Table 4 Therapies for patients with fulminant hepatitis (FH) and late-onset hepatic failure (LOHF)

	FH			LOHF ($n = 28$)
	Total ($n = 460$)	Acute type ($n = 227$)	Subacute type ($n = 233$)	
Plasma exchange	90.9 (418/460)	92.5 (210/227)	89.3 (208/233)	71.4 (20/28)**,***
Hemodiafiltration	75.0 (342/456)	75.1 (169/225)	74.9 (173/231)	57.1 (16/28)
Glucocorticosteroids	72.4 (333/460)	68.3 (155/227)	76.4 (178/233)	89.3 (25/28)*
Glucagon/insulin	14.6 (67/459)	13.7 (31/227)	14.7 (34/232)	17.9 (5/28)
BCAA-rich solution	19.1 (87/456)	14.3 (32/223)	23.6 (55/233)*	39.3 (11/28)**
Prostaglandin E_1	7.0 (32/458)	6.7 (15/225)	7.3 (17/233)	3.6 (1/28)
Cyclosporin A	10.0 (46/460)	7.0 (16/227)	12.9 (30/233)*	10.7 (3/28)
Interferon	14.1 (65/460)	15.4 (35/227)	12.9 (30/233)	10.7 (3/28)
Nucleoside analog	38.9 (179/460)	50.9 (115/226)	27.5 (64/233)**	32.1 (9/28)
Lamivudine	25.5 (116/455)	40.0 (76/224)	30.4 (40/231)	12.5 (6/28)
Entecavir†	22.4 (70/312)	27.7 (41/148)	17.7 (29/164)	33.3 (5/15)
Anticoagulation therapy‡	47.2 (216/458)	43.2 (98/227)	51.1 (118/231)	39.3 (11/28)
Liver transplantation	23.5 (108/460)	15.9 (36/227)	30.9 (72/233)	17.9 (5/28)

*$P < 0.05$, **$P < 0.01$ vs acute type, ***$P < 0.05$ vs subacute type.
†Cases between 2006 and 2009.
‡Drugs such as antithrombin III concentrate and protease inhibitor compounds, gabexate mesylate and nafamostat mesilate.
Data in parentheses indicate patient numbers.
BCAA, branched-chain amino acid.

DISCUSSION

IN THIS SURVEY, 488 patients were enrolled over 6 years. In the previous 6-year survey, 697 patients (634 for FH and 64 for LOHF) were enrolled.[7] The incidence ratio of LOHF to FH was decreased from 9:1 to 16:1. In national epidemiology research, the annual incidence of FH was estimated at 1050 cases in 1996 and 429 cases in 2004.[11] Therefore, the incidence of FH and LOHF could be decreasing longitudinally. In this survey, the mean age of patients with FH and LOHF was older than that in the previous survey. More patients with complications received daily medication compared with the previous survey. Changes in demographic features of the patients may affect the etiology and prognosis of FH. A relationship between daily dose of oral medication and idiosyncratic drug-induced liver injury has been reported.[12] Additionally, older age is considered a poor prognostic factor in acute liver failure and may be considered a relative contraindication for LT.[13,14]

The current study showed that HBV still remains a major cause of FH and LOHF. Notably, almost half of acute exacerbations in HBV carriers occurred in patients with HBV reactivation owing to immunosuppressive or cytotoxic therapy. Approximately 80% of patients with transiently infected patients had received rituximab plus steroid combination therapy for non-Hodgkin's lymphoma. This combination therapy has been identified as a risk factor for HBV reactivation in HBsAg positive/negative patients with non-Hodgkin's lymphoma.[15,16] Our survey revealed that careful attention is necessary for transiently infected patients, as well as for inactive HBV carriers using intensive immunosuppressive agents.

The frequency of HAV infection in patients with FH was decreased compared with the previous survey. This result is compatible with no occurrence of outbreak of acute hepatitis A during this period. In Japan, zoonotic transmission from pigs, wild boar and deer, either foodborne or otherwise, is the cause of HEV infection.[17,18] In the currently studied survey, two-thirds of the patients were from endemic areas (Hokkaido Island and the northern part of mainland Honshu) in Japan.

The other principal finding in this survey was that the etiology was indeterminate in approximately 40% of patients with FH. One of the reasons for this result may be the failure of diagnosis for autoimmune hepatitis or drug-induced liver injury. Although the diagnosis of autoimmune hepatitis relies on the presence of serum autoantibodies, with higher IgG levels (>2 g/dL), acute-onset autoimmune hepatitis does not always show typical clinical features.[19–21] Additionally, the sensitivity of the drug-induced lymphocyte stimulation test for diagnosis is not completely reliable.

Table 5 Survival rates and etiology of patients with fulminant hepatitis (FH) and late-onset hepatic failure (LOHF) who did not have liver transplantation

	FH			LOHF ($n = 23$)
	Total ($n = 352$)	Acute type ($n = 191$)	Subacute type ($n = 161$)	
Viral infection	39.8 (70/176)	49.2 (58/118)	20.7 (12/58)**	14.3 (1/7)
HAV	57.1 (8/14)	61.5 (8/13)	0 (0/1)	–
HBV	36.2 (55/152)	46.1 (47/102)	16.0 (8/50)**	14.3 (1/7)
(1) Transient infection	52.6 (40/76)	54.4 (37/68)	37.5 (3/8)	–
(2) Acute exacerbation in HBV carrier	15.1 (8/53)	21.4 (3/14)	12.8 (5/39)	14.3 (1/7)
(i) Inactive carrier, without drug exposure	29.2 (7/24)	27.3 (3/11)	30.8 (4/13)	0 (0/1)
(ii) Reactivation in inactive carrier†	7.7 (1/13)	0 (0/3)	10.0 (1/10)	20.0 (1/5)
(iii) Reactivation in transiently infected patients‡	0 (0/16)	–	0 (0/16)	0 (0/1)
(3) Indeterminate infection patterns	30.4 (7/23)	35.0 (7/20)	0 (0/3)	–
HCV	50.0 (2/4)	100 (1/1)	33.3 (1/3)	–
HEV	75.0 (3/4)	100 (2/2)	50 (1/2)	–
Other viruses	100 (2/2)	–	100 (2/2)	–
Autoimmune hepatitis	32.4 (9/28)	40.0 (2/5)	30.4 (7/23)	12.5 (1/8)
Drug allergy-induced	32.8 (19/58)	43.3 (13/30)	21.4 (6/28)	0 (0/3)
Indeterminate§	37.6 (32/85)	54.5 (18/33)	26.9 (14/52)*	20.0 (1/5)
Unclassified¶	1.5 (7)	40.0 (2/5)	–	–

**$P < 0.01$ vs acute type.
†Reactivation in inactive carrier by immunosuppressant and/or anticancer drugs.
‡Reactivation in transiently infected patients by immunosuppressant and/or anticancer drugs (de novo hepatitis).
§Indeterminate etiology despite sufficient examinations.
¶Unclassified due to insufficient examinations.
Data in parentheses indicate patient numbers.
HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HEV, hepatitis E virus.

Recently, powerful HDF using large buffer volumes (HF-HDF or HF-CHDF), or on-line HDF has been used. HF-HDF or HF-CHDF has a high recovery rate from a coma.[22–24] On-line HDF has an excellent recovery rate from a coma and is useful as a liver support system.[25] However, only 16% of patients with FH received these powerful HDF in the survey examined in the current study. The frequency of brain edema, gastrointestinal bleeding and congestive heart failure was decreased compared with that in the previous survey. Advances in artificial liver support and management may contribute to prevent these complications. Further evaluation is required to determine whether a new powerful support system can improve the prognosis of FH. The survival rate for FH patients with autoimmune hepatitis improved 17.1% in the previous survey to 32.4% in the 2004–2009 survey. Early commencement of corticosteroids may improve the prognosis. However, the efficacy of these drugs has not been evaluated statistically.

Recently, in patients with acute liver failure due to HBV, entecavir has been used more frequently than lamivudine because of its high potency and extremely low rates of drug resistance.[26] Entecavir beneficially affects the course of acute liver failure as lamivudine.[27,28] Despite the use of entecavir, the prognosis of HBV-infected patients, especially in HBV carriers, has not improved. In the case of HBV reactivation, it is difficult to prevent development of liver failure, even when nucleoside analogs are administrated after the onset of hepatitis. Because these agents require a certain amount of time to decrease HBV DNA in serum, they need to be administrated in the early phase of hepatitis. Guidelines for preventing HBV reactivation recommend the administration of nucleoside analogs before the start of immunosuppressive therapy in inactive carriers and at an early stage of HBV reactivation during or after immunosuppressive therapy in transiently infected patients.[29]

Despite new therapeutic approaches and intensive care, the prognosis of patients without LT with both types of FH and LOHF appeared similar to that in the previous survey. In contrast, the prognosis of patients receiving LT was good in the present survey. Yamashiki

et al. reported that the short-term and long-term outcomes of living-donor LT for acute liver failure were good, irrespective of the etiology and disease types.[30] In the current survey, the implementation rate of receiving LT was almost equivalent to that in the previous survey, irrespective of disease type. Notably, only two patients received deceased-donor LT in the current survey. Recently, patients with FH who received deceased-donor LT have been increasing since the new organ transplant bill passed in 2009. Hepatologists should realize that more donor action to increase deceased-donor LT is necessary to improve the prognosis of patients with FH or LOHF. Determining appropriate judgment to move forward to LT is the most important step. The indications for LT in cases of FH are determined according to the 1996 Guidelines of the Acute Liver Failure Study Group of Japan.[31] To improve the low sensitivity and specificity of assessment in patients with acute and subacute types,[32] new guidelines for using a scoring system have been established by the Intractable Hepato-Biliary Disease Study Group of Japan.[33] This novel scoring system showed sensitivity and specificity of 0.80 and 0.76, respectively, and greater than those in the previous guideline.[33] Recently, new prediction methods using data-mining analysis has been established.[34,35]

In conclusion, the demographic features and etiology of FH and LOHF have been gradually changing. HBV reactivation due to immunosuppressive therapy is a particular problem because of poor prognosis. The subacute types of FH and LOHF have a poor prognosis, irrespective of the etiology. Despite recent advances in therapeutic approaches, the implementation rate for LT and survival rates of patients without LT are similar to those in the previous survey.

ACKNOWLEDGMENT

THIS STUDY WAS performed with the support of the Ministry of Health, Labor and Welfare as an official project by the Intractable Hepato-Biliary Diseases Study Group of Japan.

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INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Clinical Features and Hepatitis B Virus (HBV) Genotypes in Pregnant Women Chronically Infected with HBV

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Abstract

Objective The purpose of this study was to clarify the clinical features and hepatitis B virus (HBV) genotypes in pregnant women chronically infected with HBV.

Methods Among 1,489 pregnant women who visited our hospital in 2010, 26 were positive for hepatitis B surface antigens (HBsAg). Of these subjects, 21 from whom informed consent was obtained were included in this study. The clinical features and HBV markers, including genotypes, were investigated.

Results No adverse events were observed in the subjects or the neonates during pregnancy or the perinatal period. The HBV genotypes were C in 14 cases, D in six cases, and undetermined in one case. Hepatitis B e antigens and a high viral load (>7.0 log copies/mL) were found in four and six subjects with genotype C, respectively, and in none of subjects with genotype D. The alanine aminotransferase (ALT) levels and platelet counts were within the normal ranges during pregnancy in all subjects except two and three subjects with genotype C, respectively. Three subjects with genotype C showed transient elevations of ALT after delivery.

Conclusion The majority of subjects were anti-HBe-positive with normal ALT levels; however, some subjects with genotype C showed a high viral load, elevated ALT levels and/or low platelet counts. The pregnancies and deliveries were safe; however, transient elevations of ALT after delivery were observed in some subjects with genotype C.

Key words: hepatitis B virus, genotype, pregnancy

(Intern Med 51: 3317-3322, 2012)
(DOI: 10.2169/internalmedicine.51.8596)

Introduction

Hepatitis B virus (HBV) is a DNA virus with approximately 3,200 base pairs. HBV induces a variety of liver diseases, ranging from acute or fulminant hepatitis to liver cirrhosis and hepatocellular carcinoma. Approximately 350 million people are chronically infected and two billion people are transiently infected worldwide. HBV can be classified into at least eight genotypes, with a divergence of more than 8% in the nucleotide sequences (1-3). It has been reported that there are differences in the clinical features and routes of transmission between genotypes (4, 5).

In Japan, genotypes B and C are the predominant genotypes; however, the distribution of genotypes is changing (6-8). It has been reported that the main transmission route is vertical in genotypes B and C and horizontal in genotypes A and D (4, 9). Furthermore, the viral states of mothers are closely related to transmission to neonates and children (10-12). There is a paucity of information regarding

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Received for publication July 13, 2012; Accepted for publication August 26, 2012

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HBV markers including HBV genotypes and infectious routes in pregnant women chronically infected with HBV. Moreover, little is known about the differences in clinical features of pregnant women infected with HBV in relation to HBV genotypes. In the present study, we attempted to clarify these issues among patients in the north-western area of Shikoku Island in Japan.

Materials and Methods

Subjects

In 2010, 1,489 pregnant women visited our hospital located in the north-western area of Shikoku Island in Japan. Hepatitis B surface antigens (HBsAg) were assayed in all of the subjects. The aim and protocol of the study were explained to the HBsAg-positive pregnant women, and those from whom written informed consent was obtained were included in the study. This study was conducted prospectively.

Methods

HBsAg were screened using a chemoluminescence immunoassay (CLIA). Hepatitis B e antigens (HBeAg) and anti-HBe were assayed with CLIA, and the HBV-DNA levels were assayed using a real-time polymerase chain reaction (PCR) method. The HBV genotypes were determined using a serial invasive signal amplification reaction assay (Invader® assay; BML Inc, Saitama, Japan) (13). When genotype could not be identified with this method, it was also assayed using an enzyme immunoassay (EIA) method (Immunis®, HBV genotype EIA; Institute of Immunology Co., Ltd, Tokyo, Japan) (14). The platelet counts and the levels of alanine aminotransferase (ALT), AFP, HBeAg, anti-HBe and HBV-DNA were assayed during pregnancy, including in the third trimester and one to two months after delivery. Ultrasonography was performed to determine the findings of the liver, especially to screen for findings suspicious of liver cirrhosis or hepatocellular carcinoma.

The clinical diagnoses were determined according to liver function tests, hemograms and ultrasonography findings. In the present study, HBeAg-positive asymptomatic HBV carrier (immune tolerant phase) and inactive HBsAg carrier state were defined as an ALT level within the normal range during pregnancy, a platelet counts over 150,000/μL, no ultrasonographic findings suspicious of liver cirrhosis or hepatocellular carcinoma and HBeAg positive and negative status, respectively. Chronic hepatitis was defined as an elevated ALT level and a platelet count within the normal range (>150,000/μL) with no ultrasonographic findings suspicious of liver cirrhosis. ALT <40 IU/L was defined as the normal range according to the normal range used in our institute. It is known that the level of platelet is related to fibrosis of the liver, and patients with a platelet count < 150,000/μL may have fibrosis of the liver. Therefore, regarding the diagnosis of subjects with normal ALT levels and low platelet counts, the diagnoses of these cases were classified as "undetermined".

Information regarding infectious routes was obtained with medical interviews. When the mothers of the subjects had no history of hepatitis B infection, the infectious route was classified as horizontal; when the mothers had a history of chronic HBV infection, the infectious route was classified as vertical.

Statistical analysis

The statistical analyses were performed using the chi-square test, the Wilcoxon signed-rank test and paired *t*-test. $p<0.05$ was considered significant.

Results

Twenty-six of the 1,489 (1.7%) pregnant women were proven to be positive for HBsAg. The details of the study were explained to the 26 women, and written informed consent for participation in the study was obtained from 21 of them. These 21 women were involved in the study. No subjects showed ultrasonographic findings suspicious of liver cirrhosis or hepatocellular carcinoma. The clinical data of the subjects are shown in Table. The route of infection was vertical in six cases (28.6%), horizontal in nine cases (42.9%), and undetermined in six cases (28.6%). HBeAg status was positive in four of the 21 subjects, whereas 16 subjects were positive for anti-HBe and one subject was negative for both HBeAg and anti-HBe. The HBV-DNA level was >7 log copies (LC)/mL in six subjects, 3-5 LC/mL in five subjects, and lower than 3 LC/mL in 10 subjects. The ALT level was within the normal range in 19 subjects and elevated in two subjects during pregnancy. In the present study, the normal range of ALT was defined as <40 IU/L. No subjects showed an ALT level with a range between 30 and 40 IU/L during pregnancy; therefore, the data described above would not have changed if the normal range of ALT was defined as ≤30 IU/L, as has been reported elsewhere (15).

The platelet count was lower than the normal range in three cases (108,000-115,000/μL). These three women had normal ALT levels and were positive for anti-HBe. The clinical diagnosis was HBeAg-positive asymptomatic HBV carrier in four cases, chronic hepatitis in two cases, and inactive HBsAg carrier state in 12 cases. The HBV genotype was determined in 20 subjects: genotype C in 14 subjects and genotype D in six subjects. The genotype was undetermined in one subject due to low levels of HBV-DNA and HBsAg. The infectious route in the genotype C-infected subjects was vertical in six cases and horizontal in four cases, whereas no subjects with genotype D were infected vertically. HBeAg-positive asymptomatic HBV carrier and chronic hepatitis were found in genotype C-infected women only. All of the six genotype D-infected women were diagnosed with inactive HBsAg carrier state. The HBsAg levels ranged from 30.5 to 74,591 IU/mL in genotype C-infected women and 880 to 20,343 IU/mL in the genotype D-

Table. The Clinical Data of the Subjects according to the HBV Genotypes

		HBV genotype			Total
		C	D	Undetermined	
Number of subjects		14	6	1	21
Age (median)		25 - 40 (34)	32 - 37 (34)	36	25 - 40 (34)
Infectious route	Vertical	6	0	0	6
	Horizontal	4	4	1	9
	undetermined	4	2	0	6
Diagnosis*	HBeAg+ASC	4	0	0	4
	CH	2	0	0	2
	ICS	5	6	1	12
	undetermined**	3	0	0	3
HBeAg/ anti-HBe	+/-	4	0	0	4
	-/-	1	0	0	1
	-/+	9	6	1	16
HBV-DNA (log copies/mL)	0 – 3	6	3	1	10
	3 - 5	2	3	0	5
	>7	6	0	0	6
HBsAg (IU/mL)	Median (mim-max)	9,511 (30.5-74,591)	4,090 (880-20,343)	2,281	7,070 (30.5-74,591)
ALT (IU/L)	≥40	2	0	0	2
	<40	12	6	1	19
Platelet (/μL)	<150,000	3	0	0	3
	≥150,000	11	6	1	18
Delivery	vaginal delivery	13	5	1	19
	Cesarean section	1	1	0	2

*HBeAg+ASC: HBeAg-positive asymptomatic HBV carrier, CH: chronic hepatitis, ICS: inactive HBsAg carrier state

**Anti-HBe+, a normal level of ALT, a low levels of HBV-DNA (<4.0 LC/mL) with a low platelet count (<150,000/μL)

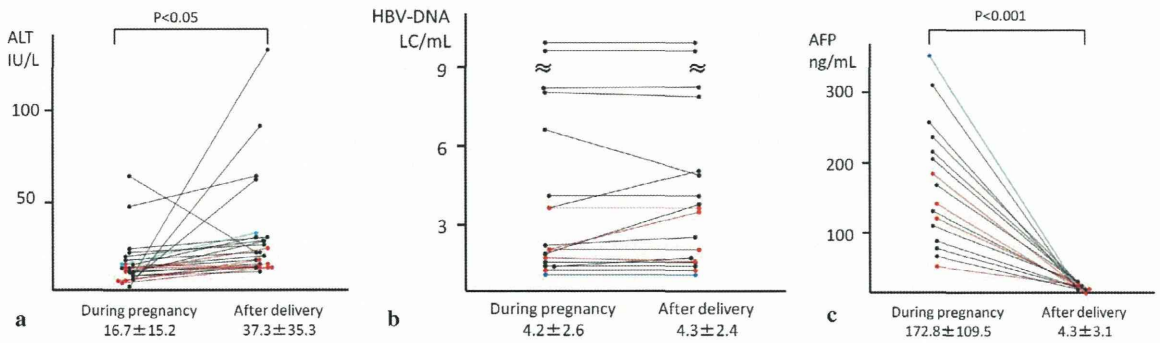

Figure 1. Changes in the levels of ALT, HBV-DNA and AFP before and after delivery. (a) ALT, (b) HBV-DNA, (c) AFP. Infected genotypes are shown by colors; black: genotype C, red: genotype D, blue: undetermined.

infected women. Although the median level was higher in genotype C-infected women (9,511 vs. 4,090 IU/mL), the difference was not significant. No subjects with genotype D infection showed elevated ALT levels or low platelet counts; however, two and three subjects with genotype C showed elevated ALT levels and low platelet counts, respectively. No adverse events were observed in the subjects or neonates during pregnancy or the perinatal period regardless of HBV genotype. Cesarean sections were performed in one of 15 women infected with genotype C and one of the six women infected with genotype D.

The changes in the levels of ALT, HBV-DNA and AFP before and after delivery are shown in Fig. 1. The ALT levels were significantly higher after delivery (16.7±15.2 IU/L vs. 37.3±35.3 IU/L, $p<0.05$, paired *t*-test). There were no significant differences in the HBV-DNA levels before and after delivery. The AFP levels were elevated in all subjects during pregnancy (172.8±109.5 ng/μL), and returned to normal levels after delivery (4.3±3.1 ng/μL) in all subjects regardless of genotype ($p<0.001$, paired *t*-test). Three subjects,

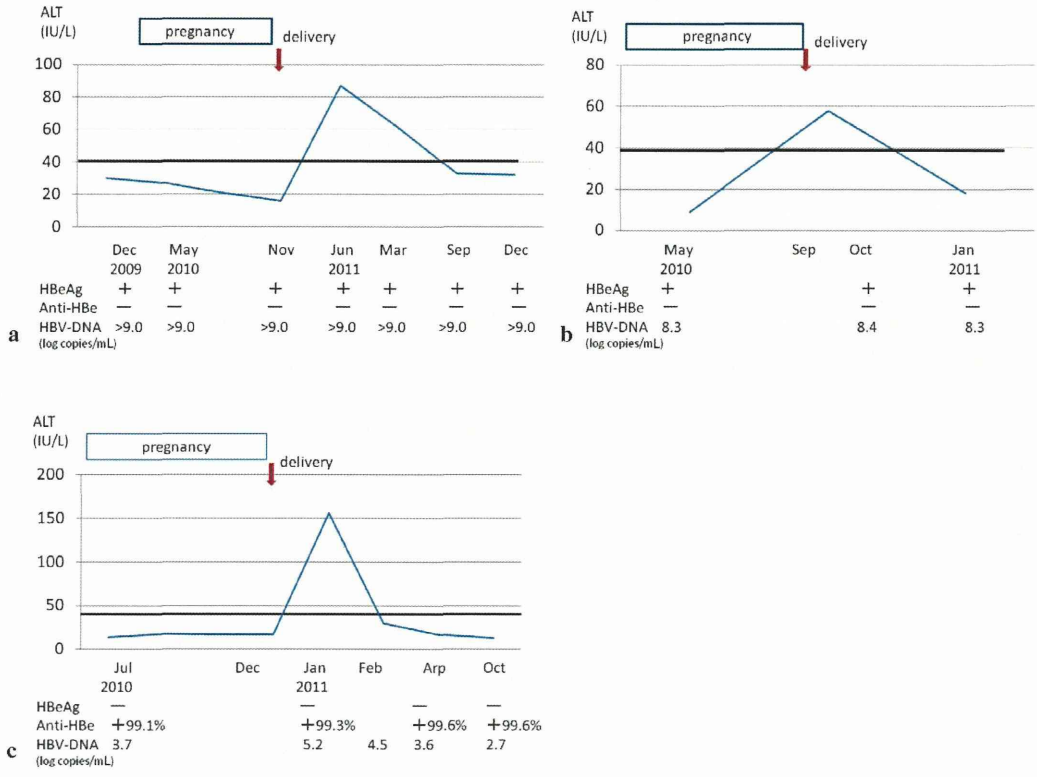

Figure 2. The clinical courses of the women infected with hepatitis B virus genotype C who showed transient elevations of ALT after delivery. (a) A 35-year-old woman, (b) A 25-year-old woman, (c) A 39-year-old woman. The bold line indicates the upper limit of the normal range of ALT.

with normal ALTs level before delivery, showed transient elevation of ALT after delivery (Fig. 2). Two of these three subjects were positive for HBeAg, and the remaining subject was positive for anti-HBe with a low platelet count. All three subjects were infected with HBV genotype C, with HBsAg levels ranging from 2,893 to 74,591 IU/mL (median: 20,977 IU/mL) and HBV-DNA levels ranging from 3.7 to >9.0 LC/mL (median:8.3 LC/mL). Although no significant differences in the levels of HBsAg and HBV-DNA were found between these three subjects and those with no elevations of ALT after delivery, two of the three subjects with ALT elevation after delivery showed high levels of HBsAg and HBV-DNA. The HBV-DNA levels were elevated from 3.7 LC/mL to 5.2 LC/mL in one woman and > 8.0 LC/mL in other 2 women both before and after delivery. The ALT levels returned to normal several months after delivery in all cases. No changes in the HBV-DNA level were observed in 18 of the 21 women, whereas the HBV-DNA levels were transiently elevated in three women after delivery (two women with genotype C and one woman with genotype D). One subject showed an elevation in the HBV-DNA level from 3.7 LC/mL to 5.2 LC/mL accompanied by an elevation in the ALT level, as shown in Fig. 2(c). The other two women showed elevations in the HBV-DNA level from <2.1 to 3.9 LC/mL and 2.4 to 3.6 LC/mL, respectively; however, no elevations in the ALT levels were observed. No changes in HBeAg or anti-HBe status were observed in any of the subjects.

Discussion

The prevalence of HBsAg-positive status in pregnant women was 1.7% in the present study. This is higher than the prevalence in the whole population of Japan, which has been estimated to be approximately 1%. The high rate observed in our study may be associated with the nature of our hospital to which many pregnant women at high risk or with complications are referred; therefore, the present data are probably not indicative of a generally high prevalence of HBV carrier status among pregnant women in this district.

In the majority of the subjects, the clinical diagnosis was inactive HBsAg carrier state, whereas two of the 21 subjects had chronic hepatitis. Three of the 19 women whose ALT levels were within the normal range during pregnancy showed transient elevations in ALT after delivery. Exacerbation of hepatitis B during pregnancy and the postpartum period, accompanied by clearance of HBeAg has been reported elsewhere (16-18). Although no changes in the HBeAg/anti-HBe were observed in the present study, the changes in the ALT levels observed in some of the subjects are consistent with previous reports that disease exacerbation may occur in some patients. Our findings confirm that it is necessary to monitor the ALT levels carefully before as well as after delivery, even if the ALT levels are within the normal range

during pregnancy.

HBV genotypes C and D were identified in the present subjects. In Japan, genotype C is most common followed by genotype B (6). However, in the district where our hospital is situated, the presence of genotype D has also been reported, with a prevalence of genotypes B, C, and D of approximately 5%, 88%, and 6%, respectively (19). The rate of genotype D-infected subjects in the present study was higher than that reported in previous studies, however, another report indicated that the proportion of genotype D in this district is high among HBV carriers born in the 1970s, in which decade the HBV genotype D was supposed to have spread in this area (20). Duong et al. investigated the clinical features of patients infected with genotypes C and D in this district, and reported a high virulence of genotype C compared with genotype D, with the rate of anti-HBe state being higher in patients with genotype D than in those with genotype C (21). In the present study, chronic hepatitis or HBeAg-positive cases were found only in genotype C-infected women, and all women who showed elevations in the ALT levels after delivery were also genotype C-infected subjects. These data are compatible with those of previous reports.

The route of transmission was vertical in six cases and horizontal in nine cases, which may indicate that the most common major route of transmission is horizontal in pregnant women in Japan. Vertical transmission has been reported to be the major route of transmission in East Asia; however, this trend may be changing in Japan. The prevention program against vertical HBV transmission introduced in 1986 in Japan (22) and improvements in hygiene may be associated with this change in transmission. Regarding the infectious routes in relation to the HBV genotype, the majority (or all) of the subjects with genotype D included in this study were presumed to be horizontally infected. Genotype C is known as a genotype for which the main transmission route is vertical. However, in the present study, four of 10 patients with genotype C for whom information regarding transmission route was obtained were presumed to be infected horizontally. Inui et al. reported that the rate of vertical transmission in children is 77% in Japan and that the rate was high in patients infected with genotype C compared with those infected with other genotypes. This report indicates that the major transmission route is vertical; however, there exist a considerable number of HBV carriers infected horizontally, especially those infected with genotypes other than genotype C (23). The infectious routes were determined according to medical interviews in the present study; therefore, the data regarding infectious routes in the present study may not be precise. However, the ratio of vertical transmission to horizontal transmission may be decreasing, especially in areas where genotypes other than genotype C are circulated. These data may indicate that existing prevention programs for vertical infection are not sufficient for preventing HBV transmission, and projects for preventing horizontal infection should be discussed as a national prophylactic strategy in areas or countries where universal vaccination has not been introduced.

In conclusion, HBV genotypes C and D are prevalent among pregnant women in this district. All of the subjects infected with genotype D exhibited the clinical features of the inactive HBsAg carrier state. On the other hand, all of the subjects with elevated ALT levels during pregnancy, transient elevations of ALT after delivery, high viral loads and/or low platelet counts were infected with genotype C.

The authors state that they have no Conflict of Interest (COI).

Acknowledgement

This study was supported by a Grand-in-Aid from the Ministry of Health, Labor, and Welfare of Japan.

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10.5005/jp-journals-10018-1050

CASE REPORT

A Case of *de novo* Hepatitis B Complicated due to Lack of Comprehensive Interventional Approach

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ABSTRACT

Here, we report a case of *de novo type B hepatitis* in a patient with hepatitis B surface antigen (HBsAg) negative but positive for low titer of anti-HBc antibody (anti-HBc titer; dilution 200; negative). As the disease was anticipated in advance, the patient received nucleos(t)ide analogs, but *de novo type B hepatitis* was developed, because of discontinuation of antiviral drugs.

A 59-year-old male with a history of T cell rich diffuse large B-cell lymphoma (DLBCL) and was treated with rituximab plus cyclophosphomide, doxorubicin, vincristine and prednisolone (R-CHOP). The patient responded to anticancer therapy and his complete responder status was confirmed by PET-CT on October 4, 2010. As the patient was expressing low levels of anti-HBc (anti-HBc titer; dilution 200-negative), he was given lamivudine to block HBV reactivation, but the drug was continued after 1 year due to apparent improvement. Stoppage of antiviral drug resulted in detectable HBV DNA and evidences of liver damages and he was referred to our department for specialized consultation about liver-related complications. He was given entecavir at a dose of 1 gm/day from May 2012. However, the parameters of liver function test showed anomaly indicating progressive liver damages. Subsequently, he was given steroid pulse therapy with 1,000 mg of prednisolone and tapered successively. The levels of HBV DNA decreased and parameters of liver function test were improved. A biopsy specimen taken in July 2012 showed the findings compatible with resolved acute hepatitis. To prevent *de novo type B hepatitis*, critical observation and timely management of the patients are necessary. The administration with nucleoside analogs at least 1 year after R-CHOP therapy is recommended in guideline of Japanese Society of Hepatology. However, we should reconsider the term of administration with nucleoside analogs after R-CHOP therapy.

Keywords: HBV, *de novo* hepatitis, Rituximab, HBV reactivation, Nucleoside analogs, Interdisciplinary approach.

How to cite this article: Onji H, Koizumi Y, Hanayama M, Akbar SMF, Hirooka M, Tokumoto Y, Abe M, Hiasa Y, Aoto M, Mitsuda N, Onji M. A Case of *de novo* Hepatitis B Complicated due to Lack of Comprehensive Interventional Approach. Euroasian J Hepato-Gastroenterol 2012;2(2):122-125.

Source of support: Nil

Conflict of interest: None

INTRODUCTION

It is estimated that 2 billion people worldwide have been infected with Hepatitis B Virus (HBV).[1] In Japan, it is reported that 23.2% of blood donors are positive for HBc antibody and/or HBs antibody.[2] Reactivation of HBV is a well-recognized complication in hepatitis B surface antigen (HBsAg) positive patients who are undergoing immunosuppressive chemotherapy including anti-CD20 antibody for malignancies. The clinical manifestation ranges from subclinical hepatitis to severe and potentially fatal fulminant hepatic failure.[3-6] In this decade, HBV reactivation has been observed in patients with resolved infection who have undergone intensive immunosuppressive chemotherapy, such as rituximab plus steroid-containing chemotherapy. This usually happens in patients expressing HBsAg and HBV DNA in peripheral blood. Here, we report a case of *de novo* type B hepatitis in a patient with HBsAg negative but positive for low titer of anti-HBc antibody (anti-HBc titer; dilution 200; negative). The patient developed *de novo* type B hepatitis even after the prophylactic administration of nucleos(t)ide analogs for more than 1 year and 4 months after stopping of R-CHOP therapy. The present case report would contribute about importance of comprehensive approach to prevent *de novo* hepatitis. Also, some insights would be provided about duration of antiviral therapy in these circumstances.

CASE REPORT

A 59-year-old male with a history of T cell rich diffuse large B-cell lymphoma (DLBCL) is presented. The patient revealed a history of general malaise, low-grade fever, skin itching and weight loss from February 2010. Lymph node enlargement was shown in the right cervix, and biopsy specimen of right cervical region showed T-cell rich DLBCL (stage III). The patient was administered an anticancer therapy with rituximab plus cyclophosphomide, doxorubicin, vincristine and prednisolone (R-CHOP) for eight courses from March 2010 to August 2010. The patient responded to anticancer therapy and his complete responder status was confirmed by PET-CT on October 4, 2010. As the patient was expressing low levels of anti-HBc (anti-HBc titer; dilution 200 negative), he was given lamivudine to block HBV reactivation from May 2010. Administration of lamivudine was stopped in October 26, 2011 after the prophylactic administration of lamivudine for more than 1 year and 4 months after stopping of R-CHOP therapy. Subsequently, HBV DNA became detectable in the sera from February 29, 2012. The amount of HBV DNA was increased to 8.4 log copies/ml on May 30, 2012. Just after

consulting with hepatologist, he was administered entecavir at a dose of 1 gm/day. However, the parameters of liver function test showed downhill terns and referred to us. Physical findings on admission: Height: 174 cm; weight: 58.3 kg, BMI: 19.3 he was not icteric; lung; no abnormality detected. The abdomen was flat, smooth, soft and non-tender. The liver and spleen was not palpable. There was no flapping tremor. Laboratory data was shown in Table 1. The patient showed general malaise and loss of appetite. Hepatomegaly and yellowing of the eyes were not shown. The parameters of liver function test in June 25, 2012, was AST, 322 U/l; ALT, 390 U/l; LDH, 373 U/l; ALP, 283 U/l, γ-GTP, 38 U/l, total bilirubin; 0.8 gm/dl; direct bilirubin, 0.1 mg/dl and prothrombin time, 76.8 %. The level of HBV DNA was 6.1 log copies/ml (HBV genotype B). The patient was expressing HBsAg (36,360 IU/ml) and HBeAg in the sera. HBsAg became for positive on June 30, 2012. His HBV was wild-type. Positivity of HBV-DNA was observed after 1 year and 7 months, HBs antigen became positive after 1 year and 10 months, abnormality of AST/ALT was after 1 year and 11 months after cease of R-CHOP therapy. Clinical course of present case was shown in Figure 1. In spite of antiviral therapy, progressive liver damages continued as evident from values of ALT and AST. In June 2012, steroid pulse therapy was started with 1,000 mg of prednisolone and tapered successively. The levels of HBV DNA, AST and ALT fell due to integrated therapy. The levels of HBV-DNA decreased and subjective symptoms disappeared, and the parameters of liver function test became normalized. A liver biopsy was done in July 30, 2012, the liver specimen showed in Figures 2A and B. Liver-cell damage, cell death and inflammatory cells infiltration were seen predominantly in central area, but there was no bridging necrosis. There was few abnormal finding in periportal area. These findings indicate resolved acute hepatitis. The patient discharged from hospital on 15th August 2012.

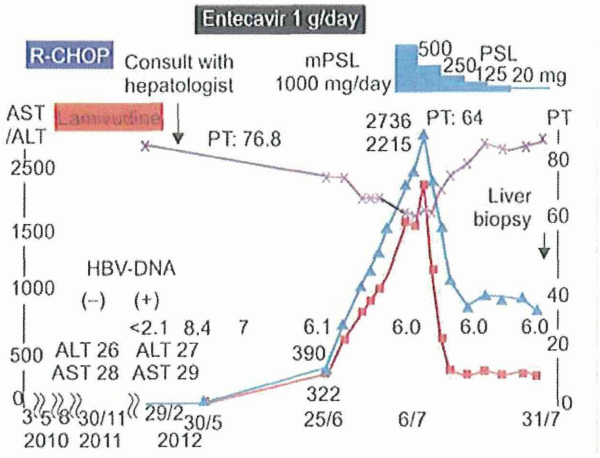


Fig. 1: Clinical course of present case

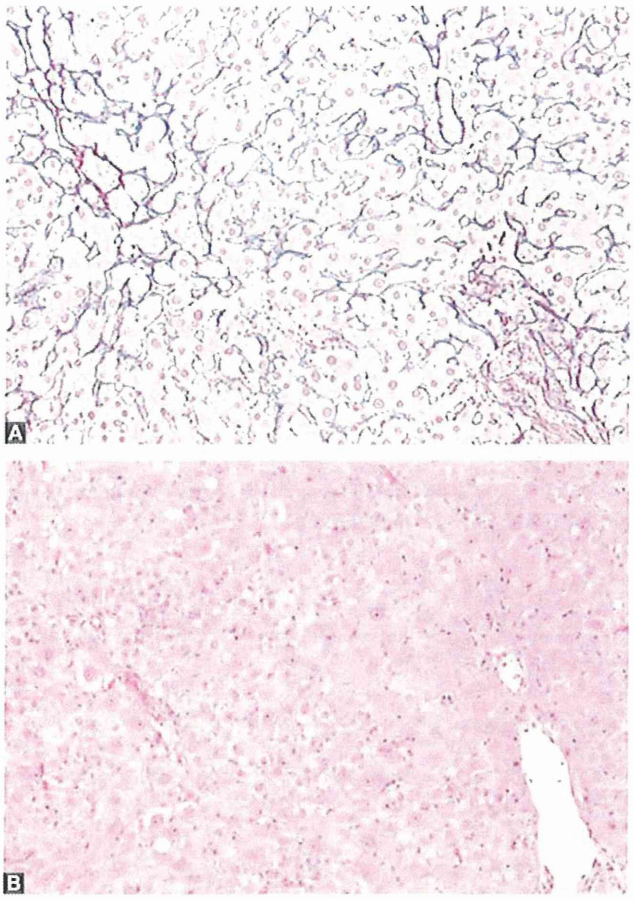
DISCUSSION

With the advent of immunosuppressive agents, the uses of these drugs have been related to some incidences of death because of reactivation of HBV and acute hepatic failure. Previous reports have clarified that this combination therapy can lead to acute hepatic failure and even death. For this reason, clinicians need to be aware of HBV reactivation not only in patients with current infection but also in those with resolved infection who are undergoing intensive immunosuppressive therapy.

The *de novo* hepatitis B could occur after the reactivation of HBV, when HBs antigen and HBV-DNA negative, but antipositive patients are treated with immunosuppressive agents. It becomes evident that the prophylactic administration of nucleos(t)ide analogs is very effective against the reactivation of HBV and occurrence of *de novo* hepatitis.[7-9] To prevent *de novo* type B hepatitis, critical observation and timely management of the patients are necessary. Also, multidisciplinary approach is necessary. A guideline of Japanese Society of Hepatology to manage *de novo* hepatitis and reactivation of HBV during and after

Table 1: Laboratory findings on administration day (June 25th, 2012)

WBC	4200/μl	AST	322 U/l	HBV-DNA	6.1 log copies/ml
RBC	3.82 × 10^6/μl	ALT	390 U/l	HBs Ag	(+)
HGB	12.9 g/dl	LDH	373 U/l	HBe Ag	(+) 245
HCT	37.7%	ALP	283 U/l	HBe Ab	(–)
PLT	15.3 × 10^4/μl	γ-GTP	38 U/l	HBc Ab	X1 (±) 53%
PT	76.8%	Na	142 mEq/l		X200 (–)
		K	3.6 mEq/l	HBV genotype	B
TP	6.3 g/dl	Cl	103 mEq/l	HBV YMDD	
Alb	4.0 g/dl	BUN	14 mg/dl	Lamivudine	Mutant (–)
TB	0.8 g/dl	Cre	0.78 mg/dl	HBV precore	Wild type
DB	0.1 g/dl	UA	6.7 mg/dl	Core promoter	Wild type
CHE	344 U/l	CRP	0.33 mg/dl		
		Glu	102 mg/dl		



Figs 2A and B: Biopsy specimen on the 63rd clinical day. Liver-cell damage, cell death and inflammatory cells infiltration were seen predominantly in central area, but there was no bridging necrosis. These findings indicate resolved acute hepatitis

immunosuppressive drugs, was reported and became popular to use it in Japan.[10,11] Japanese guideline that prevents reactivation *de novo* hepatitis of HBV is widely used not only by hepatologist but also by hematologist and rheumatologist. Similar guidelines were reported from Europe and USA.[12-14] Japanese guideline described that prophylactic administration should be continued for at least 1 year. In present case, the patient was treated with lamivudine for 1 year and 4 months after the case of R-CHOP therapy. But after stopping administration of lamivudine, HBV-DNA became positive within 4 months, and HBsAg became positive in 7 months, then acute hepatitis occurred. It means that, in this case, the occurrence of acute hepatitis was not prevented with the administration of lamivudine for 1 year and 4 months after stopping R-CHOP therapy. Though the Japanese guideline described that nucleos(t)ide analogs should be administered 'at least 1 year', the present case showed that nucleoside analogs for more than 1 year. We think that this case is valuable to know when we should stop the prophylactic administration of nucleos(t)ide analogs and how we should observe the patient after the stop of administration. This has become an emerging problem in every field of clinical medicine.[15]

CONCLUSION

Prolonged use of antiviral drugs seems to be necessary in immune suppressed patients with previous history of HBV infection. Also, a comprehensive approach is needed to tackle these patients. Periodic updating of therapeutic recommendations is also a necessity.

ACKNOWLEDGMENT

This study was supported in part by a Grant-in-Aid from the Japanese Ministry of Health, Welfare, and Labor, about Research Group for the long-term prognosis of anti-HBe antibody positive asymptomatic HBV carriers in Japan.

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Quantification of Hepatic Iron Concentration in Chronic Viral Hepatitis: Usefulness of T2-weighted Single-Shot Spin-Echo Echo-Planar MR Imaging

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Abstract

Objective: To investigate the usefulness of single-shot spin-echo echo-planar imaging (SSEPI) sequence for quantifying mild degree of hepatic iron stores in patients with viral hepatitis.

Methods: This retrospective study included 34 patients with chronic viral hepatitis/cirrhosis who had undergone histological investigation and magnetic resonance imaging with T2-weighted gradient-recalled echo sequence (T2-GRE) and diffusion-weighted SSEPI sequence with *b*-factors of 0 s/mm^2 (T2-EPI), 500 s/mm^2 (DW-EPI-500), and 1000 s/mm^2 (DW-EPI-1000). The correlation between the liver-to-muscle signal intensity ratio, which was generated by regions of interest placed in the liver and paraspinous muscles of each sequence image, and the hepatic iron concentration (μmol/g dry liver), which was assessed by spectrophotometry, was analyzed by linear regression using a spline model. Akaike information criterion (AIC) was used to select the optimal model.

Results: Mean ± standard deviation of the hepatic iron concentration quantified by spectrophotometry was 24.6±16.4 (range, 5.5 to 83.2) μmol/g dry liver. DW-EPI correlated more closely with hepatic iron concentration than T2-GRE (R square values: 0.75 for T2-EPI, 0.69 for DW-EPI-500, 0.62 for DW-EPI-1000, and 0.61 for T2-GRE, respectively, all $P<0.0001$). Using the AIC, the regression model for T2-EPI generated by spline model was optimal because of lowest cross validation error.

Conclusion: T2-EPI was sensitive to hepatic iron, and might be a more useful sequence for quantifying mild degree of hepatic iron stores in patients with chronic viral hepatitis.

Citation: Tonan T, Fujimoto K, Qayyum A, Kawaguchi T, Kawaguchi A, et al. (2012) Quantification of Hepatic Iron Concentration in Chronic Viral Hepatitis: Usefulness of T2-weighted Single-Shot Spin-Echo Echo-Planar MR Imaging. PLoS ONE 7(3): e33868. doi:10.1371/journal.pone.0033868

Editor: James Fung, The University of Hong Kong, Hong Kong

Received August 16, 2011; **Accepted** February 23, 2012; **Published** March 16, 2012

Funding: This study was partially supported by a Health and Labour Sciences Research Grants for Research on Hepatitis from the Ministry of Health, Labour and Welfare of Japan. No additional external funding received for this study. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Abnormalities of iron metabolism are frequently observed in patients with chronic liver diseases such as viral hepatitis, nonalcoholic fatty liver disease, and cirrhosis [1,2]. Iron excess, which increases oxidative stress via the formation of hydroxyl radicals and other highly reactive oxidizing molecules, leads to hepatotoxicity; it is related to the fibrogenesis and hepatocarcinogenesis associated with chronic viral hepatitis [1,3].

In recent years, several research groups have reported on the efficacy of iron reduction therapies by phlebotomy [4–10]. Yano et al. [6] reported that phlebotomy therapy contributed to improvement of biochemical markers in patients with hepatitis C virus infection. Kato et al. [10] stated that phlebotomy therapy may potentially lower the risk of progression to hepatocellular carcinoma (HCC) in patients with hepatitis C virus infection. Therefore, precise quantification of hepatic iron overload might be beneficial for managing iron reduction therapy in patients with chronic viral hepatitis.

Assessment of body iron stores by measurement of serum ferritin concentration has poor specificity [11]. Liver biopsy, the most reliable method to measure hepatic iron stores, is an invasive procedure. Magnetic resonance imaging (MRI) is sensitive to hepatic iron because iron leads to a decline of MR signal due to T2-shortening effect related to paramagnetic properties. MRI has recently been recognized as a suitable noninvasive technique for

quantifying hepatic iron overload [12]. Quantification of hepatic iron overload by MRI is useful in that it obviates the need for invasive liver biopsy and allows for repeat performance.

Generally, it is accepted that gradient-recalled echo (GRE) sequences are the most sensitive sequence to quantify mild degree of hepatic iron overload [13–20]. However, many studies evaluating GRE sequence with different echo-time and flip angle report variable results in the quantification of hepatic iron overload. Although the reproducibility of the technique and the quantification algorithm has been validated in various centers, these results are complicated.

Diffusion-weighted (DW) single-shot spin-echo echo-planar imaging (DW-EPI) has become a sequence used routinely in many institutions since the image quality was improved by recent technical progress such as parallel imaging and respiratory triggering [21–23]. In previous studies, it was reported that single-shot spin-echo EPI (SSEPI) sequence also had a high susceptibility effect [24,25].

We postulate that DW-EPI sequence might be superior to GRE sequence for quantifying mild degree of hepatic iron stores. To our knowledge, the investigation of hepatic iron overload by DW-EPI sequence has not been examined. The aim of this study was to investigate the usefulness of SSEPI sequence for quantifying mild degree of hepatic iron stores in patients with viral hepatitis.

Materials and Methods

Patients

The institutional review board (the Ethics Committee of Kurume University) approved this retrospective study (Approval No. 09112), which complied with the principles of the Declaration of Helsinki (2008 version). All included patients gave written informed consent to participate.

Our study was targeted at patients with viral chronic hepatitis/cirrhosis and HCC because such patients with chronic liver impairment may have increased liver iron and would have undergone both liver MR imaging and hepatic surgery.

We reviewed the patients who admitted use of both liver specimens and MR images before hepatic surgery at our institution between January 2007 and April 2008 and identified patients who met the following inclusion criteria: (*a*) patients had both chronic viral hepatitis/cirrhosis and HCC; (*b*) patients underwent abdominal MR imaging with T2-weighted GRE sequence and DW-EPI sequence with *b*-factors of 0 s/mm^2, 500 s/mm^2, and 1000 s/mm^2 (these sequences were part of our standard abdominal MR imaging protocol during this period); and (*c*) patients underwent an operation for HCC and received a histopathologic diagnosis of either chronic hepatitis or cirrhosis that was based on findings at surgical resection, performed within a month after MR imaging.

Forty-six patients fulfilled these criteria. Twelve of these 46 patients were excluded on the basis of the following reasons: *(a)* Available imaging data did not correspond to available histopathologic data because of interval surgery ($n = 5$), *(b)* MR studies were incomplete ($n = 3$), *(c)* an artifact was observed on MR images and precluded accurate measurement of signal intensity ($n = 1$), and *(d)* other causes of chronic liver disease such as alcoholic hepatitis ($n = 2$) and non-alcoholic steatohepatitis ($n = 1$). Thirty-four patients formed the final study group (21 men and thirteen women; median age, 65 years; range, 52–83 years). Histopathologic sampling of all patients included in the study was performed after MR imaging (median, 5 days; range, 1–30 days). The cause of chronic liver disease was hepatitis C virus infection (n = 26) or hepatitis B virus infection (n = 8). None of the patients had a clinical diagnosis of hemochromatosis that was based on review of medical records.

Hepatic iron concentration and histological analysis

A partial hepatic resection was performed in all patients with HCC. For each patient, 50 mg of wet liver tissue was extracted from the surgically removed specimen by a MLS1200 MEGA microwave digestion system (Milestone General Co. Ltd., Kawasaki, Japan) for 1 min at 250 W, 1 min at 0 W, 5 min at 250 W, 5 min 400 W, and 5 min at 500 W. For determination of hepatic iron concentration (μmol/g dry liver), the resulting extracts were analyzed by spectrophotometry with a graphite atomic absorption camera (Polarized Zeeman Atomic Absorption Spectrophotometer, Hitachi, Ltd., Tokyo, Japan) and were converted to the units shown above [26].

For histological analysis, fibrosis stage and necroinflammation grade were evaluated semiquantitatively using the METAVIR scoring system [27]. Fibrosis stage graded on a scale of 0 to 4, as follows: F0 = no fibrosis; F1 = portal fibrosis without septa; F2 = portal fibrosis and few septa; F3 = numerous septa without cirrhosis; and F4 = cirrhosis. The necroinflammatory activity score was graded on a scale of 0 to 3, as follows: A0 = none; A1 = mild; A2 = moderate; A3 = severe. Distribution of steatosis was also retrospectively evaluated as the overall impression of the percentage of fat-containing hepatocytes on hematoxylin and eosin–stained specimens [28,29]. Steatosis grade was scored on a scale of 0 to 2, as follows: grade 0 = absence of steatosis; grade 1 = steatosis <5%; and grade 2 = steatosis ≥5%.

MRI technique and analysis

Within one month prior to surgery, MR imaging was performed at field strength of 1.5 T (Magnetom Symphony Advanced; Siemens, Erlangen, Germany) with use of a body phased-array surface coil. A series of DWIs and T2-weighted GRE sequence were obtained using parallel imaging with generalized auto calibrating partially parallel acquisition (GRAPPA) of acceleration factor 2 in all patients. DWI was performed in the transverse plane by respiratory-triggered combining SSEPI sequence with a chemical shift–selective pulse (CHESS). Any antiperistalsis drug was not used.

The imaging parameters for DW-EPI were as follows: repetition time (TR), 2000 msec; echo time (TE), 81 msec; directions of the motion-probing gradient, three orthogonal axes; gradient factor *b* values of 0 sec/mm^2 (T2-weighted SSEPI, hereafter T2-EPI), 500 sec/mm^2 (DW-EPI-500), and 1000 sec/mm^2 (DW-EPI-1000); 2170-Hz per pixel bandwidth; 350-mm field of view; 128×88 rectangular matrixes; 9-mm-thick sections; 1-mm intersection gap; six signals acquired; and acquisition time of approximately 1 minute 30 seconds.

T2-weighted GRE sequence (hereafter, T2-GRE) was performed in the transverse plane by fast low angle shot (FLASH) with one signal acquired during a 22-second breath hold. The imaging parameters for T2-GRE were as follows: TR, 246 msec; TE, 9.5 msec; flip angle (FA), 30°; 350-mm field of view; 9-mm-thick sections; 1-mm intersection gap; 16-number of sections; 256×192 matrix; and 130-Hz per pixel bandwidth.

Quantitative image analysis was conducted by measuring the signal intensities of the liver parenchyma and paraspinous muscles. Image analysis was performed by two independent radiologists using plug-in software developed in-house by one of the authors [30,31] (Figure 1). Five separate regions of interest (ROIs) were carefully placed manually in the anterior and posterior segments of the right hepatic lobe at the level of the porta hepatis (whenever possible) on each sequence; care was taken to avoid focal lesions,

major vascular structures, and artifacts such as chemical shifts, magnetic susceptibility, and cardiac motion. Liver signal intensities were recorded as the mean values generated from the five measurements (total liver ROI area sampled, 500 mm^2). The procedure was repeated to measure muscle signal intensity by placing two separate ROIs on the right and left paraspinous muscles in the same slice section used to measure liver signal intensity; care was taken to avoid artifacts such as chemical shifts, magnetic susceptibility, and motion on each sequence.

Muscle signal intensities were recorded as the mean values generated from the two measurements (total muscle ROI area sampled, 200 mm^2). We calculated the liver-to-muscle signal intensity ratio (LMR) by dividing mean liver signal intensity by mean muscle signal intensity for each sequence [15].

Statistical analysis

A Bland-Altman plot was used to analyze the 95% limits of interobserver agreement for the LMR on each sequence [32]. The correlation of the LMR obtained by the two observers on each sequence was determined using the Pearson correlation coefficient (r).

The relationship between the LMR on each sequence and hepatic iron concentration was analyzed by means of scatter plots. These results were inspected for linearity and goodness of fit. The relationship between the LMR on each sequence and hepatic iron concentration was modeled by regression techniques using a spline model. Details of spline models are given in the next section.

To investigate effects of each LMR on hepatic iron concentration, we applied the linear models containing not only a main term but also knot terms which play a role as an inflection point. The Akaike information criterion (AIC) was used to evaluate these alternate models [33]. The number and location of knots were determined objectively with the minimum AIC among their prespecified candidates, which were 20, 40, 60, and 80 percentiles of each LMR. To evaluate the predictive accuracy, a leave one out cross validation (CV) error [34] was computed.

The Kruskal–Wallis test was used to determine significant differences in the LMR on each sequence among category classification in each histological finding (i.e. necroinflammation grade, fibrosis stage, and steatosis grade). All analyses were performed using SPSS statistical software (version 12.0 J; SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered statistically significant.

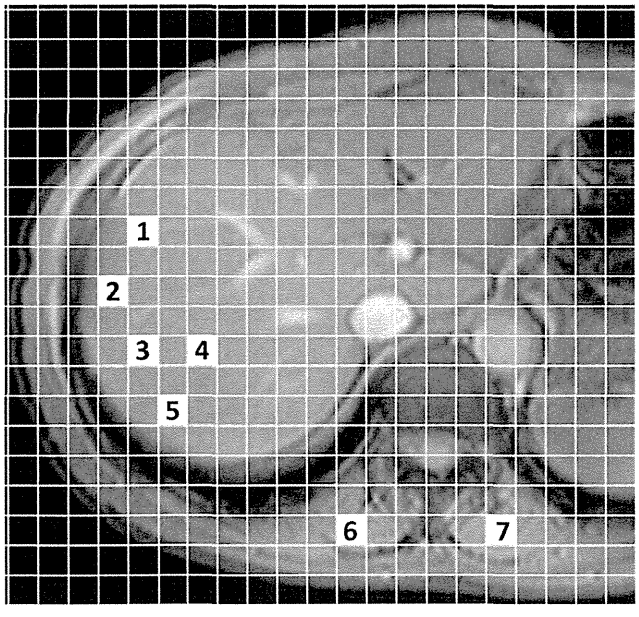

Figure 1. Illustration of the method used to measure regions of interest on an MR image. With use of computer software (developed in-house by the authors), two independent observers freely and easily selected a region of interest by clicking a mesh unit on the right hepatic lobe of an image while avoiding the large vessels, focal hepatic lesions, or artifacts. Seven regions of interest were chosen for liver parenchyma (1–5, total liver ROI area sampled, 500 mm^2) and paraspinous muscles (6 and 7, total muscle ROI area sampled, 200 mm^2) in the same slice section of each sequence.
doi:10.1371/journal.pone.0033868.g001

Details of the spline models used in statistical analysis

Response and predictor variables are denoted by y and x, respectively. The general form of the univariate (first order) spline model is

$$y=\alpha+\beta x+\sum_{j=1}^{m}\gamma_j(x-r_j)_+ +\varepsilon \quad (1)$$

where α, β, and γ_j ($j=1, 2, \ldots, m$) are parameters to be estimated, $(z)_+ =\max(0,z)$, $r_1,r_2,\cdots,r_m$ are called knots which play a role as an inflection point, and ε is an error following a normal distribution with mean 0 and a constant variance. Note that in the case of $\gamma_1=\gamma_2=\cdots=\gamma_m=0$ the model can be identified as a simple linear regression model. The parameters in the model (1) are estimated by an ordinary least squares method to minimize squared residuals Q in (2) from samples (x_i,y_i) ($i=1, 2, \ldots, n$) from n patients.

$$Q=\sum_{i=1}^{n}\left(y_i-\alpha-\beta x_i-\sum_{j=1}^{m}\gamma_j(x_i-r_j)_+\right)^2 \quad (2)$$

To illustrate the interpretation of parameters in the spline model, we consider the model as with only one knot as in (3). This model contains two lines whose slope and intercept are changed at $x=r$.

$$y=\alpha+\beta x+\gamma(x-r)_+ +\varepsilon \quad (3)$$

In the range $x\le r$, the slope is β and the intercept is α. In the other range $x>r$, the slope is $\beta+\gamma$ and the intercept is $\alpha-\gamma r$.This modeling can be easily implemented by standard software such as SAS, SPSS, and R. Supposing that the data set has two columns corresponding to response (y) and predictor (x) variables, one can add the computed $(x-r)_+$ as the third column. Then, the multiple regression model can be applied with the response y and two predictors, x and $(x-r)_+$. If you want more knots, you can add the corresponding columns and predictors in the regression model.

The essential point in the use of this spline model is to select the number and location of knots. As used in this paper, one choice for candidates for knots is the quantiles for continuous variables taking into account the sample size. Once one specifies the candidates, the problem turns to the variable selection for predictor variables used in the multiple regression model, which can also be implemented by standard software. One effective method is to use information criteria such as AIC. This kind of modeling [35] is useful to investigate the flexible relationship between the response and predictor.

Results

Hepatic iron concentration and histological findings

Mean ± SD of the hepatic iron concentration quantified by spectrophotometry was 24.6±16.4 (range, 5.5 to 83.2) μmol/g dry liver. Histological necroinflammation grade was A1 in 21 patients and A2 in 13 patients. Fibrosis stage was F1 in 13 patients, F2 in 4 patients, F3 in 5 patients, and F4 (i.e. cirrhosis) in 12 patients. Steatosis grade was 0 in 14 patients, grade 1 in 11 patients, and grade 2 in 9 patients.

Interobserver agreement for the LMR on each sequence

There was no significant difference between measurements made by the two observers for the two parameters; the interclass Pearson correlation coefficients were 0.96 (95% confidence interval [CI]: 0.86, 1.00) for T2-GRE, 0.99 (95% CI: 0.92, 1.00) for T2-EPI, 0.97 (95% CI: 0.85, 1.00) for DW-EPI-500, and 0.98 (95% CI: 0.97, 1.00) for DW-EPI-1000; the mean difference (± standard deviation) was −0.0027±0.054 for T2-GRE, −0.0069± 0.052 for T2-EPI, 0.017±0.11 for DW-EPI-500, and 0.013±0.16 for DW-EPI-1000; and the coefficients of repeatability were 0.108 for T2-GRE, 0.105 for T2-EPI, 0.213 for DW-EPI-500, and 0.316 for DW-EPI-1000. Bland-Altman plots with 95% limits of agreement for each sequence are shown in Figure 2. There was no proportional bias or fixed bias in each Bland-Altman plot for the two parameters.

Correlation between the LMR on each sequence and hepatic iron concentration

Figure 3 shows results for the line fit by the selected regression model. Created simple regression models to estimate the hepatic iron concentration in each sequence are as follows:

$$\mathrm{T2-GRE}: y = 103.7 - 85.7 \times \mathrm{LMR} + 58.2 \times (\mathrm{LMR} - 1.05)_+$$

$$\mathrm{T2-EPI}: y = 131.0 - 139.7 \times \mathrm{LMR} + 106.5 \times (\mathrm{LMR} - 0.73)_+ + 27.4 \times (\mathrm{LMR} - 1.24)_+$$

$$\mathrm{DW-EPI-500}: y = 80.2 - 51.8 \times \mathrm{LMR} + 43.0 \times (\mathrm{LMR} - 1.24)_+$$

$$\mathrm{DW-EPI-1000}: y = 66.7 - 29.3 \times \mathrm{LMR} + 25.7 \times (\mathrm{LMR} - 1.76)_+$$

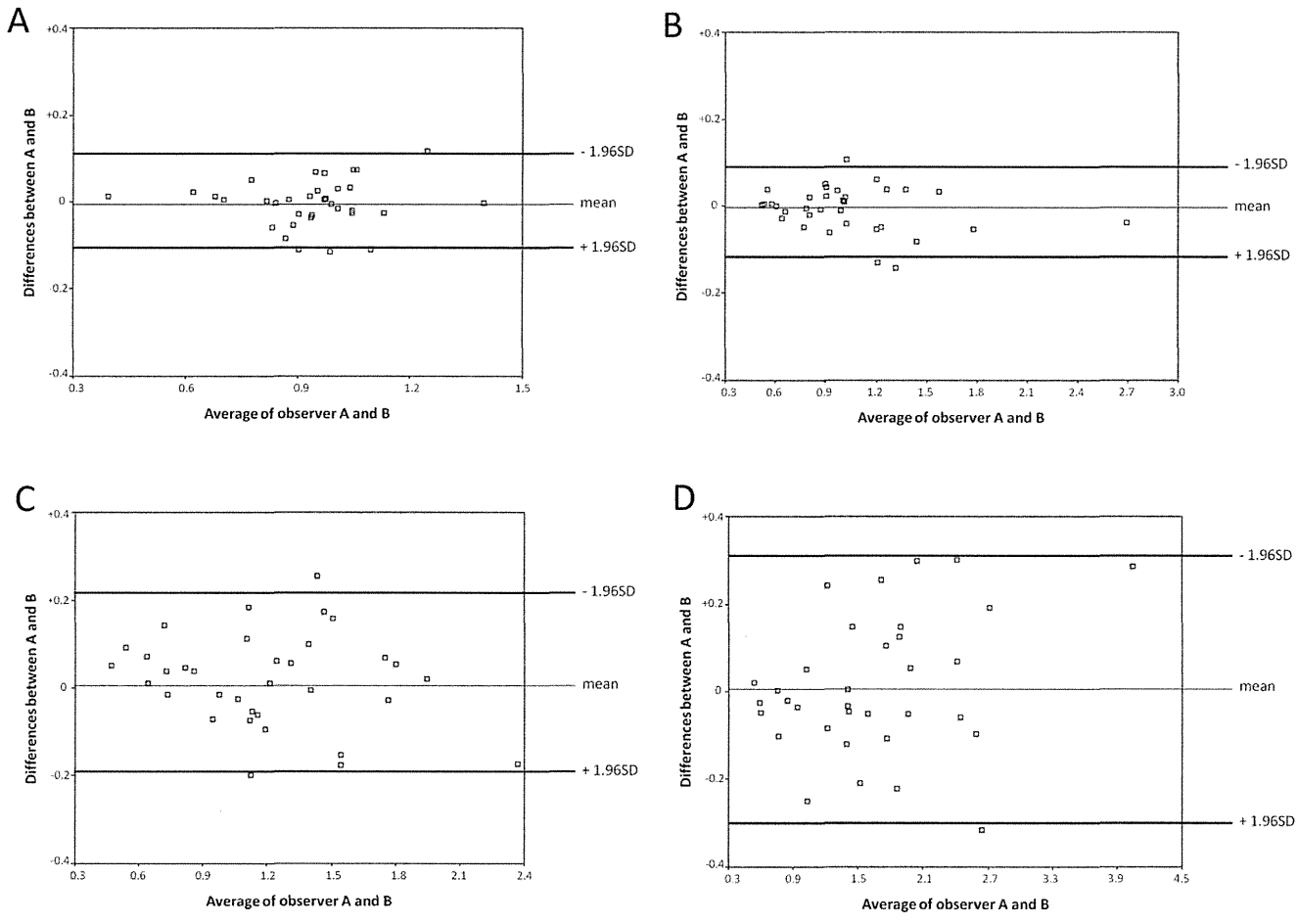

Figure 2. Bland-Altman plots for measurements of T2-GRE (A), T2-EPI (B), DW-EPI-500 (C), and DW-EPI-1000 (D) in liver parenchyma. Each Bland-Altman plots demonstrates good interobserver agreement and lack of proportional bias or fixed bias. The average of the measurements made by the two observers is plotted against the difference between the measurements made by the two observers. The thin lines represent the mean value of all differences between the two observers, and the thick lines represent the 95% limits of agreement. *SD* = standard deviation.

doi:10.1371/journal.pone.0033868.g002

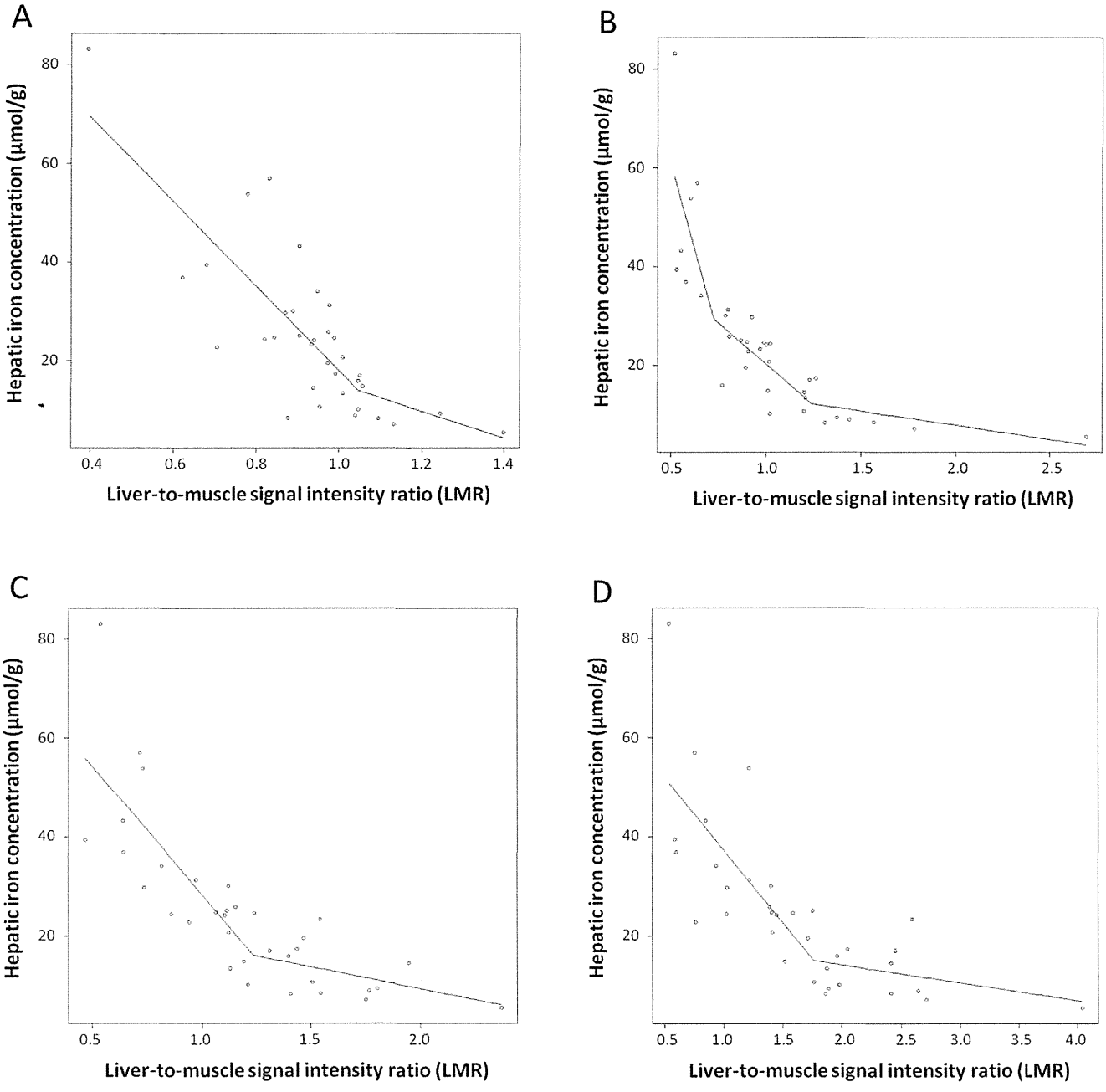

Figure 3. Scatter plots of LMR and hepatic iron concentration (μmol/g dry liver) on T2-GRE (A), T2-EPI (B), DW-EPI-500 (C), and DW-EPI-1000 (D). Correlation between LMR and hepatic iron concentration for linear regression with spline models are shown as solid lines on each sequence. The linear regression model [$y = 131.0 - 139.7 \times LMR + 106.5 \times (LMR - 0.73)_+ + 27.4 \times (LMR - 1.24)_+$] on T2-EPI was optimal.
doi:10.1371/journal.pone.0033868.g003

where LMR is the measurement value on each sequence (appendix).

The regression analyses showed an excellent overall negative correlation on each sequence. Particularly, T2-EPI correlated most closely with hepatic iron concentration. R square values on each sequence were as follows: 0.75 for T2-EPI, 0.69 for DW-EPI-500, 0.62 for DW-EPI-1000, and 0.61 for T2-GRE (F-test, $P<0.0001$, respectively).

Using the AIC, the linear regression model on T2-EPI [$y = 131.0 - 139.7 \times LMR + 106.5 \times (LMR - 0.73)_+ + 27.4 \times (LMR - 1.24)_+$] was chosen as having the best fit, since it had the lowest CV error. The corresponding CV errors were as follows: 14161.3 for T2-GRE, 11357.4 for T2-EPI, 12220.0 for DW-EPI-500, and 14376.2 for DW-EPI-1000.

Correlation between the LMR on each sequence and histological findings

No significant differences were found for the LMR on each sequence among category classification of histological findings (i.e. necroinflammation grade, fibrosis stage, and steatosis grade). *P* values (Kruskal-Wallis test) were as follows: (a) necroinflammation grade: $P = 0.4$ for T2-GRE, $P = 0.89$ for T2-EPI, $P = 0.68$ for DW-EPI-500, and $P = 0.6$ for DW-EPI-1000; (b) fibrosis stage: $P = 0.39$

for T2-GRE, $P=0.29$ for T2-EPI, $P=0.19$ for DW-EPI-500, and $P=0.38$ for DW-EPI-1000; (c) steatosis grade: $P=0.75$ for T2-GRE, $P=0.77$ for T2-EPI, $P=0.69$ for DW-EPI-500, and $P=0.95$ for DW-EPI-1000.

Discussion

In the present study, we found good correlation between DW-EPI and hepatic iron concentration in patients with chronic viral hepatitis, and also demonstrated that SSEPI sequence was more sensitive than T2-GRE sequence for quantifying small amount of hepatic iron overload; this is in concordance with prior studies reporting a high susceptibility effect with SSEPI sequence [24,25].

A lot of studies have evaluated the correlation between hepatic iron concentration and MRI measurements [13–20]. Particularly, GRE sequences, which are more sensitive to field heterogeneities than spin-echo sequences [15,16,18], were used for quantifying mild degree of hepatic iron stores in many studies. It was reported that the best means to evaluate mild degrees of hepatic iron overload was T2-GRE sequences with long TE (i.e. >15 ms) and with low FA (i.e. 20°–30°) [15,19]. Alternatively, Bonkovsky et al. [18] reported that GRE sequence with shortest TR and TE, which results in a short breath hold time, was useful to minimize motion artifact and other sources of noise. Results from studies of GRE sequence were variable in terms of quantification of hepatic iron overload [13–20].

The sensitivity to iron on T2-GRE sequences varies significantly with various different TE and FA [15]. Marked signal loss from proton dephasing will occur at longer TEs, and once signal intensity falls to the level of image noise, inaccuracies in signal intensity measurement can be expected [36]. From these points, in the routine examination, we employed the conventional TE which corresponds to second in-phase on T2-GRE sequence for quantifying mild degree of hepatic iron overload.

SSEPI sequences are very fast and have a high susceptibility effect, but suffer from limited image quality. This is mostly related to limited signal to noise ratio (SNR), especially at higher b-values, and limited spatial resolution, which constitute an obstacle for its widespread use in clinical practice [37]. However, techniques such as parallel imaging and pulse triggering improve image quality of SSEPI sequences by correcting magnetic field heterogeneity [21–23]. Recent data showed that respiratory triggering improved the image quality with SNR on SSEPI sequences. This method attempts to avoid motion artifacts prospectively by using respiratory signals to synchronize image acquisition with the patient's breathing cycle and by acquiring the imaging data during the relative quite end expiration phase [38–40].

In the present study, we employed SSEPI sequence with techniques such as parallel imaging and respiratory triggering. This sequence, which has the advantage of high susceptibility effects, was useful to assess mild degree of hepatic iron stores in patients with viral hepatitis. Of DW-EPIs, it was suggested that T2-EPI was the most suitable sequence because DW-EPI-500 and DW-EPI-1000 had loss of SNR caused by application of the motion-probing gradients pulse.

In patients with chronic viral hepatitis, steatosis is a common secondary phenomenon. Westphalen et al. [41] reported that iron stores in background liver complicated measurement of steatosis by opposed-phase MR imaging. Alternatively, a recent study reported that concomitant steatosis lowers the diagnostic performance of T2-GRE sequence and chemical shift imaging for quantifying mild degree of hepatic iron stores because intravoxel constructive and destructive interference between fat and water spins due to chemical shift effect of the second kind potentially affect the signal intensity measurements for T2-GRE sequence [36]. Therefore, it might be important to consider the influence of each factor in background liver tissue in the quantification of steatosis and iron stores using MR imaging.

On DW-EPI, we found no significant differences in LMR among histological steatosis grades. Use of fat saturation pulse (i.e., CHESS) on DW-EPIs could eliminate the influence of steatosis, which might support the better utility of this sequence for quantifying mild degree of hepatic iron stores. On the other hand, although previous studies reported that liver fibrosis decreased the diffusion signal [30,42,43], no significant differences were found in LMR on DW-EPIs among histological fibrosis stages, which suggest that influence of liver fibrosis to the signal of DW-EPIs was low as a result. The quantification of iron stores by DW-EPIs may have suffered potential influence by fibrosis, which might be one of the reasons that T2-EPI was most accurate sequence for quantifying mild degree of iron stores. Therefore, we recommend the T2-EPI with b values of 0 sec/mm^2, which is not affected to the diffusion signal, for quantifying mild degree of iron stores.

Several limitations of the present study warrant mention. First, the study was conducted retrospectively and sample size was small. Although a major effort was made to exclude sample bias, there was limited sample size for examination of liver iron concentration using spectrophotometry because of its retrospective nature. Second, all measurements for the LMR were obtained in the right lobe of the liver to avoid motion-related artifact. Because the pathologic specimens were obtained at surgery for an HCC, histologically sampled areas did not completely correspond to radiologically sampled areas. A prospective study with a substantially larger sample is needed to further validate our findings.

In conclusion, DW-EPI (especially, T2-weighted SSEPI) was sensitive to hepatic iron, and might be a more useful sequence for quantifying mild degree of hepatic iron stores in patients with chronic viral hepatitis.

Author Contributions

Conceived and designed the experiments: TT KF TK MS. Performed the experiments: TT KF TK ON. Analyzed the data: TT KF AK. Contributed reagents/materials/analysis tools: KF TK ON KO NH MS. Wrote the paper: TT KF AQ AK. Designed the software used in analysis: KF. Drafting the article or revising it critically for important intellectual content and final approval of the version to be published: TT KF AQ TK AK ON KO NH MS.

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