28 Akuta N, Suzuki F, Kawamura Y *et al.* Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: Amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. *J Hepatol* 2007; 46: 403–10.
29 Okanoue T, Itoh Y, Hashimoto H *et al.* Predictive values of amino acid sequences of the core and NS5A regions in antiviral therapy for hepatitis C: a Japanese multi-center study. *J Gastroenterol* 2009; 44: 952–63.
30 Mori N, Imamura M, Kawakami Y *et al.* Randomized trial of high-dose interferon-alpha-2b combined with ribavirin in patients with chronic Hepatitis C: correlation between Amino acid substitutions in the Core/NS5A region and virological response to interferon therapy. *J Med Virol* 2009; 81: 640–9.
31 Ishii K, Shinohara M, Sawa M *et al.* Interferon alpha receptor 2 expression by peripheral blood monocytes in patients with a high viral load of Hepatitis C virus genotype 1 showing substitution of Amino acid 70 in the core region. *Intervirology* 2010; 53: 105–10.
32 Larrea E, Garcia N, Qian C, Civeira MP, Prieto J. Tumor necrosis factor alpha gene expression and the response to interferon in chronic hepatitis C. *Hepatology* 1996; 23: 210–17.
33 Reiberger T, Aberle JH, Kundi M *et al.* IP-10 correlates with hepatitis C viral load, hepatic inflammation and fibrosis and predicts hepatitis C virus relapse or non-response in HIV-HCV coinfection. *Antivir Ther* 2008; 13: 969–76.
34 Romero-Gomez M, Viloria MD, Andrade RJ *et al.* Insulin resistance impairs sustained response rate to peginterferon plus ribavirin in chronic hepatitis C patients. *Gastroenterology* 2005; 128: 636–41.
35 Zografos TA, Liaskos C, Rigopoulou EI *et al.* Adiponectin: a new independent predictor of liver steatosis and response to IFN-alpha treatment in chronic Hepatitis C. *Am J Gastroenterol* 2008; 103: 605–14.
36 Welzel TM, Morgan TR, Bonkovsky HL *et al.* Variants in interferon-alpha pathway genes and response to pegylated interferon-alpha2a plus ribavirin for treatment of chronic Hepatitis C virus infection in the Hepatitis C antiviral long-term treatment against cirrhosis trial. *Hepatology* 2009; 49: 1847–58.
37 Hijikata M, Ohta Y, Mishiro S. Identification of a single nucleotide polymorphism in the MxA gene promoter (G/T at nt -88) correlated with the response of hepatitis C patients to interferon. *Intervirology* 2000; 43: 124–7.
38 Knapp S, Yee L, Frodsham A *et al.* Polymorphisms in interferon-induced genes and the outcome of hepatitis C virus infection: roles of MxA, OAS-1 and PKR. *Genes Immun* 2003; 4: 411–19.
39 Matsuyama N, Mishiro S, Sugimoto M *et al.* The dinucleotide microsatellite polymorphism of the IFNAR1 gene promoter correlates with responsiveness of hepatitis C patients to interferon. *Hepatol Res* 2003; 25: 221–5.
40 Naito M, Matsui A, Inao M *et al.* SNPs in the promoter region of the osteopontin gene as a marker predicting the efficacy of interferon-based therapies in patients with chronic hepatitis C. *J Gastroenterol* 2005; 40: 381–8.
41 Tsukada H, Ochi H, Maekawa T *et al.* A polymorphism in MAPKAPK3 affects response to interferon therapy for chronic hepatitis C %J. *Gastroenterology* 2009; 136: 1796–805, e6.
42 Ge DL, Fellay J, Thompson AJ *et al.* Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* 2009; 461: 399–401.
43 Suppiah V, Moldovan M, Ahlenstiel G *et al.* IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nat Genet* 2009; 41: 1100–4.
44 Tanaka Y, Nishida N, Sugiyama M *et al.* Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat Genet* 2009; 41: 1105–9.
45 Thomas DL, Thio CL, Martin MP *et al.* Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature* 2009; 461: 798–801.
46 Charlton MR, Pockros PJ, Harrison SA. Impact of obesity on treatment of chronic hepatitis C. *Hepatology* 2006; 43: 1177–86.
47 Gopal K, Johnson TC, Gopal S *et al.* Correlation between beta-lipoprotein levels and outcome of hepatitis C treatment. *Hepatology* 2006; 44: 335–40.
48 Guidi M, Muratori P, Granito A *et al.* Hepatic steatosis in chronic hepatitis C: impact on response to anti-viral treatment with peg-interferon and ribavirin. *Aliment Pharmacol Ther* 2005; 22: 943–9.
49 Bergmann JF, Vrolijk JM, van der Schaar P *et al.* g-Glutamyltransferase and rapid virological response as predictors of successful treatment with experimental or standard peginterferon-alpha-2b in chronic hepatitis C non-responders. *Liver Int* 2007; 27: 1217–25.
50 Sezaki H, Suzuki F, Kawamura Y *et al.* Poor response to pegylated interferon and ribavirin in older women infected with hepatitis C virus of genotype 1b in high viral loads. *Dig Dis Sci* 2009; 54: 1317–24.
51 Kogure T, Ueno Y, Fukushima K *et al.* Pegylated interferon plus ribavirin for genotype Ib chronic hepatitis C in Japan. *World J Gastroenterol* 2008; 21: 7225–4230.
52 Watanabe S, Enomoto N, Koike K *et al.* Prolonged treatment with pegylated interferon alpha 2b plus ribavirin improves sustained virological response in chronic hepatitis C genotype 1 patients with late response in a clinical real-life setting in Japan. *Hepatol Res* 2010; 40: 135–44.
53 Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis – diagnosis, grading and staging. *Hepatology* 1994; 19: 1513–20.

54 Akuta N, Suzuki F, Hirakawa M *et al.* Amino acid substitutions in the hepatitis C virus core region of genotype 1b affect very early viral dynamics during treatment with telaprevir, peginterferon, and ribavirin. *J Med Virol* 2010; 82: 575–82.
55 Izopet J, Payen JL, Alric L *et al.* Baseline level and early suppression of serum HCV RNA for predicting sustained complete response to alpha-interferon therapy. *J Med Virol* 1998; 54: 86–91.
56 Poynard T, Marcellin P, Lee SS *et al.* Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). *Lancet* 1998; 31: 1426–32.
57 Izumi N, Enomoto N, Uchihara M *et al.* Hepatic iron contents and response to interferon-alpha in patients with chronic hepatitis C. Relationship to genotypes of hepatitis C virus. *Dig Dis Sci* 1996; 41: 989–94.
58 Syed GH, Amako Y, Siddiqui A. Hepatitis C virus hijacks host lipid metabolism. *Trends Endocrinol Metab* 2010; 21: 33–40.

J Gastroenterol (2009) 44:952–963
DOI 10.1007/s00535-009-0087-x

Predictive values of amino acid sequences of the core and NS5A regions in antiviral therapy for hepatitis C: a Japanese multi-center study

Takeshi Okanoue · Yoshito Itoh · Hiroaki Hashimoto · Kohichiroh Yasui · Masahito Minami · Tetsuo Takehara · Eiji Tanaka · Morikazu Onji · Joji Toyota · Kazuaki Chayama · Kentaro Yoshioka · Namiki Izumi · Norio Akuta · Hiromitsu Kumada

Received: 31 March 2009 / Accepted: 20 April 2009 / Published online: 11 June 2009

Abstract
Background Chronic hepatitis C (CHC) genotype 1b patients with high viral load are resistant to peginterferon (PEG-IFN) and ribavirin (RBV) combination therapy, especially older and female patients.
Methods To elucidate the factors affecting early and sustained viral responses (EVR and SVR), 409 genotype 1b patients CHC with high viral loads who had received 48 weeks of PEG-IFN/RBV therapy were enrolled. The amino acid (aa) sequences of the HCV core at positions 70 and 91 and of the interferon sensitivity determining region (ISDR) were analyzed. Host factors, viral factors, and treatment-related factors were subjected to multivariate analysis.
Results Male gender, low HCV RNA load, high platelet count, two or more aa mutations of ISDR, and wild type of core aa 70 were independent predictive factors for SVR. In patients with over 80% adherences to both PEG-IFN and RBV, male gender, mild fibrosis stage, and wild type of core aa 70 were independent predictors for SVR.
Conclusions Independent predictive factors for SVR were: no aa substitution at core aa 70, two or more aa mutations in the ISDR, low viral load, high values of platelet count, mild liver fibrosis and male gender.

T. Okanoue (✉)
Hepatology Center, Saiseikai Suita Hospital,
1-2 Kawazonocho, Suita 564-0013, Japan
e-mail: okanoue@suita.saiseikai.or.jp

T. Okanoue · Y. Itoh · H. Hashimoto · K. Yasui · M. Minami
Department of Gastroenterology and Hepatology,
Kyoto Prefectural University of Medicine,
Kawaramachi-Hirokoji Kamigyo-Ku,
Kyoto 602-8566, Japan

Y. Itoh
e-mail: yitoh@koto.kpu-m.ac.jp

H. Hashimoto
e-mail: road1820@yahoo.co.jp

K. Yasui
e-mail: yasuik@koto.kpu-m.ac.jp

M. Minami
e-mail: minami@koto.kpu-m.ac.jp

T. Takehara
Department of Gastroenterology and Hepatology,
Osaka University Graduate School of Medicine,
2-2 Yamadaoka, Suita 565-0871, Japan
e-mail: takehara@gh.med.osaka-u.ac.jp

E. Tanaka
Department of Medicine, Shinshu University School
of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan
e-mail: etanaka@shinshu-u.ac.jp

M. Onji
Department of Gastroenterology, Ehime University,
454 Shizukawa, Tohon, Ehime 791-0295, Japan
e-mail: onjimori@m.med.ehime-u.ac.jp

J. Toyota
Departmet of Gastroenterology, Sapporo-Kosei General
Hospital, Kita Sanjyo, Chuo-ku, Sapporo 060-0033, Japan
e-mail: joji.toyota@ja_hokkaidoukouseiren.or.jp

K. Chayama
Department of Medicine and Molecular Science,
Graduate School of Science, Hiroshima University,
1-2-3 Kasumi, Minami-ku, Hiroshima,
Hiroshima 734-8551, Japan
e-mail: chayama@hiroshima-u.ac.jp

K. Yoshioka
Department of Hepato-Biliary-Pancreas, Fujita Health Science,
Kutsukake-cho, Toyoake 470-1192, Japan
e-mail: kyoshiok@fujita-hu.ac.jp

Keywords Chronic hepatitis C · Peginterferon and ribavirin · Core amino acid · Interferon sensitivity determining region

Abbreviations

CHC	Chronic hepatitis C
PEG-IFN	Peginterferon
RBV	Ribavirin
RVR	Rapid viral response
cEVR	Complete early viral response
LVR	Late viral response
ETR	End of treatment response
NR	Non response
SVR	Sustained viral response
ISDR	Interferon sensitivity determining region
Aa	Amino acid
ALT	Alanine aminotransferase
PLT	Platelet
HCC	Hepatocellular carcinoma

Introduction

A combination of pegylated interferon (PEG-IFN) and ribavirin (RBV) therapy for 48 weeks achieves a sustained viral response (SVR) rate of 40–50% in chronic hepatitis C (CHC) patients with a high viral load of genotype 1 [1–4]. The dose-reduction rate and the frequency of discontinuation of this treatment are high in aged patients [5]. The SVR rate of the therapy is lower in females than males, especially in older patients in Japan [6].

Around 30% of HCV carriers have serum alanine aminotransferase (ALT) levels within the upper limit of normal ranges [7, 8] and HCV carriers with persistently normal serum ALT (PNALT) and serum platelet (PLT) counts of over $15 \times 10^4/mm^3$ show low grade hepatic fibrosis and good prognosis [9]. Before treating HCV carriers, it is very important to predict non-response to PEG-IFN plus RBV therapy because of its medical cost, adverse effects, and its impact on the long term quality of life.

N. Izumi
Department of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Sakaiminamimachi, Musashino 180-8610, Japan
e-mail: nizumi@musashino.jrc.or.jp

N. Akuta · H. Kumada
Department of Hepatology, Toranomon Hospital, Kajigaya, Takatsu-ku, Kawasaki 213-8587, Japan

N. Akuta
e-mail: akuta-gi@umin.ac.jp

H. Kumada
e-mail: kumahiro@toranomon.gr.jp

There are many factors affecting response to IFN monotherapy and PEG-IFN/RBV therapy, including body mass index (BMI) [10, 11], steatosis [12, 13], insulin resistance [14], stage of liver fibrosis [15, 16], total cholesterol (T. Chol), triglyceride (TG), adherence to both PEG-IFN and RBV [17], race [18, 19], age [1, 2, 20], and viral factors including serum quantity of HCV RNA, HCV genotype and substitution of amino acids (aa) in the interferon sensitivity determining region (ISDR, 2209–2248) of the nonstructural protein 5A (NS5A) [21] and in the core protein [22, 23]. Early viral response is an important predictive factor in PEG-IFN/RBV therapy for CHC patients with genotype 1 and high viral loads [24–27].

The aim of this study was to elucidate the valuable predictive factors of SVR in Japanese patients with HCV genotype 1b high viral loads following 48 weeks of PEG-IFN/RBV therapy, focusing on the relationship between aa substitutions in the ISDR and at core aa 70 and 91 and early viral kinetics.

Patients and methods

Selection of patients

This retrospective study was conducted at 15 clinical sites in Japan which are part of the Study Group of Optimal Treatment of Viral Hepatitis supported by the Ministry of Health, Labor and Welfare, Japan. Eligible subjects were CHC patients, who (1) had received liver biopsy; (2) were genotype 1b with high viral load ($\geq$100 KIU/ml by Cobas Amplicor Hepatitis C Virus Test, version 2.0) at the start of PEG-IFN/RBV therapy; (3) received weekly injections of PEG-IFN-α-2b (PEG-INTRON; Shering-Plough, Kenilworth, NJ) of 1.5 μg/kg bw and oral administration of RBV (Rebetol; Shering-Plough) for 48 weeks. The amount of RBV was adjusted based on the subject's body weight; (600 mg for $\leq$60 kg bw, 800 mg for 60–80 kg bw, 1,000 mg for $>$ 80 kg bw); (4) were examined serially for quantitative and qualitative HCV RNA; and (5) the aa sequences at positions 70 and 91 in the core region and of the ISDR in the NS5A had been determined in pretreatment sera.

Hepatitis B virus (HBV) infection, human immunodeficiency virus (HIV) infection, autoimmune hepatitis, primary biliary cirrhosis, hemochromatosis, and Wilson's disease were excluded. Histopathological diagnosis was based on the scoring system of Desmet et al. [28]. The definition of alcohol abuse included patients having a history of more than 100 kg of total ethanol intake. Complete blood counts, liver function tests, serum lipids, serum ferritin, serum fibrosis markers, fasting plasma glucose (FPG), and immune reactive insulin (IRI) were examined in most cases. Written informed consent was obtained from all

patients before treatment, and the protocol was approved by the ethics committees in each site.

Study design

Four hundred and nine patients who completed 48 weeks of treatment and were followed for more than 24 weeks after treatment were enrolled in the first study (*Study design 1*).

To elucidate the effect of aa substitution of HCV core and in the ISDR on HCV dynamics, including a rapid viral response (RVR), complete early viral response (cEVR), a late viral response (LVR) and SVR, according to gender and age (<60 years $\geq$ 60 years), 201 of the 409 patients maintaining over 80% adherences to both PEG-IFN and RBV were enrolled in the second study (*Study design 2*).

Nucleotide sequencing of the core and NA5A gene

The nucleotide sequences encoding aa 1–191 (HCV core) and aa 2209–2248 (ISDR) were analyzed by direct sequencing as described by Akuta et al. [22, 27] and Enomoto et al. [21]. In brief, RNA was extracted from the sera and converted to cDNA and two nested rounds of polymerase chain reaction (PCR) were performed. Primers used in the PCR were as follows; (a) Nucleotide sequences of the core region: the first-round PCR was performed with CC11 (sense) and e14 (antisense) primers [22, 27], and the second-round PCR with CC9 (sense) and e14 (antisense) primers [22, 27]. (b) Nucleotide sequences of the ISDR in NS5A: the first-round PCR was performed with ISDR1 (sense) and ISDR2 (antisense) primers [21], and the second-round PCR with ISDR3 (sense) and ISDR4 (antisense) primers [21]. These sequences were compared with the consensus sequence of genotype 1b (HCV-J) [29]. Wild types virus encoded arginine and leucine at aa 70 and 91, respectively, and the aa substitutions were glutamine or histidine at aa 70 and methionine at aa 91.

Viral kinetic study

Serum HCV RNA levels were measured by PCR (Amplicor HCV RNA kit, version 2.0, Roche Diagnostics) using samples taken before treatment and at 4, 12, 24, and 48 weeks after the therapy. SVR was defined as HCV RNA negativity by qualitative analysis by PCR at 24 weeks after the treatment. RVR was defined as HCV RNA negativity at 4 weeks, cEVR as HCV RNA negativity at 12 weeks, LVR as HCV RNA negativity during 13–24 weeks and an end of treatment response (ETR) as HCV RNA negativity at the end of treatment. Patients who remained positive for HCV RNA at the end of the treatment and at 24 weeks after the therapy were defined as non-responders (NR).

Adherences to PEG-IFN and RBV

Adherences to PEG-IFN and RBV were assessed by separately calculating the actual doses of PEG-IFN and RBV received as percentages of the intended dosages. Adherences to PEG-IFN and RBV were divided into two groups; 80%$\leq$ and <80%.

Statistical analysis

All data analyses were conducted using the SAS version 9.1.3 statistical analysis packages (SAS Institute, Cary, NC, USA). Individual characteristics between groups were evaluated by Mann–Whitney U test for numerical variables or Fisher's exact test for categorical variables. Variables exhibiting values of $p < 0.1$ in the univariate analysis were subjected to stepwise multivariate logistic regression analysis. The grade of steatosis and iron deposition in liver tissue, BMI, albumin (Alb), low density lipoprotein-cholesterol (LDL-C), homeostasis model assesment-insulin resistance (HOMA-IR), ferritin, and hyaluronic acid were excluded from multivariate logistic regression analysis because of the absence of those data in more than 10% of the patients. All p values of $p < 0.05$ by the two-tailed test were considered statistically significant.

Results

Study design 1

Baseline backgrounds, characteristics and adherences of peginterferon and ribavirin in males and females

The treatment outcome of PEG-IFN and RBV combination therapy depends on gender in Japanese patients, so in addition to aa substitutions in the ISDR in NS5A [21] or at HCV core 70 and 91 [22, 27], we compared the baseline characteristics according to gender (Table 1). Males were younger and the grade of hepatic inflammation was milder in males. The serum levels of LDL-C, PLT count, and aa substitutions of ISDR and at core 70 and 91 did not differ significantly different between males and females. The frequency of no alcohol abuse was significantly ($p < 0.0001$) higher in females than males (Some of them are not described in Table 1).

The rates of over 80% adherences to PEG-IFN and RBV were significantly lower ($p = 0.0066$, $p < 0.00001$, respectively) in females than males. Only in those above 60 years did the rate of over 80% adherence to PEG-IFN not differ significantly between males and females, but the rate of over 80% adherence to RBV was significantly lower ($p = 0.035$) in females than males (Table 1).

Table 1 Backgrounds and characteristics of male and female patients

Factors	Gender		*p* value
	Male	Female	
No. of patients	256 (62.6%)	153 (37.4%)	
Age			
Median (range)	53 (18–73)	59 (23–75)	0.00001
F stage			
F0–2	206 (80.5%)	119 (77.8%)	0.592
F3–4	50 (19.5%)	34 (22.2%)	
Grade (A factor)			
A0–1	163 (63. 7%)	79 (51.6%)	0.026
A2–3	93 (36.3%)	74 (48.4%)	
HCV RNA load 0 week (KIU/mL)			
Median (range)	1500 (100–5000 <)	1280 (100–5000<)	0.384
ALT 0 week (IU/L)			
Median (range)	74.5 (16–504)	59 (19–391)	0.001
BMI			
Median (range)	23.6 (17.5–31.2)	22.1 (16.1–33.9)	0.00033
Alb (g/dL)			
Median (range)	4.0 (3.0–5.2)	3.8 (3.0–4.8)	0.011
LDL-C (mg/dL)			
Median (range)	97 (30–185)	90 (34–174)	0.612
T-Chol (mg/dL)			
Median (range)	167 (85–273)	176 (114–261)	0.0016
PLT count ($\times 10^4$/mm^3)			
Median (range)	17.0 (8.0–31.9)	16.4 (8.1–39.9)	0.350
Amino acid mutation of ISDR			
0–1	200 (78.1%)	121 (79.1%)	0.608
2≤	56 (21.9%)	32 (20.9%)	
Amino acid substitution of core 70			
Wild	177 (69.1%)	114 (74.5%)	0.261
Mutant	79 (30.9%)	39 (25.5%)	
Amino acid substitution of core 91			
Wild	153 (59.8%)	98 (64.1%)	0.403
Mutant	103 (40.2%)	55 (35.9%)	
PEG-IFN adherence			
<80%	41 (17.7%)	42 (30.4%)	0.0066
80%≤	190 (82.3%)	96 (69.6%)	
Ribavirin adherence			
<80%	54 (23.6%)	73 (52.1%)	<0.00001
80%≤	175 (76.4%)	67 (47.9%)	
Age: <60 years			
PEG adherence			
<80%	30 (17.8%)	23 (31.5%)	0.027
80%≤	139 (82.2%)	50 (68.5%)	
Ribavirin adherence			
<80%	27 (16.2%)	31 (42.5%)	0.000029
80%≤	140 (83.8%)	42 (57.5%)	
Age: 60 years≤			
PEG adherence			
<80%	11 (17.7%)	19 (29.2%)	0.147
80%≤	51 (82.3%)	46 (70.8%)	
Ribavirin adherence			
<80%	27 (43.5%)	42 (62.7%)	0.035
80%≤	35 (56.5%)	25 (37.3%)	

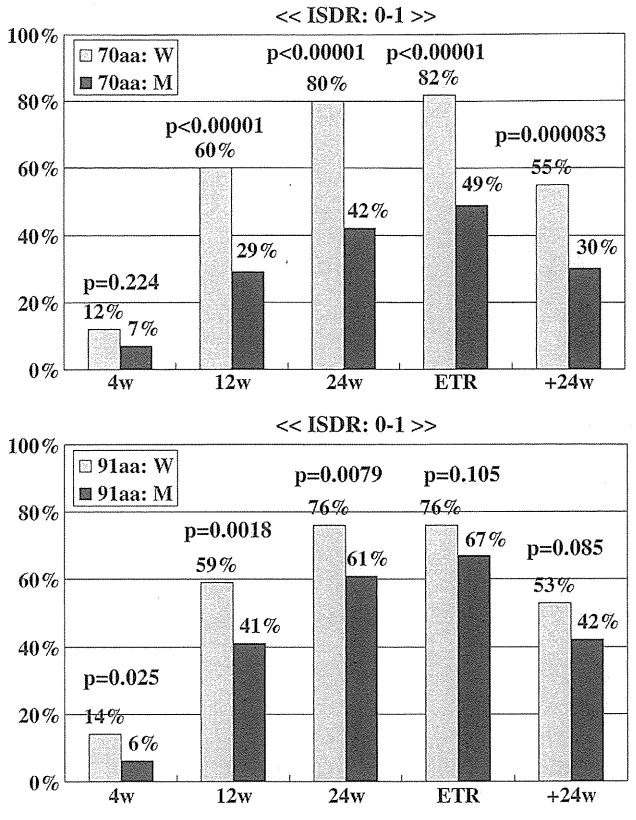

Fig. 1 Relationship between time course of serum HCV RNA negativity and amino acid substitutions in the ISDR and core amino acids 70 and 91. For cases with no or only one amino acid (aa) change in the ISDR, the rates of cEVR, LVR, ETR and SVR were significantly higher in patients with wild type core aa 70 but only the rates of RVR, cEVR, and LVR were significantly higher in patients with wild type core aa 91

Amino acid substitutions

There were no significant differences in the frequency of aa substitutions in the ISDR between males and females. Core aa substitutions at positions 70 and 91 were as follows; 291 (71.1%) were wild type and 118 (28.9%) were mutant at core aa 70, and 251 (61.4%) were wild type and 158 (38.6%) were mutant at core aa 91. There were no significant differences between males and females and between patients below and above 60 years of age.

Virological responses and aa substitutions

The rate of RVR did not differ significantly between males and females. However, more male patients showed HCV RNA negativity at 12 weeks (males vs. females; 60.7 vs. 48.4%, $p = 0.018$), 24 weeks (76.8 vs. 64.2%, $p = 0.0078$) and 48 weeks (78.2 vs. 68.6%, $p = 0.049$), and the proportion of male patients in SVR was significantly higher than that of females (61.3 vs. 37.3%, $p < 0.00001$).

RVR, cEVR and SVR rates were significantly higher in patients with two or more aa mutations in the ISDR compared to patients having no or one aa substitution in that region (20 vs. 11%, $p = 0.044$; 71 vs. 52%, $p = 0.0021$; 66 vs. 49%, $p = 0.0054$, respectively). AA substitution at core position 70 resulted in significantly lower rate of cEVR, LVR, ETR, and SVR (40 vs. 63%, $p = 0.000037$; 51 vs. 81%, $p < 0.00001$; 56 vs. 83%, 41 vs. 57%; $p < 0.00001$, $p = 0.0031$, respectively). Although the patients with the wild type aa at core 91 showed significantly higher rates of RVR and cEVR, the rate of SVR was not significantly higher in those patients ($p = 0.054$).

SVR rates were 30% for patients with no or one aa substitution in the ISDR and the core aa 70 substitution, and were significantly lower compared to those with the wild type aa core 70 (Fig. 1). These findings were not confirmed in patients with no or one aa substitution in the ISDR and the core aa 91 substitution (Fig. 1).

Factors affecting SVR by univariate analysis

Univariate analysis identified nine parameters that influenced non-SVR significantly: female gender, older age, advanced staged liver fibrosis, high viral load, low serum Alb level, low PLT count, no or one aa substitution in the ISDR, aa substitution at core aa 70, and low adherence to RBV (Table 2). The frequency of steatosis and HOMA-IR were significantly ($p = 0.0057$, $p < 0.00001$, respectively) lower in patients with SVR compared with non-SVR (data not shown). However, these factors were not entered in the multivariate analysis because of the absence of the data in many cases.

Factors affecting RVR, cEVR, and SVR by multivariate logistic regression analysis

Multivariate analysis identified four parameters that influenced RVR independently: low HCV RNA load, low serum ALT level, two or more aa mutations in the ISDR and the wild type aa at core position 91 (Table 3).

Concerning cEVR, male gender, mild fibrosis stage, low HCV RNA load, two or more aa mutations in the ISDR, and the wild type aa at core positions 70 and 91 were independent predictors (Table 3).

Concerning SVR, male gender ($p < 0.0001$), low HCV RNA load ($p = 0.013$), high PLT count ($p = 0.0019$), two or more aa mutations in the ISDR ($p = 0.024$), and wild type core aa 70 ($p = 0.0045$) were found to be independent predictors (Table 3).

The predictive values of the combination of gender, PLT count, ISDR and core aa 70 are shown in Fig. 2a. In male patients having PLT of $<15 \times 10^4/mm^3$, and, no or one aa substitution in the ISDR, the SVR rate was 68% when core 70

Table 2 Univariate analysis to identify the factors of SVR

Factors	Negative of HCV RNA after 24 weeks		*p* value
	(−)	(+)	
No. of patients	214 (52.3%)	195	
Gender			
Male	157 (61.3%)	99	<0.00001
Female	57 (37.3%)	96	
Age			
Median (range)	52.5 (18–75)	58 (20–74)	<0.00001
<60 years	155 (58.1%)	112	0.0018
60 years≤	59 (41.5%)	83	
Age: <60 years			
Male	118 (63.4%)	68	0.010
Female	37 (45.7%)	44	
Age: 60 years≤			
Male	39 (55.7%)	31	0.0011
Female	20 (27.8%)	52	
F stage			
F0–2	190 (58.5%)	135	0.000013
F3–4	25 (29.8%)	59	
Grade (A factor)			
A0–1	138 (56.8%)	104	0.130
A2–3	81 (48.5%)	86	
HCV RNA load 0 week (KIU/mL)			
Median (range)	1300 (100–5000<)	1700 (130–5000<)	0.016
ALT 0 week (IU/L)			
Median (range)	66 (16–391)	67 (19–504)	0.892
BMI			
Median (range)	23.0 (17.3–32.4)	23.25 (16.1–33.9)	0.714
Alb (g/dL)			
Median (range)	4.0 (3.2–5.2)	3.8 (3.0–4.8)	0.0088
LDL-C (mg/dL)			
Median (range)	94.5 (31–185)	97.5 (30–182)	0.611
T-Chol (mg/dL)			
Median (range)	169.5 (85–257)	170 (103–273)	0.511
PLT count ($\times 10^4$/mm^3)			
Median (range)	18.2 (8.7–39.9)	15.1 (8.0–31.9)	<0.00001
<15	54 (36.5%)	94	<0.00001
15≤	160 (61.3%)	101	
Amino acid mutation of ISDR			
0–1	156 (48.6%)	165	0.0054
2≤	58 (65.9%)	30	
Amino acid substitution of core 70			
Wild	166 (57.0%)	125	0.0031
Mutant	48 (40.7%)	70	
Amino acid substitution of core 91			
Wild	141 (56.2%)	110	0.054
Mutant	73 (46.2%)	85	
PEG-IFN adherence			
<80%	35 (42.2%)	48	0.063
80%≤	154 (53.8%)	132	
Ribavirin adherence			
<80%	55 (43.3%)	72	0.048
80%≤	132 (54.5%)	110	

Table 3 Multivariate logistic regression analysis to identify independent predictive factors of RVR, cEVR, and SVR

	Odds ratio	95% CI	*p* value
RVR factors selected by stepwise method			
F stage			
F0–2/F3–4	2.924	0.988–8.696	0.053
HCV RNA load 0 week (KIU/mL)			
<1000/1000≤	2.151	1.130–4.082	0.020
ALT 0 week (IU/L)			
<60/60≤	2.165	1.127–4.149	0.020
Amino acid mutation of ISDR			
2≤/0–1	2.371	1.187–4.735	0.014
Amino acid substitution of core 91			
W/M	2.137	1.021–4.464	0.044
cEVR factors selected by stepwise method			
Gender			
Male/female	1.912	1.209–3.021	0.0055
F stage			
F0–2/F3–4	2.079	1.133–3.817	0.018
HCV RNA load 0 week (KIU/mL)			
<1000/1000≤	1.608	1.002–2.577	0.049
PLT count ($\times 10^4$/mm^3)			
15≤/<15	1.427	0.882–2.309	0.148
Amino acid mutation of ISDR			
2≤/0–1	2.512	1.407–4.485	0.0018
Amino acid substitution of core 70			
W/M	2.513	1.508–4.184	0.0004
Amino acid substitution of core 91			
W/M	1.965	1.241–3.115	0.004
SVR factors selected by stepwise method			
Gender			
Male/female	3.704	2.132–6.410	<0.0001
F stage			
F0–2/F3–4	1.812	0.888–3.690	0.103
HCV RNA load0 week (KIU/mL)			
<1000/1000≤	2.024	1.163–3.534	0.013
PLT count ($\times 10^4$/mm^3)			
15≤/<15	2.469	1.394–4.372	0.0019
Amino acid mutation of ISDR			
2≤/0–1	2.148	1.107–4.170	0.024
Amino acid substitution of core 70			
W/M	2.415	1.316–4.444	0.0045
Amino acid substitution of core 91			
W/M	1.433	0.828–2.481	0.199
PEG adherence (%)			
80≤/<80	1.562	0.834–2.926	0.164

W Wild, *M* Mutant

was a wild type but only 16% in patients with mutant at core 70. In female patients, no or one aa substitution in ISDR and $<15 \times 10^4$/mm^3 of PLT count, the SVR rates were as low as 10 or 8%, irrespective of aa substitution at core 70. SVR was only 24% in patients with substitution of core aa 70 even when the PLT count was $\geq 15 \times 10^4$/mm^3. In this study, the combination analysis of PLT count, ISDR, and core aa substitution was useful for predicting non-SVR.

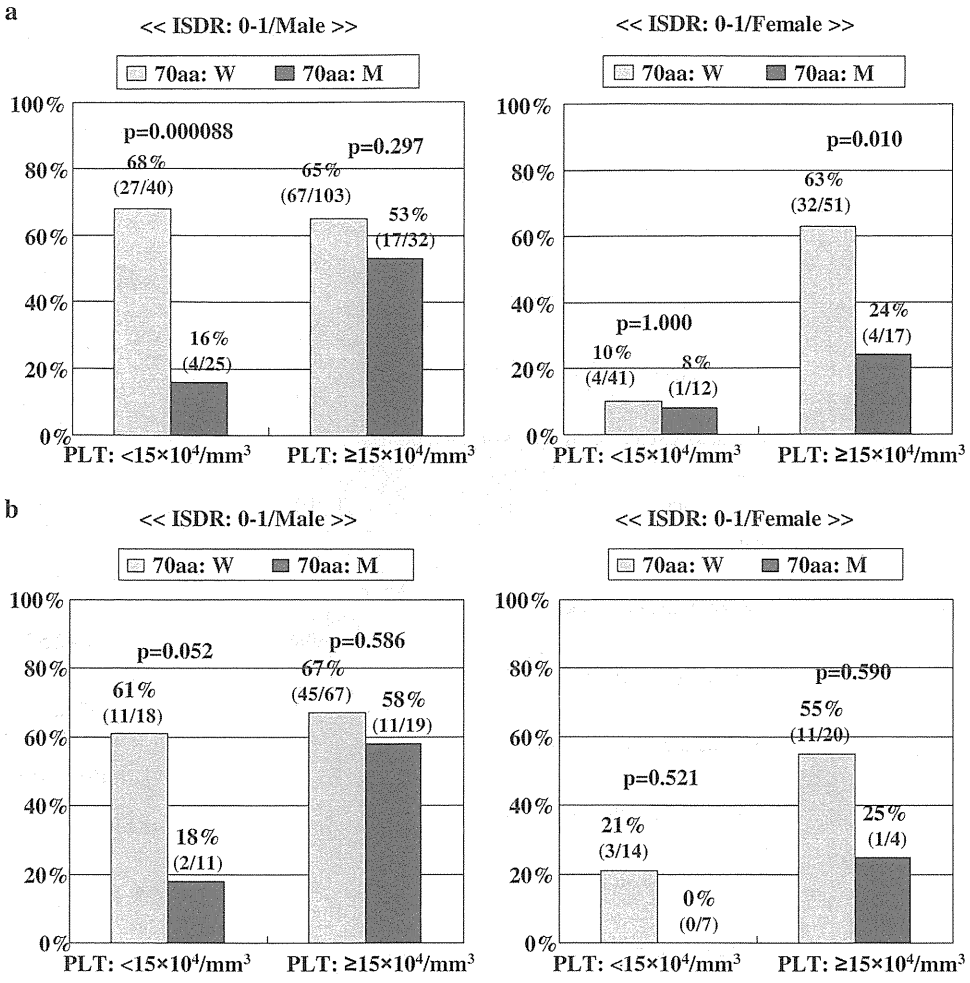

Fig. 2 Relationship between SVR rate and amino acid substitutions in the ISDR and core amino acids 70 and 91, PLT counts and gender difference. The two figures of **a** show the results of *Study 1* and the two figures of **b** show the results of *Study 2*. In male patients with no or only one amino acid (aa) substitution in the ISDR and PLT count of less than $15 \times 10^4/mm^3$, the SVR rate was 68% in those with wild type core aa 70, but only 16% in patients with mutant type of core aa 70, which is significantly different ($p = 0.000088$). There were no significant differences between wild type and mutant type of core aa 70 in the patients with no or one aa substitution in the ISDR and PLT count of over $15 \times 10^4/mm^3$. By contrast, in female patients with no or one aa substitution in the ISDR, there were no significant differences between wild type and mutant type of core aa 70 with PLT count of less than $15 \times 10^4/mm^3$, but there were significant differences between wild type and mutant type of core aa 70 with PLT counts of less than $15 \times 10^4/mm^3$ (**a**). For the patients maintaining over 80% adherences to both PEG-IFN and RBV, in males having no or one aa substitution in the ISDR and PLT counts of less than $15 \times 10^4/mm^3$, a wild type of core aa 70 could predict SVR with a positive predictive value (PPV) of 61% and negative predictive value (NPV) of 82% ($p = 0.052$). However, in male patients with PLT counts of over $15 \times 10^4/mm^3$, core aa 70 was not a useful marker for predicting SVR and non-SVR. The number of female patients with no or one aa substitution in ISDR was too small to reach a definite conclusion (**b**)

Study design 2

The basic features of 201 patients achieving 80% adherences to both PEG-IFN and RBV are as follows: the females were significantly ($p = 0.00006$) older than the males. Iron deposition in liver tissue, alcohol abuse, BMI, serum albumin level, serum ferritin level, and PLT count were significantly higher in males than females. Inflammatory activity was significantly ($p = 0.046$) higher in females than males (data not shown).

AA substitutions in the ISDR were as follows; in males 33 (22.3%) had two or more aa substitutions, in females 8 (15.1%) had two or more aa substitutions. The analysis of core aa position 70 and 91 sequences showed no significant differences in aa substitutions of either core aa 70 or 91 between males and females (data not shown).

In patients less than 60 years of age, SVR rate was significantly higher ($p = 0.0042$) in males than females, but no significant difference was noted between males and females over 60 years old. However, the number of patients over 60 years was small (Table 4).

Table 4 Univariate analysis to identify the significantly different factors between SVR and non-SVR (201 patients received over 80% adherences of both PEG-IFN and RBV)

Factors	Negative of HCV RNA after 24 weeks		*p* value
	(−)	(+)	
No. of patients	111 (55.2%)	90	
Gender			
Male	93 (62.8%)	55	0.00037
Female	18 (34.0%)	35	
Age			
Median (range)	51 (18–70)	56 (23–74)	0.00025
<60 years	91 (60.3%)	60	0.014
60 years≤	20 (40.0%)	30	
Age: <60 years			
Male	79 (66.4%)	40	0.0042
Female	12 (37.5%)	20	
Age: 60 years≤			
Male	14 (48.3%)	15	0.243
Female	6 (28.6%)	15	
F stage			
F0–2	103 (60.9%)	67	0.0012
F3–4	8 (25.8%)	23	
Grade (A factor)			
A0–1	80 (59.3%)	55	0.189
A2–3	31 (47.0%)	35	
HCV RNA load 0 week (KIU/mL)			
Median (range)	1300 (110–5000<)	1280 (130–5000<)	0.351
ALT 0 week (IU/L)			
Median (range)	74 (16–268)	67.5 (19–504)	0.752
BMI			
Median (range)	23.1 (17.3–31.0)	23.6 (16.1–33.9)	0.626
Alb (g/dL)			
Median (range)	3.95 (3.3–5.2)	3.9 (3.0–4.8)	0.079
LDL-C (mg/dL)			
Median (range)	96 (31–185)	97.5 (30–182)	0.865
T-Chol (mg/dL)			
Median (range)	170 (85–248)	170 (105–273)	0.624
PLT count ($\times 10^4/mm^3$)			
Median (range)	18.9 (8.7–30.9)	15.55 (7.2–28.4)	0.00003
<15	23 (35.9%)	41	0.00024
15≤	88 (64.2%)	49	
Amino acid mutation of ISDR			
0–1	84 (52.5%)	76	0.159
2≤	27 (65.9%)	14	
Amino acid substitution of core 70			
Wild	91 (61.5%)	57	0.0037
Mutant	20 (37.7%)	33	
Amino acid substitution of core 91			
Wild	73 (60.3%)	48	0.083
Mutant	38 (47.5%)	42	

Virological responses and aa substitution

The rates of RVR, cEVR, LVR, ETR and SVR in males and females were 12.5 versus 11.3% ($p = 1.000$), 59.6 versus 43.4% ($p = 0.053$), 74.3 versus 50.0% ($p = 0.0018$), 76.2 versus 66.7% ($p = 0.198$), and 62.8 versus 34.0% ($p = 0.00037$), respectively (data not shown). The backgrounds and characteristics of SVR and non SVR patients are shown in Table 4. There were significant differences in gender (male vs. female; $p = 0.00037$), age (<60 years vs. ≥60 years; $p = 0.014$), F stage (F0-2 vs. F3,4; $p = 0.0012$), PLT count ($<15 \times 10^4/mm^3$ vs. $15 \times 10^4/mm^3 \leq$; $p = 0.00024$), and substitution of core aa 70 (wild type vs. mutant, $p = 0.0037$) between SVR and non-SVR patients. The distribution of fatty change in liver tissue (≤10% vs. 11–33% vs. 34%≤; $p = 0.046$) and the grade of HOMA-IR (1.7 vs. 3.9, $p = 0.0018$) were significantly different between SVR and non-SVR (data not described in Table 4).

Factors affecting SVR by multivariate logistic regression analysis

Male gender ($p = 0.0006$), mild fibrosis stage ($p = 0.027$), and wild type of core aa 70 ($p = 0.043$) were independent predictors of SVR.

Valuable markers for predictions of sustained virological response to peginterferon and ribavirin therapy

Two or more aa mutations in the ISDR, wild type core aa 70, $\geq 15 \times 10^4/mm^3$ of PLT count, and male gender were selected statistically as independent predictors of SVR. We show here SVR rates of the patients having over 80% adherences to both PEG-IFN and RBV (Fig. 2b). In males having no or one aa substitution in the ISRD and PLT count of $<15 \times 10^4/mm^3$, wild type core aa 70 could predict SVR with a positive predictive value (PPV) of 61% and negative predictive value (NPV) of 82% ($p = 0.052$). In females, the SVR rate was very low in those who had substitution of core aa 70, but there was no significant difference between patients with wild type and substitution of core aa 70. The number of female patients was too small to provide a definite conclusion.

Discussion

The present multivariate logistic regression analysis revealed that male gender, low HCV RNA load, high PLT count, and two or more aa mutations in the ISDR and wild type core aa 70 were independent predictors for SVR. PLT count significantly decreased corresponding to the progression to the stage of liver fibrosis in CHC [9, 30, 31].

It has been considered that the low adherence level to PEG-IFN/RBV is a major cause of a significantly lower SVR rate in females and older patients [32]. The percentage of patients having over 80% adherences to both PEG-IFN and RBV was significantly lower in females than males, however, differences in the adherence to PEG-IFN/RBV between males and females were not independent predictive factors of non-SVR.

A recent report from Japan showed six or more mutations in the variable region 3 (V3) of nonstructural protein 5A (NS5A) plus upstream flanking region NS5A (aa 2334–2379), referred to as the IFN/RBV resistance determining region (IRRDR), was a useful marker for predicting SVR, but the ISDR sequence was not valuable for predicting SVR [33]. However, the number of subjects in that study was too small ($n = 45$) to reach an acceptable conclusion.

To elucidate the factors affecting low SVR rate in older female patients, we performed a multivariate logistic regression analysis using patients who achieved ≥80% adherence to both PEG-IFN and RBV. Male gender, stage of mild liver fibrosis, and wild type core aa 70 were independent predictors of SVR. In this study, blood concentration of RBV was determined in fewer than 50% of cases during treatment. Thus we cannot exclude the possibility of the effect of the blood concentration of RBV during treatment on the low SVR rate in females and older patients.

From the present analysis, it was clear that ALT, BMI, Alb, T. Chol, and adherence to RBV differed significantly between males and females, however, these factors were not independent predictors of SVR. There is a report that steatosis is an important cofactor that reduces the SVR rate in genotype 1 infected patients [34], however, such an effect was not seen in this study. Thus we could not identify the factors associated with a significantly lower SVR rate in females than males.

In the present multivariate logistic regression analyses, patients having wild type core aa 91 had significantly higher rates of RVR and cEVR, but not SVR, and patients with wild type core aa 70 had significantly higher rates of cEVR and SVR, but not RVR. Patients having two or more aa substitutions in the ISDR had significantly higher rates of RVR, cEVR, and SVR. Although several possibilities have been considered concerning the effects of aa substitutions of core protein on SVR in PEG-IFN/RBV therapy for CHC patients, the exact mechanisms have not yet been elucidated.

Recent reports have indicated that low serum IP-10 (interferon-γ inducible protein 10 kDa) [35], a higher HCV-specific CD8 cell proliferation potential [36], and a high ratio of Th1/Th2 [37] are good predictors of SVR to

PEG-IFN/RBV therapy. These results indicate the importance of immunological status and immunological response to treatment in patients difficult to treat with PEG-IFN/RBV therapy for CHC.

The present univariate analyses revealed that there were many factors relating to RVR, cEVR, and SVR including LDL-C, HOMA-IR, fatty change in liver tissue, and hyaluronic acid, however some of these factors had not been examined in some participating institutes. We consider that we must perform a prospective mass study using many factors including immunological aspects, viral factors, disease status, and therapeutic aspects to elucidate the reason that older female patients are resistant to a combination of PEG-IFN and RBV therapy in CHC with a high viral load genotype 1b.

In conclusion, our results demonstrated that wild type core aa 70, two or more aa mutations in the ISDR, low viral load, high PLT counts, and male gender are useful markers for predicting SVR.

Acknowledgments We express our thanks to other members of the Study Group of Optimal Treatment of Viral Hepatitis; Hideyuki Nomura, Shin-Kokura Hospital; Yoshiyuki Ueno, University of Tohoku; Hisataka Moriwaki, Gifu University; Makoto Oketani, Kagoshima University Graduate School of Medical and Dental Sciences; Masataka Seike, Oita University; Hiroshi Yotsuyanagi, The University of Tokyo. This study was supported in part by a Grant-in-Aid from the Ministry of Health, Labor and Welfare, Japan.

References

1. Manns MP, McHutchinson JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, et al. Peginterferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomized trial. Lancet. 2001;358:958–65.
2. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Gonzales FL, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med. 2002;347:975–82.
3. Hadziyannis S, Sette H Jr, Morgan TR, Balan V, Diago M, Marcellin P, et al. Peginterferon-alfa-2a plus ribavirin combination therapy in chronic hepatitis C. a randomized study of treatment duration and ribavirin dose. Ann Intern Med. 2004;40:346–55.
4. Hiramatsu N, Kurashige N, Oze T, Takehara T, Tamura S, Kasahara A, et al. Early decline of hemoglobin can predict progression of hemolytic anemia during pegylated interferon and ribavirin combination therapy in patients with chronic hepatitis C. Hepatol Res. 2008;38:52–9.
5. Honda T, Katano Y, Urano F, Murayama M, Hayashi K, Ishigami M, et al. Efficacy of ribavirin plus interferon-α in patients aged 60 years with chronic hepatitis C. J Gastroenterol Hepatol. 2007;22:989–95.
6. Sezaki H, Suzuki F, Kawamura Y, Yatsuji H, Hosaka T, Akuta N, et al. Poor response to pegylated interferon and ribavirin in older women infected with hepatitis C virus of genotype 1b in high viral load. Dig Dis Sci 2009;54:1317–24
7. Puoti C, Castellacci R, Montagness F, Zaltron S, Stornaiuolo G, Bergami N, et al. Histological and virological features and follow-up of HCV carriers with normal aminotransferase levels: the Italian Study of the Asymptomatic C Carriers (ISACC). J Hepatol. 2002;37:117–23.
8. Hui CK, Belaye T, Montegrande K, Wright TL. A comparison in the progression of liver fibrosis in chronic hepatitis C between persistently normal and elevated transaminase. J Hepatol. 2003;38:511–7.
9. Okanoue T, Makiyama A, Nakayama M, Sumida Y, Mitsuyoshi H, Nakajima T, et al. A follow-up study to determine the value of liver biopsy and need for antiviral therapy for hepatitis C virus carriers with persistently normal serum aminotransferase. J Hepatol. 2005;43:599–605.
10. Bressler BL, Guindi M, Tomlinson G, Heathcote J. High body mass index in an independent risk factor for non response to antiviral treatment in chronic hepatitis C. Hepatology. 2003;38:639–44.
11. Walsh MJ, Jonsson JR, Richardson MM, Lipka GM, Purdi DM, Clouston AD, et al. Non-response to antiviral therapy is associated with obesity and increased hepatic expression of suppressor of cytokine signaling 3 in patients with chronic hepatitis C, viral genotype 1. Gut. 2006;55:604–9.
12. Patton HM, Patel K, Behling C, Bylund C, Blatt LM, Vallee M, et al. The impact of steatosis on disease progression and early and sustained treatment response in chronic hepatitis C patients. J Hepatol. 2004;40:484–90.
13. Asselah T, Rubbia-Brandt L, Marcellin M, Negro F. Steatosis in chronic hepatitis C: why does it really matter? Gut. 2006; 55:123–30.
14. Romero-Gomez M, Del Mar Viloria M, Andrade RJ, Salmeron J, Diago M, Fernandez-Rodriguez CM, et al. Insulin resistance impairs sustained response rate to peginterferon plus ribavirin in chronic hepatitis C patients. Gastroenterology. 2005;128: 636–41.
15. Bruno S, Camma C, Di Marco V, Rumi M, Vinci M, Cammozzi M, et al. Peginterferon alfa-2b plus ribavirin for native patients with genotype 1 chronic hepatitis C: a randomized controlled trial. J Hepatol. 2004;41:474–81.
16. Everson GT, Hoefs JC, Seeff LB, Bonkovsky HL, Naishadham D, Shiffman ML, et al. Impact of disease severity on outcome of antiviral therapy for chronic hepatitis C: lessons from the HALT-C trial. Hepatology. 2006;44:1675–84.
17. McHutchison JG, Manns M, Patel K, Poynard T, Lindsay KL, Trepo C, et al. Adherence to combination therapy enhances sustained response in genotype-1-infected patients with chronic hepatitis C. Gastroenterology. 2002;123:1061–9.
18. Jeffers LJ, Cassidy W, Howell CD, Hu S, Reddy R. Peginterferon alfa-2a (40kd) and ribavirin for black American patients with chronic HCV genotype 1. Hepatology. 2004;39:1702–6.
19. Muir AJ, Bornstein JD, Killenberg PG, Atlantic Coast Hepatitis Treatment Group. Peginterferon alfa 2b and ribavirin for the treatment of chronic hepatitis C in blacks and non-Hispanic whites. N Engl J Med. 2004;350:2265–71.
20. Poynard T, Marcellin P, Lee SS, Niederau C, Minuk GS, Ideo G, et al. Randomized trial of interferon alpha 2b plus ribavirin for 48 weeks or 24 weeks versus interferon alpha 2b plus placebo for 48 weeks for treatment for chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). Lancet. 1998;352:1426–32.
21. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, et al. Mutations in the nonstructural protein 5 A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med. 1996;334:77–81.
22. Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, et al. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response in interferon-ribavirin combination therapy. Intervirology. 2005;48:372–80.
23. Donlin MJ, Cannon NA, Yao E, Li J, Wahed A, Taylor MW, et al. Pretreatment sequence diversity differences in the

full-length hepatitis C virus open reading frame correlate with early response to therapy. J Virol. 2007;81:8211–24.
24. Davis GL, Wong JB, McHutchison JG, Manns MP, Harvey J, Albrecht J. Early virologic response to treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C. Hepatology. 2003;38:645–52.
25. Ferenci P, Fried MW, Shiffman ML, Smith CI, Marinos G, Goncales FL, et al. Predicting sustained virological responses in chronic hepatitis C patients treated with peginterferon alfa-2a ribavirin. J Hepatol. 2005;43:425–33.
26. Moucari R, Ripault M-P, Oules V, Martinot-Peignoux M, Asselah T, Boyer N, et al. High predictive value of early viral kinetics in retreatment with peginterferon and ribavirin of chronic hepatitis C patients non-responders to standard combination therapy. J Hepatol. 2007;46:596–604.
27. Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1a: amino acid substitutions in the core region and low-density lipoprotein cholesterol level. J Hepatol. 2007;46:403–10.
28. Desmet VJ, Gerber M, Hoofnagle JH, Manna M, Scheuer PJ. Classification of chronic hepatitis: grading and staging. Hepatology. 1994;19:1513–20.
29. Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, et al. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc Natl Acad Sci USA. 1990;87:9524–8.
30. Okanoue T, Itoh Y, Minami M, Sakamoto S, Yasui K, Sakamoto M, et al. Interferon therapy lowers the rate of progression to hepatocellualr carcinoma in chronic hepatitis C but not significantly in an advanced stage: a retrospective study in 1148 patients. J Hepatol. 1999;30:653–9.
31. Okanoue T, Itoh Y, Minami M, Hashimoto H, Yasui K, Yotsuyanagi H, et al. Guidelines for the antiviral therapy of hepatitis C virus carriers with normal serum aminotransferase based on platelet count. Hepatol Res. 2008;38:27–36.
32. Iwasaki Y, Ikeda H, Araki Y, Osawa T, Kita K, Ando M, et al. Limitation of combination therapy of interferon and ribavirin for older patients with chronic hepatitis. Hepatology. 2006;43:54–63.
33. El-Shamy A, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H. Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. Hepatology. 2008;48:38–47.
34. Patton HM, Patel K, Behling C, Bylund D, Blatt LM, Vallee M, et al. The impact of steatosis on disease progression and early and sustained treatment response in chronic hepatitis C patients. J Hepatol. 2004;40:484–90.
35. Lagging M, Romero A, Westin J, Norkrans G, Dhillon AP, Palwlosky JM, et al. IP-10 predicts viral response and therapeutic outcome in difficult-to-treat patients with HCV genotype 1 infection. Hepatology. 2006;44:1617–25.
36. Pilli M, Zerbini A, Penna A, Orlandini A, Lukasiewicz E, Pawlotsky JM, et al. HCV-specific T-cell response in relation to viral kinetics and treatment outcome (DITTO-HCV Project). Gastroenterology. 2007;133:1132–43.
37. Shirakawa H, Matsumoto A, Joshita S, Komatsu M, Tanaka N, Umemura T, et al. Pretreatment prediction of virological response to peginterferon plus ribabirin therapy in chronic hepatitis C patients using viral and host factors. Hepatology. 2008; 48:1753–60.

Hepatology Research 2009; 39: 213–222 doi: 10.1111/j.1872-034X.2008.00442.x

Review Article

Role of hepatic iron in non-alcoholic steatohepatitis

Yoshio Sumida,[1] Toshikazu Yoshikawa[2] and Takeshi Okanoue[3]

[1]*Center for Digestive and Liver Diseases, Nara City Hospital, Nara,* [2]*Department of Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, Kyoto,* [3]*Center of Gastroenterology and Hepatology, Saiseikai Suita Hospital, Suita, Japan*

Non-alcoholic fatty liver disease (NAFLD) includes a spectrum of clinical entities ranging from simple steatosis to non-alcoholic steatohepatitis (NASH) with possible evolution to cirrhosis and hepatocellular carcinoma. Iron is considered a putative element that interacts with oxygen radicals in inducing liver damage and fibrosis. The role of hepatic iron in the progression of NASH remains controversial, but in some patients, iron may have a role in the pathogenesis of NASH. Though genetic factors, insulin resistance, dysregulation of iron-regulatory molecules, erythrophagocytosis by Kupffer cells may be responsible for hepatic iron accumulation in NASH, exact mechanisms involved in iron overload remain to be clarified. Iron reduction therapy such as phlebotomy or dietary iron restriction may be promising in patients with NASH/NAFLD to reduce insulin resistance as well as serum transaminase activities.

Key words: insulin resistance, iron, nonalcoholic fatty liver disease, non-alcoholic steatohepatitis, oxidative stress, phlebotomy

INTRODUCTION

IRON IS A potent catalyst of oxidative stress and may act synergistically with other promoters of lipid peroxidation by catalyzing these reactions. Iron overload can also directly cause lipid peroxidation, and one of the subsequent products, malondialdehyde, has been shown to activate hepatic stellate cells in vitro, the major source of fibrogenesis in liver injury.[1]

Non-alcoholic liver disease (NAFLD) is defined as a constellation of clinical conditions characterized by predominantly macrovesicular steatosis of the liver. The histologic spectrum of this disease ranges broadly from simple steatosis and non-alcoholic steatohepatitis (NASH) through to cirrhosis. Although simple steatosis seems to be benign, NASH can have a progressive course. Diagnosis of NASH is defined by liver histology, which typically shows macrovesicular steatosis and lobular inflammation with or without fibrosis. Mallory bodies are occasionally seen in the absence of a history of excessive ethanol ingestion.[2] The exact mechanism of this progression is not known but probably involves two steps.[3] Excessive triglyceride accumulation is the most likely first step. The second step may relate to an increase in oxidative stress, which, in turn, triggers liver cell necrosis and activation of hepatic stellate cells, both leading to fibrosis and ultimately to the development of cirrhosis. One of the potential cofactors suspected to enhance this oxidative stress is excessive hepatic iron accumulation.

This article explores the role of hepatic iron in NASH/NAFLD, and the possible therapeutic implications of iron reduction therapy.

Iron indices and hepatic iron deposition in NASH/NAFLD

There is controversial evidence that hepatic iron may play a role in the pathogenesis of NASH/NAFLD. Bacon *et al.*[4] documented abnormal iron indices (serum ferritin and/or transferrin saturation), and elevated hepatic iron concentration in NASH. George *et al.*[5] first proposed the hypothesis of iron-related liver injury in NASH. In their study of 51 patients, increased hepatic iron was present in 41%, and 23% had hepatic iron concentration (HIC)

Correspondence: Dr Yoshio Sumida, Center for Digestive and Liver Diseases, Nara City Hospital, Higashi Kidera-cho 1-50-1, Nara 630-8305, Japan. Email: sumida@nara-jadecom.jp
Received 5 July 2008; revision 1 August 2008; accepted 4 August 2008.

above the upper limit of normal. Their most significant conclusion was that increased hepatic iron had the greatest association with the severity of fibrosis; Perl's stain grade was assigned a relative risk of 5.5 (95% CI, 2.5–13.6). We also previously reported high frequencies of hyperferritinemia and increased hepatic iron stores in Japanese patients with NASH.[6,7] Serum thioredoxin (TRX) levels, an indicator of oxidative stress, were increased in proportion of the grade of hepatic iron accumulation.[6] Our data imply that the presence of excessive hepatic iron may be one of the possible cofactors for the induction of oxidative stress in NASH. Iron could potentially play a supporting role in the lipid peroxidation and fibrogenesis central to the development and progression of NASH.

In contrast, Younossi *et al.*,[8] Angulo *et al.*,[9] and Chitturi *et al.*[10] documented that significant iron accumulation is not seen in most patients with NASH. Younossi *et al.*[8] found no significant iron accumulation in NAFLD patients and no association between hepatic iron and aggressive histological or clinical outcome. Angulo *et al.*[9] studied 132 patients with NASH from Mayo Clinic database to find independent predictors of hepatic fibrosis. HIC and hepatic iron index were normal in all patients with abnormal iron indices (53% had elevated serum ferritin; 11% had elevated transferrin saturation). They found no association between increased iron indices and degree of fibrosis in multivariate analysis. Chitturi *et al.*[10] demonstrated that hyperferritinemia was present in 38 (40%) of 93 patients with NASH, but that only nine (10%) patients showed increased iron: 7 with grade 2 and 2 with grade 3. In India, Duseja *et al.*[11] showed that 71% of thirty-one NASH patients had negative iron staining. There was no association the degree of iron staining and fibrosis stage. These authors conclude that hyperferritinemia in NASH is a nonspecific effect of hepatic necroinflammation, reflecting its function as an acute phase protein. Serum ferritin is known to increase because of release from damaged hepatocytes. We also previously suggested that serum ferritin levels reflect oxidative stress as well as hepatic iron concentration and hepatocyte damage in chronic liver disease,[12] because the synthesis of ferritin seems to be influenced by oxidative stress or reduction/oxidation (redox) state.[13,14] It is possible that elevated serum ferritin in NASH may be derived from iron-unrelated oxidative stress,[12] such as free fatty acid, lipid peroxide, cytokines, and induction of cytochrome P450 enzymes (CYP2E1 and CYP4A).[15]

In this way, the role of hepatic iron in the pathogenesis of NASH or abnormal iron indices in NASH remains controversial and unsettled as of this time.[16]

The role of HFE mutation in hepatic iron deposition of NASH/NAFLD

The significance of hemochromatosis gene (HFE) mutations in the pathogenesis and progression of NASH/NAFLD also remains controversial.[5,10,17] George *et al.*[5] demonstrated a higher prevalence of the Cys282Tyr mutation of the HFE gene in patients with NASH, although there was no difference in the frequency of the His63Asp mutation. The presence of the Cys282Tyr mutation was associated with increased hepatic iron staining and hepatic iron concentration. Bonkovsky *et al.*[18] also reported a significantly higher prevalence of certain HFE mutation in patients with NASH. These authors found that Cys282Tyr heterozygotes had significantly more hepatic fibrosis than those without HFE mutations. However, the HIC was normal in all subjects and did not differ between those with and without HFE mutations. According to data from 126 NASH patients which were collected from 6 North American conters,[19] Cys282Tyr heterozygotes is associated with advanced hepatic fibrosis and stainable hepatic iron in Caucasians with NASH. They speculate that the mechanism is related to increased oxidative stress in the liver due to increased iron deposition. Chitturi *et al.*[10] found a trend toward higher serum ferritin levels among Cys282Tyr heterozygotes with NASH. Neither hepatic iron nor the presence of HFE mutations were identified as risk factors for fibrotic severity. Similarly, no evidence of an association of hepatic iron overload and HFE mutations with NASH was found in Brazilian or Asian Indian patients.[11,20,21] In Japan, there were no NASH/NAFLD patients with HFE mutations,[22] in agreement with a previous report indicating that the frequencies of HFE mutations in the Japanese population are extremely low (Cys282Tyr, 0%; and His63Asp, 0.99%).[23]

In this way, HEF gene mutation which is related to ethnicity[10] does not seem to have important roles in hepatic iron deposition in NASH/NAFLD.

Possible mechanisms involved in hepatic iron overload in NASH/NAFLD

As mentioned above, the precise mechanisms underlying hepatic iron deposition in NASH remain unknown. However, several possible mechanisms have been suggested in clinical or experimental studies (Fig. 1).

An association between insulin resistance (IR) and hepatic iron overload has been recently described.[24] First, it is quite plausible that the unhealthy diets contribute to IR not only through excess fat intake but also through excess iron supply (for example, in meat or in

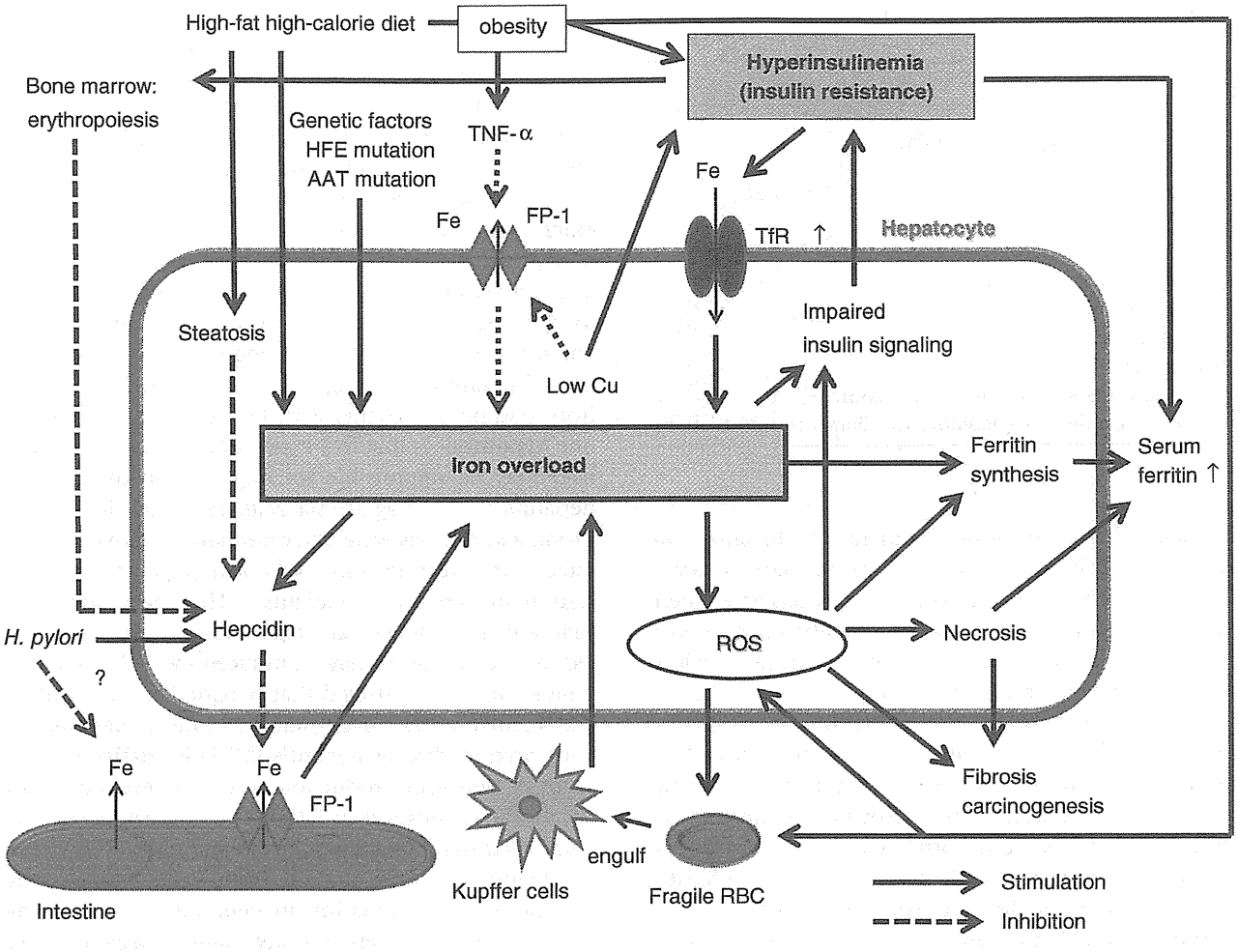

Figure 1 Possible mechanisms of hepatic iron deposition and pathogenetic roles of iron in nonalcoholic steatohepatitis/nonalcoholic fatty liver disease. AAT, alpha 1-antitrypsin; FP-1, ferroporitn-1; *H. pylori*, *Helicobacter pylori*; RBC, red blood cell; ROS, reactive oxygen species; TfR, transferrin receptor; TNF-α, tumor necrosis factor-α.

iron-supplemented food). Second, iron overload can interfere with insulin signaling through the induction of reactive oxygen species (ROS) (Fig. 1), the latter impairing insulin uptake through a direct effect on insulin receptor function,[25] by inhibiting the translocation of glucose transporter 4 (GLUT4) to the plasma membrane and iron induces IR of glucose transport in adipocytes through a mechanism independent of fatty acids.[26] Moreover, iron has been found to reduce hepatic extraction/metabolism of insulin and to interfere with insulin action on the liver, leading to peripheral hyperinsulinemia.[27,28] Another mechanism is the enhancement of GLUT 1 and 4 activities in skeletal muscle after iron depletion.[29] By contrast, hyperinsulinemia may cause rapid stimulation of iron uptake into the liver, because insulin is known to redistribute transferrin receptors from an intracellular membrane compartment to the cell surface (Fig. 1).[30,31] The association of unexplained hepatic iron overload with metabolic disorders has recently been coined as the insulin resistance-associated hepatic iron overload syndrome (IR-HIO) proposed by Mendler *et al.*[32] Although patients with IR-HIO have a high prevalence of IR-related metabolic disorders, the relationship of IR-HIO to NASH is unclear. It is plausible that IR, which is often associated with NASH,[33] may be directly responsible for the accumulation of iron in the liver.[34] In general population, the association of elevated serum ferritin concentrations and IR was already demonstrated.[35–37] Several studies have demonstrated that serum ferritin is a good indica-

Table 1 Similarities between chronic hepatitis C and non-alcoholic steatohepatitis

1. Histological findings
Steatosis
Iron deposition (hyperferritinemia)
2. Pathogenesis
Oxidative stress
Insulin resistance
3. Progression
Cirrhosis or hepatic failure
Hepatocellular carcinoma
4. Treatments
Diet therapy
Medication (ursodeoxycholic acid, vitamin E)
Iron reduction therapy (phlebotomy, dietary iron restriction)

tor of IR also in hepatitis C patients.[38,39] In Japan, we also previously reported that serum ferritin levels were positively correlated with homeostasis assessment model for IR (HOMA-IR) in non-diabetic patients with chronic hepatitis C.[40] One problem of these previous studies is that the iron concentration of liver tissue was not measured, because serum ferritin cannot always reflect total body iron stores as mentioned above. We recently studied 56 non-obese non-diabetic patients with chronic hepatitis C to investigate whether hepatic iron deposition is really correlated with IR. HOMA-IR was significantly correlated not only with serum ferritin but also with the grade of hepatic iron deposition.[41] Our results suggest that chronic hepatitis C may be one of the IR-HIO. Marcheisini *et al.*[42] have reported that serum indices of iron overload are present in 10 or 43% patients with NAFLD, but that those do not correlate with measures of insulin sensitivities. We demonstrated that serum ferritin levels and HOMA-IR in patients with NASH were significantly higher than in those with simple steatosis.[43] Our study did not show the correlation of HOMA-IR with serum ferritin levels or hepatic iron concentrations in patients with NASH/NAFLD,[43] in contrast with previous studies showing the positive correlation of IR with hepatic iron deposition in hepatitis C.[40,41] In this way, further studies are required to clarify the association of IR with iron deposition in NASH/NAFLD. On the other hand, high frequencies of hepatic steatosis in hepatitis C as previously shown[44] have suggested that chronic hepatitis C may be named a virus-associated steatohepatitis (VASH).[45] Between chronic hepatitis C and NASH, there are several similarities such as steatosis, insulin resistance, hepatic iron accumulation, and oxidative stress (Table 1).

Hepcidin is a disulfide-bonded peptide that was first identified as an antimicrobial peptide and was subsequently shown to be central player in systemic iron homeostasis.[46,47] Hepcidin is believed to be a negative regulator of dietary iron absorption and of iron release by macrophages via inducing internalization and degradation of the iron exporter ferroportin in absorptive enterocytes and reticuloendothelial cells.[48] The synthesis of hepcidin, which is specifically produced by the liver,[49] is greatly stimulated by inflammation or by iron overload.[50] The expression is down-regulated by hypoxia, anemia, iron deficiency, erythropoietin, and erythropoietic stimulation.[51] Though the role of hepcidin in the iron loading of patients with hepatitis C is unknown, one hypothesis is that low expression of hepcidin in the liver may be responsible for hepatic iron overload in hepatitis C.[52–54] Nagashima *et al.* reported that serum prohepcidin levels were decreased and negatively correlated with serum ferritin levels or hepatic iron concentration in hepatitis C patients.[52] They have suggested that failure of homeostatic regulation of serum prohepcidin concentrations may be induced by HCV infection. Fujita *et al.* demonstrated that hepatic hepcidin mRNA was relatively low in chronic hepatitis C patients, as compared to chronic hepatitis B.[53] This relative impairment of hepcidin production was fully reversible after successful eradication of HCV.[54] In contrast, Aoki *et al.* concluded that liver hepcidin, whose mRNA was correlated with iron concentration, cannot play a role in the hepatic iron accumulation in hepatitis C.[55] According to a recent experimental study using transgenic mice expressing HCV polyprotein by Nishina *et al.*, HCV-induced ROS may down-regulate hepcidin transcription, which in turn leads to increased duodenal iron transport and macrophage iron release, causing hepatic iron accumulation.[56] In alcoholic liver disease, hepatic iron loading seems to be contributed to the down-regulated hepcidin leading to the increase of iron absorption from the intestine.[57,58] The expression of hepcidin in NASH/NAFLD patients remains unknown. In animal model of IR, IR can lead to a downregulation of hepcidin expression via stimulation of erythropoiesis, which in turn increases the needs for iron (Fig. 1).[59] It has been recently shown that hepcidin is expressed, at both the mRNA and protein levels, in adipose tissue and that this expression is enhanced in severely obese patients.[60] Human studies focused on iron absorption rates and hepcidin expression in NASH/NAFLD or metabolic syndrome should be performed to unravel the mechanisms behind the iron metabolism disturbance.[61] According to a recent study from Australia,[62] analysis of

iron-regulatory molecules in liver tissue revealed a striking down-regulation of the liver iron exporter ferroporitn-1 (FP-1) and the iron sensing molecule hemojuvelin (HJV). They suggest that TNF-α, highly expressed in NAFLD patients, play a role in exerting these regulatory changes, because they found inverse correlations of TNF-α concentrations and expression of FP-1 or HVJ in vivo and decreased formation of FP-1 and HVJ in HepG2 cells on stimulation with TNF-α in vitro. Thus, they concluded that iron accumulation in NAFLD may result from decreased iron mobilization from hepatocytes due to low expression of FP-1 and HVJ (Fig. 1). The same group found that NAFLD patients with iron overload had low levels of serum and hepatic copper along with low serum ceruloplasmin levels.[63] They suggested that copper deficiency may be responsible for hepatic iron overload, because the copper-dependent ferroxidase ceruloplasmin is required for the mobilization of iron from storage sites such as the liver. Moreover, FP-1 mRNA expression and protein were found to be lowest in NAFLD patients with low hepatic copper concentrations. They detected significantly lower FP-1 protein levels in rats kept on a copper-deficient diet as compared with rats with a normal copper diet. Lower copper bioavailability causes increased hepatic iron stores via decreased FP-1 expression and ceruloplasmin ferroxidase activity thus blocking liver iron export in copper-deficient NAFLD patients (Fig. 1). These studies observed hepatic expression of hepcidin, which reflects the physiologic response to liver iron accumulation.

On the other hand, several lines of evidences have suggested an association between *Helicobacter pylori* (Hp) infection and iron deficiency.[64] If Hp infection is associated with iron deficiency anemia, then eradicating the organism should increase iron stores and resolve the anemia. Reversal of iron deficiency anemia after successful eradication of Hp has been observed not only in children[65,66] but also in adults.[67–69] These findings have supported an association between Hp infection and iron deficiency. Several possible mechanisms may correlate Hp infection with decreased iron accumulation.[70] Recent findings support the hypothesis that in patients with Hp positive gastritis, concomitant changes in intragastric pH and ascorbic acid are present that might play a role in impairing alimentary iron absorption with consequent iron deficiency.[64,71] It has also been speculated that Hp infected antrum could act as a sequestering focus for iron. Hp infection enhances gastric lactoferrin,[72,73] which captures iron from transferrin. The iron bound to lactoferrin is in turn picked up by the bacterium, by means of its outer membrane receptors,[74] for its own growth.[64] Though the exact mechanisms of iron deficiency in Hp-infected individuals are unknown, one hypothesis[75] is that up-regulated expression of hepcidin by Hp infection via releasing cytokines such as IL-6 could impair intestinal iron absorption with consequent lower iron deposit in the liver (Fig. 1). Dr Beutler hypothesizes that Hp may produce hepcidin mimicks preventing the absorption of iron in a manner analogous with the suppression of iron absorption by hepcidin associated with inflammation.[76] We previously reported that Hp infection may decrease hepatic iron deposition in hepatitis C patients.[77] Our preliminary study also demonstrated that the grades of hepatic iron deposition in NAFLD patients with Hp infection were lower than those without.[43]

Otogawa K *et al.*[78] newly proposed that hepatic iron in NASH may be derived from erythrophagocytosis by liver macrophages (Kupffer cells), using rabbits fed a cholesterol-rich high-fat diet (HFD) with IR who exhibit pathological changes very similar to those of human NASH (Fig. 1). In addition, immunohistochemistry in liver tissue from NASH patients revealed that the aggregation of erythrocytes in inflammatory hepatic sinusoids was increased, leading to hepatic iron deposition and oxidative stress. How erythrocytes in patients with NASH become easy to be engulfed by Kupffer cells is not fully understood, though ROS may be involved in the mechanism (Fig. 1).

Recently, another element has been examined that could affect iron metabolisim. Alpha 1-antitrypsin (AAT) protein, an acute-phase protein, has been demonstrated to interact with transferrin receptor inducing ferritin synthesis. Valenti *et al.*[79] demonstrated that NAFLD patients with AAT mutations had higher ferritin levels than those without. In liver histology, AAT mutations were associated with higher prevalence of sinusoidal siderosis, but not with more severe liver damage in NAFLD. AAT mutations did not affect parenchymal or portal siderosis.

Iron reduction therapy in NAFLD/NASH

Although the causes of NASH are not well defined and several therapies including diet,[80] antioxidants,[81] and approaches that improve IR[82] have been tried, the optimal therapy for NASH has not been established.

In Japan, we previously confirmed a significant improvement in serum transaminase activities after 3-month iron reduction therapy by phlebotomy for chronic hepatitis C in a multicenter, prospective, randomized, controlled trial.[83] We have also demonstrated that the efficacy of phlebotomy is superior to that of

Table 2 Phlebotomy in nonalcoholic steatohepatitis/non-alcoholic fatty liver disease

Study	Location	Study design	Patients, *n* (F/M)	Disease	ALT (IU/mL)	Ferritin (ng/mL)	HFE mutation	Insulin resistance (IRI, HOMA-IR)	Histological evaluation
Facchini *et al.* (2002)[85]	San Francisco, US	Open	17 (5/12)	NAFLD IGT (+)	From 61 ± 5 to 32 ± 2 ($P < 0.001$)	From 299 ± 41 to 15 ± 1 ($P < 0.001$)	Excluded	Improved	NA
Valenti *et al.* (2003)[86]	Milano, Italy	Open	12 (0/12)	NAFLD IGT (–)	From 62 ± 45 to 33 ± 22 ($P = 0.0074$)	From 583 ± 274 to 36 ± 22	C282Y (+/–): 3/12	Improved (independently of serum ferritin levels)	Only at entry
Riquelme *et al.* (2004)[88]	Santiago, Chile	Case report	1 (1/0)	NASH (PCT, β thalassemia minor)	From 68 to normal (<20)	From 762 to normal (<417)	H63D (+/–)	NA	Complete resolution of steatosis and inflammation
Fargion *et al.* (2005)[28]	Milano, Italy	Open	42 (9/33)	NAFLD	From 44 ± 30 to 32 ± 19 ($P = 0.03$)	From 361 ± 222 to 123 ± 100 ($P = 0.0001$)	NA	Improved (independently of serum ferritin levels)	NA
Sumida *et al.* (2006)[87]	Nara, Japan	Open	11 (5/6)	NASH	From 126 ± 47 to 56 ± 17 ($P = 0.002$)	From 563 ± 322 to 18 ± 9 ($P = 0.001$)	NA	NA	Only at entry
Valenti *et al.* (2007)[89]	Milano, Italy	Case-control	64 (11/53)	NAFLD	From 57.9 ± 47.4 to 34.3 ± 27 (NS *versus* controls)	From 438 {21–628} to 52 {27–96}	C282Y (+/–): 11/54 H63D (+/–): 19/54	Improved (especially in patients with hyperferrtinemia and carrying HFE mutation)	Only at entry

{}: interquartile range. IGT, impaired glucose tolerance; IRI, immuno-reactive insulin; HOMA-IR, homeostasis model assessment for insulin resistance; NA, not assessed; NAFLD, nonalcoholic fatty liver disease; NS, not significant; PCT, porphyria cutanea tarda.