an anti-rabbit phosphorylated eukaryotic initiation factor-2α (p-eIF2α) antibody (Cell Signaling Technology Inc.).

Histological staining

A portion of liver was immediately snap frozen in liquid nitrogen for determination of hepatic triglyceride and iron concentrations. The remaining liver tissue was fixed in 4% paraformaldehyde in phosphate-buffered saline and embedded in paraffin for histological analysis. Liver sections were stained with haematoxylin and eosin.

Electron microscopy

Liver specimens were fixed in 2.1% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in graded ethanol and propylene dioxide and embedded in Epok. Thick sections (1 μm in width) were stained with toluidine blue to identify steatosis by light microscopy. Thin sections were stained with uranyl acetate and lead citrate, and examined using a Hitachi-7000 transmission electron microscope (Hitachi Ltd.).

In situ detection of reactive oxygen species

In situ ROS production in the liver was assessed by staining with dihydroethidium, as described previously (8). In the presence of ROS, dihydroethidium (Invitrogen Corp., Carlsbad, CA, USA) is oxidized to ethidium bromide and stains nuclei bright red by intercalating with the DNA (14). Fluorescence intensity was quantified using NIH image analysis software for three randomly selected areas of digital images in each mouse.

Statistical analysis

Quantitative values are expressed as mean ± SD. Two groups among multiple groups were compared by the rank-based, Kruskal–Wallis ANOVA test, followed by the Scheffé test. Data between two groups were compared by Student's *t*-test. The statistical significance of correlation was determined by the use of a simple regression analysis. A *P* value of < 0.05 was considered to be significant.

Results

Correlation between iron and triglyceride contents in the liver

Dietary intake and body weight were measured every 4 weeks until 12 months after commencement of iron loading, and these parameters did not differ significantly

among any of the 4 groups. The hepatic iron content (267 ± 94 μg/g liver weight) of FL-N/35 transgenic mice fed the excess-iron diet was significantly greater than that of nontransgenic and FL-N/35 transgenic mice fed the control diet at 12 months after commencement of iron loading (Fig. 1A), and was comparable to that of a large number of patients with chronic hepatitis C in extensive studies (15, 16). The hepatic ferritin level of FL-N/35 transgenic mice fed the excess-iron diet was significantly greater than that of nontransgenic mice fed the control diet (Fig. 1B). The hepatic iron content was positively correlated with the hepatic triglyceride concentration when both parameters were compared for all mice (*r* = 0.63, *P* = 0.002, Fig. 1C). These results were consistent with our previous observation that FL-N/35 transgenic mice fed the excess-iron diet demonstrated the most severe steatosis in the liver among the four groups (9).
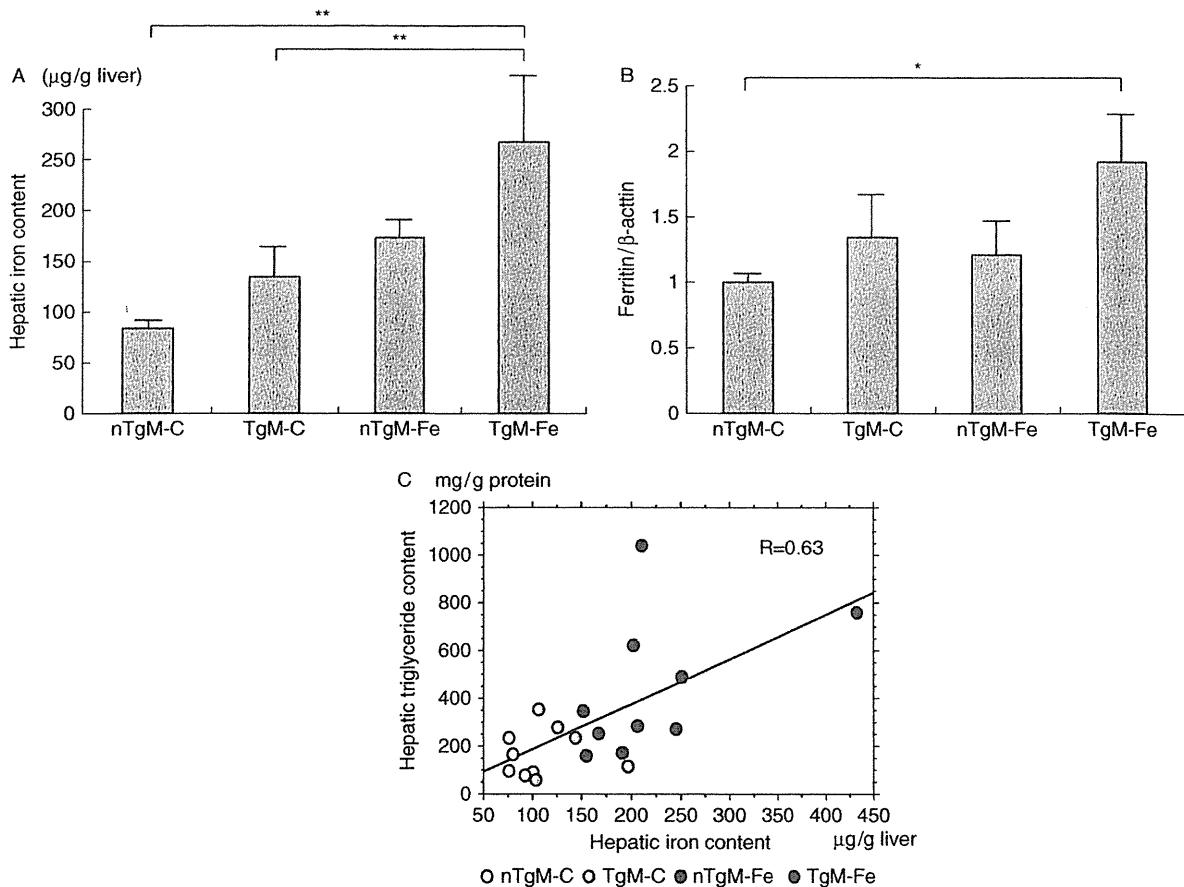

Fig. 1. Hepatic iron contents and ferritin levels, and correlation between iron and triglyceride contents in the liver. (A) The hepatic iron content was measured by atomic absorption spectrometry in five mice in each group at 12 months after initiation of iron loading. (B) Immunoblots for ferritin were performed using liver lysates obtained from four mice in each group. The protein expression was normalized with β-actin. (C) The correlation between hepatic iron and triglyceride levels was determined in 20 mice from four groups. nTgM-C: nontransgenic mice fed the control diet, nTgM-Fe: nontransgenic mice fed the excess-iron diet, TgM-C: FL-N/35 transgenic mice fed the control diet, TgM-Fe: FL-N/35 transgenic mice fed the excess-iron diet. *P < 0.05, **P < 0.01.
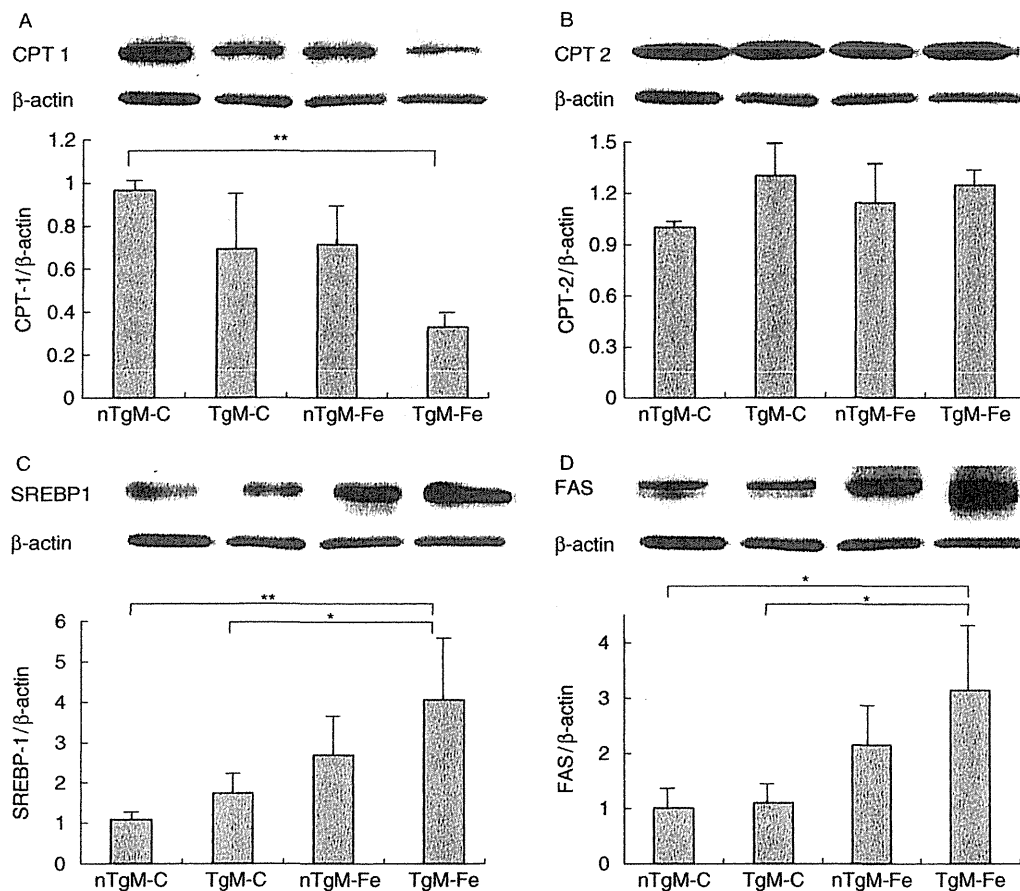
Fig. 2. Expression of carnitine palmitoyl transferase I (CPT1), carnitine palmitoyl transferase I (CPT2), sterol-regulatory element-binding protein I (SREBP1) and fatty acid synthetase (FAS) in the liver. Immunoblots for CPT1 (A), CPT2 (B), SREBP1 (C) and FAS (D) were performed using liver lysates obtained from four mice in each group at 12 months after initiation of iron loading. The protein expression was normalized with β-actin. $*P < 0.05$, $**P < 0.01$. nTgM-C, TgM-C, nTgM-Fe and TgM-Fe; see legend for Figure 1.

Decreased expression of carnitine palmitoyl transferase I and increased expression of sterol-regulatory element-binding protein 1

As we previously reported reduced oxidation activity of fatty acid in iron-overloaded transgenic mice (9), we first examined the expression levels of CPT1 and CPT2, which regulate oxidation of long-chain fatty acids in the mito-chondria. The expression of CPT1, but not CPT2, was significantly reduced in FL-N/35 transgenic mice fed the excess-iron diet compared with the nontransgenic mice fed the control diet ($P = 0.0003$, Fig. 2A and B). We next examined the expression level of SREBP1, a transcription factor that activates the genes required for lipogenesis (17), and FAS, a target gene of SREBP1. As shown in Figures 2C and D, the expression of SREBP1 and FAS was significantly greater in FL-N/35 transgenic mice fed the excess-iron diet than in nontransgenic and FL-N/35 transgenic mice fed the control diet, suggesting the involvement of activated lipogenesis in hepatic steatosis

in FL-N/35 transgenic mice fed the excess-iron diet. It should also be noted that modest iron supplementation significantly activated lipogenesis in FL-N/35 transgenic mice, but not in nontransgenic mice.

Activated unfolded protein response

Upon endoplasmic reticulum (ER) stress, the SREBP–SREBP cleavage-acting protein (SCAP) complex dissociates from the ER retention protein and subsequently translocates to the Golgi apparatus, where SREBP is cleaved and activated (18, 19). We therefore investigated whether increased expression of SREBP1 was related to ER stress. The unfolded protein response-signalling cascades are initiated by three ER-resident sensors: inositol-requiring enzyme 1 (IRE1), RNA-activated protein kinase (PKR)-like ER kinase (PERK) and activating transcription factor 6 (ATF6) (20). IRE1 activation splices unspliced XBP-1 (uXBP-1) to form spliced XBP-1 (sXBP-1) mRNA (21),