ORIGINAL PAPER

BODY IRON STORES AND IRON RESTORATION RATE IN JAPANESE PATIENTS WITH CHRONIC HEPATITIS C AS MEASURED DURING THERAPEUTIC IRON REMOVAL REVEALED NEITHER INCREASED BODY IRON STORES NOR EFFECTS OF C282Y AND H63D MUTATIONS ON IRON INDICES

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ABSTRACT

Information on the level of iron stores in chronic hepatitis C is clinically important because its reduction is technically simple and therapeutically effective. This study was performed to measure the levels of iron stores from the total amounts of hemoglobin removed during iron reduction therapy. The C282Y and H63D mutations of HFE gene were analyzed in 94 patients. All of the patients were negative for C282Y mutation. One patient was homozygous, and 4 patients were heterozygous for H63D mutation. The body iron stores and iron restoration rate were measured in 59 patients in serial courses of iron reduction therapy. Mean values of body iron stores in the two groups with and without H63D mutation were 890 and 606 mg, while those of iron restoration rate were 1.85 and 1.52 mg/day, respectively. None of the indices of iron metabolism were different from the reference values measured similarly in healthy subjects, suggesting that the iron deposition in chronic hepatitis C is limited to the liver, probably due to changes in the iron distribution in tissues.

Key Words: Hemochromatosis, Phlebotomy, Ferritin, Mucosal absorption

INTRODUCTION

The disease activity of chronic hepatitis C (CHC) is affected by various factors including biological properties of the infecting hepatitis C virus (HCV) and host defense mechanisms of the patient. Information on the levels of body iron stores is clinically important because iron reduction treatment is effective[1, 2]. Poor responders to the antiviral agent interferon should be screened for body iron status, and when the serum level of ferritin is higher than 12 ng/ml, iron reduction is indicated to suppress progressive liver fibrosis. The recently identified C282Y and H63D mutations of HFE gene disrupt the regulated mucosal uptake of iron and are responsible for hereditary hemochromatosis in Caucasians[3]. Subsequent studies indicated that these

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mutations were also involved in various iron overload disorders including CHC in Caucasians[4, 5]. Previously, we reported no subjects with C282Y mutation, and a small number of subjects with H63D mutation in a population of about 300 Japanese subjects[6, 7]. This very low prevalence of HFE mutation may reflect the rare incidence of hemochromatosis in Japan[8]. In this study, we measured body iron stores (BIS) and iron restoration rate (IRR) of patients with CHC who had undergone iron reduction therapy to elucidate whether their genetic background modified their iron metabolism and clinical course.

PATIENTS AND METHODS

Genetic analysis of HFE and iron reduction therapy for patients with CHC were approved by the Nagoya University Hospital Ethics Committee and were performed with informed consent from each patient.

CHC was confirmed by biochemical liver damage for more than 6 months and positive serum test for HCV RNA. Heavy drinkers and patients with multiple transfusions and either parenteral or oral iron supplementation were excluded from the study. The C282Y and H63D mutations of HFE gene was analyzed in 60 male patients and 34 female patients with CHC. BIS and IRR were determined in 49 patients (29 males, 20 females) who had received iron reduction therapy[1]. Forty-four patients (90%) were poor responders to pre-iron reduction interferon.

DNA was extracted from peripheral leukocytes. The HFE gene region was amplified by polymerase chain reaction, followed by restriction enzyme assay. Primers used for C282Y mutation were forward; 5′-TGGCAAGGGTAAACAGATCC, reverse; 5′-CTCAGGCACTCCTCTCAACC, and the restriction enzyme used was SnaB1[9]. Primers used for H63D were forward; 5′-ACATGGTTAAGGCCTGTTGC, reverse; 5′-GCCACATCTGGCTTGAAATT, and the enzyme used was Bcl1[10]. Direct sequence analysis of H63D mutation was performed in some patients.

Initial iron reduction by phlebotomy, 400 or 200 mL per 2 weeks, was continued until the serum levels of ferritin fell to the end point of 10 ng/mL as reported previously[1]. Subsequent maintenance therapy was performed to keep the serum ferritin level less than 20 ng/mL for at least 2 years. BIS was measured by the total amounts of hemoglobin removed by phlebotomy during the initial phlebotomy period, and IRR was determined by total amounts of hemoglobin removed by additional phlebotomy and regular blood tests during the iron deficiency maintenance treatment period. The formula used to determine iron content (mg) removed as hemoglobin was Hb (g/dL) $\times$ 0.034 $\times$ blood volume (mL). IRR was expressed as hemoglobinized iron/days in iron deficiency maintenance period (mg/day).

The correlation between BIS and IRR was evaluated after adjusting BIS by the age in years since BIS is age-dependent. The correlation between BIS and pre-treatment serum ferritin levels, and BIS and pre-treatment serum ferritin levels were also evaluated to determine whether some biochemical parameters could be of prospective value for iron overload and provide information for planning the time schedule of iron reduction therapy.

RESULTS

No C282Y mutation was found in any of the 94 CHC patients examined. One was homozygous and three were heterozygous for H63D. Direct sequencing confirmed the results of restric-

tion enzyme assay.

Clinical and laboratory features of 4 patients with H63D mutation and 45 patients without the mutation are summarized in Table 1. One patient heterozygous for H63D mutation was thought to be at the cirrhotic stage. The other 3 patients with at least one mutant H63D allele had histochemically detectable iron in the peri-portal hepatocytes and macrophages in the portal tract. Three of the 45 patients without H63D mutation had chronic persistent hepatitis, 37 chronic active hepatitis, and 5 cirrhosis. Twenty-nine of the 40 H63D-free patients whose livers were available for histological study had iron deposits as determined histochemically. Our study population included no patients with heavy iron deposits compatible with hemochromatosis. Blood parameters of 49 patients who completed iron reduction therapy are summarized in Table

Table 1. Clinical and laboratory features of chronic hepatitis C patients with and without H63D mutation

		Sex	Age[*1]	Stage	Iron Stain
H63D	homozygote	male	62	CIH	positive
	heterozygote 1	male	38	CAH	positive
	heterozygote 2	female	41	CAH	positive
	heterozygote 3	male	33	LC	no exam
		female/male		CIH/CAH/LC	positive/negative
H63D carriers (n=4)		1/3	44±13	1/2/1	3/0
H63D free patients (n=45)		19/26	55±9	3/37/5	29/11

[*1]; Age at the end point.

CIH;chronic inactive hepatitis, CAH;chronic active hepatitis, LC;liver cirrhosis.

Table 2. Blood parameters of chronic hepatitis C patients with and without H63D mutation

		ALT [U/L]		Ferritin [ng/mL]		Hb [g/dL]	
		Pre-T[*1]	Post-T[*1]	Pre-T[*1]	Post-T[*1]	Pre-T[*1]	Post-T[*1]
H63D	homozygote	31	14	190	10	14.5	11.8
	heterozygote 1	69	26	167	8	15.0	14.0
	heterozygote 2	192	96	481	10	13.7	8.6
	heterozygote 3	142	78	366	8	15.2	12.6
H63D	carriers (n=4)	109±72	54±40	301±149 (167~481)[*2]	9±1	14.6±0.7	11.8±2.3
H63D	free patients (n=45)	101±56	69±34	174±134 (28~724)[*2]	8±2	14.1±1.4	11.6±1.3

[*1]; treatment , [*2]; ranges.

ALT; alanine aminotransferase, Hb; hemoglobin.
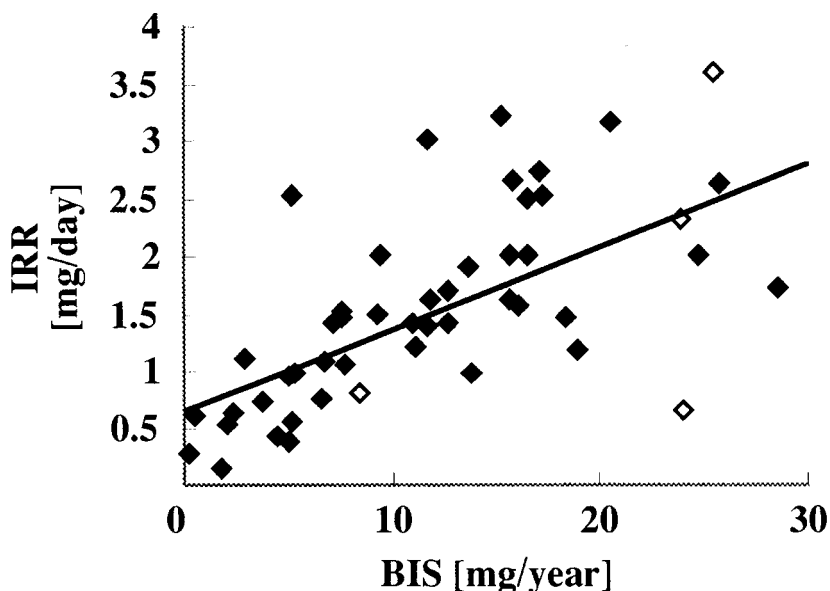
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Fig. 1. Correlation between iron restoration rate (IRR) and body iron stores (BIS).
Correlation coefficient was increased to 0.65 when BIS was divided by the age in years of patients, probably because BIS is age-dependent. Y=0.076 X + 0.64, r=0.65, p<0.01. IRR, Iron restoration rate; BIS, Body iron stores;   , H63D-free;   , H63D-positive.

2. The pre-treatment serum ferritin level of H63D-free patients was 174 ± 134 ng/mL (range; 28 – 724 ng/mL), and that of H63D-positive patients was 301 ± 149 ng/mL (range; 167 – 481 ng/mL). There was no significant difference in ferritin level between these two patient groups. Initial iron reduction therapy reduced serum alanine aminotransferase levels from 102 ± 56 U/L to 67 ± 35 U/L. The level of this enzyme in H63D mutation-free patients was reduced from 101 ± 56 U/L to 69 ± 34 U/L, while that of mutation-positive patients was reduced from 109 ± 72 U/L to 54 ± 40 U/L. The levels of this enzyme remained in a similar range in both patient groups during at least two years of iron deficiency maintenance treatment.

BIS measured from the initial phlebotomy were 551 mg in the H63D homozygote and 889, 1043 and 1077 mg in the heterozygotes, which did not differ from the control patients without the mutation (605 ± 360 mg; Fig. 1). IRR measured from total amounts of hemoglobin removed by phlebotomy or regular blood tests during iron deficiency maintenance treatment ranged between 0.13 and 3.60 mg/day. There was no difference in IRR between the two patient groups with and without H63D mutation (1.8 ± 1.4 vs. 1.5 ± 0.8 mg/day). The correlation coefficient between BIS and IRR improved from 0.61 to 0.65 when BIS was divided by the age of patients in years and expressed as mg/year. Pre-treatment levels of serum ferritin were correlated with BIS (Fig. 2), but those of aminotransferase activity were not.

DISCUSSION

All the patients studied here were natives of Honshu, the main island of Japan. In the original report of HFE, C282Y mutation was found in 85% of Caucasian subjects with hereditary hemochromatosis[3], and a close relationship between primary hemochromatosis and C282Y mu-
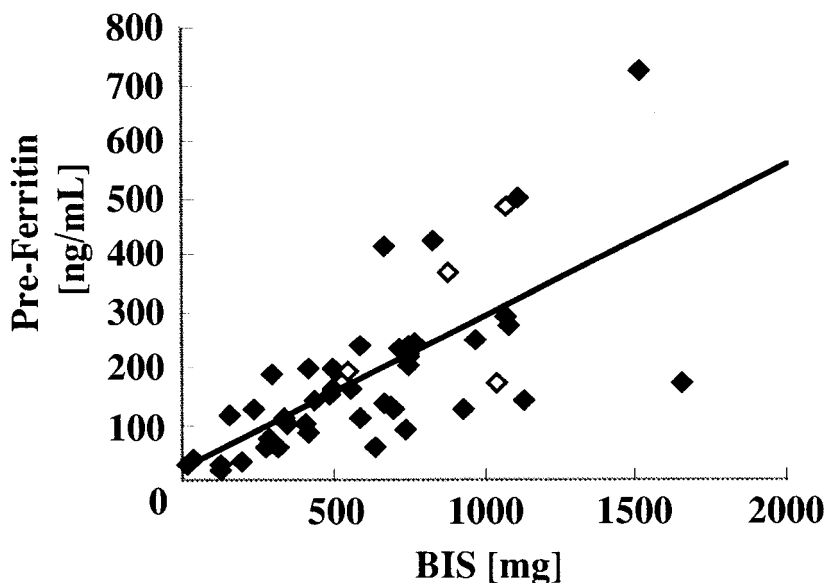
Fig. 2. Correlation between serum ferritin before treatment and body iron stores.
A good correlation was observed between serum ferritin and BIS. Y=0.26 X + 19.5, r=0.68, p<0.01. IRR, Iron restoration rate; BIS, Body iron stores;   , H63D-free;   , H63D-positive.

tation was subsequently confirmed[11]. As iron hepatotoxicity is involved in CHC disease activity, CHC patients might be a biased population with regard to iron metabolism and HFE mutation. However, none of our patients had C282Y mutation. Only 5 chromosomes with H63D mutation were found among the total of 188 chromosomes examined (2.6%). This prevalence of H63D mutation in CHC patients was similar to that of healthy residents in the same area (data not shown). Another preliminary study on HFE in the Japanese population indicated no C282Y mutation and a prevalence of H63D mutation of 1.3% in 200 healthy individuals[8]. A population study among Asians showed incidences of zero to 0.01% for C282Y mutation and 0.9 to 1.2% for H63D mutation[12]. Thus, the H63D mutation prevalence in our patients with CHC was almost twice that reported in other population studies, but this difference was not significant.

Iron absorption is regulated in the gut mucosa. Disruption of this regulation by HFE mutation leads to hemochromatosis, and defects in the duodenal divalent metal-transporter (DMT1) may lead to iron deficiency anemia[13, 14]. A recent study suggested an interaction between these proteins in that DMT1 expression is increased in patients homozygous for C282Y[15]. In this study, BIS was 0.61 ± 0.36 g including H63D mutant carriers. Only two of 49 patients, both free from H63D mutation, had excess iron storage over the reference value (0.3 – 1.2 g) measured in healthy subjects[16]. Their BIS values were 1.52 and 1.66 g, respectively, which is far less than the value of 4.0 g or more for asymptomatic hemochromatosis[17]. IRR ranged between 0.13 and 3.60 mg/day. These figures were less than the range (3 – 4 mg/day) reported as the maximum iron absorption in patients with iron deficient anemia[18]. Again, H63D mutation was not a determinant of IRR in our patients.

There is general agreement that chronic hepatitis is highly associated with hepatic iron deposits[19, 20, 21]. In fact, more than half of our patients had histochemically stainable iron in the liver. A possible explanation for the cases demonstrating hepatic iron accumulation with normal body iron stores may be that such hepatic iron deposits are from the tissues of the liver or

other than the liver and not from the dietary iron absorbed. An interesting observation supports this possibility of intra or inter-tissue iron distribution changes in CHC[22]. Pre-treatment liver iron concentration was normal or slightly elevated in patients with CHC. Interferon decreased their hepatic iron levels regardless of viral clearance. This iron decrease may be interpreted as a consequence of the anti-inflammatory effects of interferon. Combined with this information, our finding that total body iron was not increased suggested that iron is concentrated in the liver due to active inflammation by HCV infection without an expanded iron pool. The HFE gene, especially H63D mutation, might not play any role in hepatic iron storage as reported in Caucasian populations[23].

The transition element iron has a high potential to induce free radicals, and radical generation by iron has been reported to be one of the factors involved in the pathogenicity of CHC. Patients with CHC show increased hepatic levels of malondialdehyde and reduced levels of glutathione[24]. A subsequent study showed that reactive oxygen species were associated with the disease activity of CHC[25]. Small amounts of iron storage may be sufficient for such iron-induced hepatotoxicity. Thus, it is important to keep CHC patients in iron reduction treatment iron-free. As reported previously[26], the therapeutic effects of phlebotomy were not related to the iron indices but to the disease activity of patients expressed by serum aminotransferase levels. In addition to the stored iron in the liver, absorbed iron, as represented quantitatively by IRR, may generate hepatotoxic free radicals when transported to the liver. Therefore, iron restriction therapy using a low iron diet[27] may be recommended for CHC patients with a high IRR score.

As mentioned above[19, 20, 21], hepatic iron also accumulates in chronic hepatitis other than CHC. Considering that the mechanism involved in iron reduction therapy is non-specific, a similar effect of this treatment on chronic hepatitis B is highly expected, but no trials have been reported as yet, probably because a prognosis of chronic hepatitis B is not as serious as CHC.

BIS and IRR first measured in Japanese patients with CHC were mostly within the normal range regardless of HFE mutation. Chronic inflammation in the liver may promote hepatic iron accumulation without an expanded body iron pool, resulting in additional tissue damage in CHC.

REFERENCES

1) Hayashi, H., Takikawa, T., Nishimura, N., Yano, M., Isomura, T., and Sakamoto, N.: Improvement of serum aminotransferase levels after phlebotomy in patients with chronic active hepatitis C and excess hepatic iron. *Am. J. Gastroenterol.*, 89: 986–8 (1994).

2) Bonkovsky, H.L., Banner, B.F., and Rothman, A.L.: Iron and chronic viral hepatitis. *Hepatology*, 25: 759–68 (1997).

3) Feder, J.N., Gnirke, A., Thomas, W., Tsuchihashi, Z., Ruddy, D.A., Basava, A., Dormishian, F., Domingo, R.Jr., Ellis, M.C., Fullan, A., Hinton, L.M., Jones, N.L., Kimmel, B.E., Kronmal, G.S., Lauer, P., Lee, V.K., Loeb, D.B., Mapa, F.A., McClelland, E., Meyer, N.C., Mintier, G.A., Moeller, N., Moore, T., Morikang, E., Prass, C.E., Quintana, L., Starnes, S.M., Schatzman, R.C., Brunke, K.J., Drayna, D.T., Risch, N.J., Bacon, B.R., and Wolff, R.K.: A novel MHC class 1-like gene is mutated in patients with hereditary haemochromatosis. *Nat. Genet.*, 13: 399–408 (1996).

4) Piperno, A., Vergani, A., Malosio, I., Parma, L., Fossati, L., Ricci, A., Bovo, G., Boari, G., and Mancia, G.: Hepatic iron overload in patients with chronic viral hepatitis: role of HFE gene mutations. *Hepatology*, 28: 1105–9 (1998).

5) Kazemi-Shirazi, L., Datz, C., Maier-Dobersberger, T., Kaserer, K., Hackl, F., Polli, C., Steindl, P.E., Penner, E., and Ferenci, P.: The relation of iron status and hemochromatosis gene mutations in patients with chronic hepatitis C. *Gastroenterology*, 116: 127–34 (1999).

6) Hattori, K., Hayashi, H., Takikawa, T., Wakusawa, S., Ikeda, R., Shiono, Y., Yano, M., and Yoshioka, K.: No

HLA-H mutation (845G to A) in Japanese patients with hemochromatosis. *Hepatology*, 26: 522A (1997).

7) Ikeda, R., Hayashi, H., Wakusawa, S., Sanae, S., Takikawa, T., Shiono, Y., Imaizumi, Y., Okada, T., Takeda, Y., Mabuchi, H., Yano, M., and Yoshioka, K.: No involvement of HFE mutation in Japanese patients with liver disease. *Hepatology*, 28: 618A (1998).

8) Sohda, T., Yanai, J., Soejima, H., and Tamura, K.: Frequencies in the Japanese population of HFE gene mutations. *Biochem. Genet.*, 37: 63–8 (1999).

9) Jazwinska, E.C., Cullen, L.M., Busfield, F., Pyper, W.R., Webb, S.I., Powell, L.W., Morris, C.P., and Walsh, T.P.: Haemochromatosis and HLA-H. *Nat. Genet.*, 14: 249–51 (1996).

10) Roberts, A.G., Whatley, S.D., Morgan, R.R., Worwood, M., and Elder, G.H.: Increased frequency of the haemochromatosis Cys282Tyr mutation in sporadic porphyria cutanea tarda. *Lancet*, 349: 321–3 (1997).

11) Beutler, E., Gelbart, T., West, C., Lee, P., Adams, M., Blackstone, R., Pockros, P., Kosty, M., Venditti, C.P., Phatak, P.D., Seese, N.K., Chorney, K.A., Ten Elshof, A.E., Gerhard, G.S., and Chorney, M.: Mutation analysis in hereditary hemochromatosis. *Blood Cell Mol. Dis.*, 22: 187–94 (1996).

12) Merryweather-Clarke, A.T., Pointon, J.J., Shearman, J.D., and Robson, K.J.: Global prevalence of putative haemochromatosis mutations. *J. Med. Genet.*, 34: 275–8 (1997).

13) Fleming, M.D., Trenor, C.C. 3rd, Su, M.A., Foernzler, D., Beier, D.R., Dietrich, W.F., and Andrews, N.C.: Microcytic anaemia mice have a mutation in Nramp2, a candidate iron transporter gene. *Nat. Genet.*, 16: 383–6 (1997).

14) Gunshin, H., Mackenzie, B., Berger, U.V., Gunshin, Y., Romero, M.F., Boron, W.F., Nussberger, S., Gollan, J.L., and Hediger, M.A.: Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature*, 388: 482–8 (1997).

15) Zoller, H., Pietrangelo, A., Vogel, W., and Weiss, G.: Duodenal metal-transporter (DMT1, NRAMP-2) expression in patients with hereditary haemochromatosis. *Lancet*, 353: 2120–3 (1999).

16) Haskins, D., Stevens, A.R.Jr., Finch, S.C., and Finch, C.A.: Iron metabolism. Iron stores in man measured by phlebotomy. *J. Clin. Invest.*, 31: 543–7 (1952).

17) Sham, R.L., Raubertas, R.F., Braggins, C., Cappuccio, J., Gallagher, M., and Phatak, P.D.: Asymptomatic hemochromatosis subjects: genotypic and phenotypic profiles. *Blood*, 96: 3707–11 (2000).

18) Cook, J.D., Dassenko, S., and Skike, B.S.: Serum transferrin receptor as an index of iron absorption. *Br. J. Haematol.*, 75: 603–9 (1990).

19) Di Bisceglie, A.M., Axiotis, C.A., Hoofnagle, J.H., and Bacon, B.R.: Measurements of iron status in patients with chronic hepatitis. *Gastroenterology*, 102: 2108–13 (1992).

20) Isomura, T., Yano, M. Hayashi, H., and Sakamoto, N.: Excess iron in the liver of patients with chronic hepatitis C. *J. Clin. Electron Microsc.*, 25: 231–7 (1992).

21) Ishida, M., Nakagawara, G., Imamura, Y., and Fukuda, M.: Iron and copper deposition in chronic active hepatitis and liver cirrhosis; pathogenetic role in progressive live cell damage. *Eur. J. Histochem.*, 39: 221–36 (1995).

22) Boucher, E., Bourienne, A., Adams, P., Turlin, B., Brissot, P., and Deugnier, Y.: Liver iron concentration and distribution in chronic hepatitis C before and after interferon treatment. *Gut*, 41: 115–20 (1997).

23) Hezode, C., Cazeneuve, C., Coue, O., Roudot-Thoraval, F., Lonjon, I., Bastie, A., Duvoux, C., Pawlotsky, J.M., Zafrani, E.S., Amselem, S., and Dhumeaux, D.: Liver iron accumulation in patients with chronic active hepatitis C: prevalence and role of hemochromatosis gene mutations and relationship with hepatic histological lesions. *J. Hepatol.*, 31: 979–84 (1999).

24) Farinati, F., Cardin, R., De Maria, N., Della Libera, G., Marafin, C., Lecis, E., Burra, P., Floreani, A., Cecchetto, A., and Naccarato, R.: Iron storage, lipid peroxidation and glutathione turnover in chronic anti-HCV positive hepatitis. *J. Hepatol.*, 22: 449–56 (1995).

25) De Maria, N., Colantoni, A., Fagiuoli, S., Liu, G.J., Rogers, B.K., Farinati, F., Van Thiel, D.H., and Floyd, R.A.: Association between reactive oxygen species and disease activity in chronic hepatitis C. *Free Radic. Biol. Med.*, 21: 291–5 (1996).

26) Hayashi, H., Takikawa, T., Nishimura, N., and Yano, M.: Serum aminotransferase levels as an indicator of the effectiveness of venesection for chronic active hepatitis C. *J. Hepatol.*, 22: 268–71 (1995).

27) Matsumoto, M., Imamura, S., Sato, H., Takamasu, M., Okanoue, T., and Kashima, K.: Dietary iron reduction on patients with chronic hepatitis C [in Japanese with English abstract]. *Acta. Hepatol. Jpn.*, 40: 436–44 (1999).