the survey according to the exclusion criteria. Consequently, 460 patients ($n = 227$ acute type; $n = 233$ subacute type) were classified as having FH and 28 as having LOHF (Table 1). The incidence of the acute and subacute types of FH was similar and the incidence of LOHF was one-sixteenth of FH. The male : female ratio was higher for the acute type and lower for the subacute type of FH and LOHF. The mean age of patients was significantly higher for the subacute type of FH and LOHF than that for the acute type of FH. Almost half of the patients with FH and LOHF had complications which preceded the onset of acute liver failure. Furthermore, approximately 60% of patients with FH had received daily medication. This tendency for receiving medication was more obvious in patients with the subacute type of FH and LOHF.

The survival rates of patients without LT were 48.7% for the acute type and 24.2% for the subacute type of FH, and 13.0% for LOHF. The survival rates of the subacute type of FH and LOHF was worse than that of the acute type. The prognosis of both the acute type and the subacute type of FH appeared to be equivalent annually. The survival rates of patients with LT were 79.6% for FH and 100% for LOHF, with no difference in these rates among the disease types.

Clinical profile

Symptoms, imaging findings and complications are shown in Table 2. Since 2006, diagnostic criteria of systemic inflammatory response syndrome (SIRS) for fever, tachycardia and tachypnea have been adopted in the survey.[10] Icterus, flapping tremor, ascites, hepatic fetor, tachycardia, tachypnea and pretibial edema were frequently found. The frequency of patients with ascites and pretibial edema was significantly greater in the subacute type of FH and LOHF than in the acute type of FH. In contrast, fever appeared more frequently in patients with the acute type of FH. The frequency of liver atrophy was greater in the subacute type of FH, and even higher in LOHF, than in the acute type of FH.

With regard to complications, disseminated intravascular coagulation, renal failure and bacterial infection were found in more than 30% of patients with FH and LOHF. Brain edema was less frequent in the subacute type than in the acute type of FH.

Causes of FH and LOHF

The cause of FH was identified as viral infection in 46.1% of the patients (Table 3). The frequencies of viral infection were highest for the acute type of FH. HAV infection was found in 3% of patients with FH. HBV infection was found in 40.2% of patients with FH and 32.1% of patients with LOHF. Transient HBV infection was more frequent in the acute type than in the subacute type of FH, whereas the frequency of acute exacerbation in HBV carriers was greater in the subacute type than in the acute type of FH. HBV reactivation in inactive carriers and in transiently infected patients were found in 3.3% and 3.5% of patients with FH, respectively. With regard to underlying diseases in patients with HBV reactivation, non-Hodgkin's lymphoma/mucosa-associated lymphoid tissue lymphoma was most prevalent in 50% of inactive carriers and in 76% of those with transiently infected patients. Among patients with HBV

Table 1 Demographic features and survival rates of patients with fulminant hepatitis (FH) and late-onset hepatic failure (LOHF)

	FH			LOHF ($n = 28$)
	Total ($n = 460$)	Acute type ($n = 227$)	Subacute type ($n = 233$)	
Male/female	227/233	127/100	100/133**	9/19*
Age (years; mean ± SD)	51.1 ± 17.0	48.8 ± 16.9	53.4 ± 16.7**	58.0 ± 14.4**
HBV carrier (%)	13.1 (52/397)	10.5 (19/181)	15.3 (33/216)	22.2 (6/27)
Complications preceding acute liver failure (%)†	46.4 (208/448)	40.0 (88/220)	52.6 (120/228)**	50.0 (14/28)
History of medication (%)	59.9 (260/434)	51.2 (108/211)	68.2 (152/223)**	71.4 (20/28)*
Survival rates				
All patients	47.4 (218/460)	54.2 (123/227)	40.8 (95/233)**	28.6 (8/28)*
No LT	37.5 (132/352)	48.7 (93/191)	24.2 (39/161)**	13.0 (3/23)**
LT	79.6 (86/108)	83.3 (30/36)	77.8 (56/72)	100 (5/5)

*$P < 0.05$, **$P < 0.01$ vs acute type.
†Diseases such as metabolic syndrome, malignancy and psychiatric disorders.
Data in parenthesis indicate patient numbers.
HBV, hepatitis B virus; LT, liver transplantation; SD, standard deviation.

Table 2 Symptoms, imaging findings and complications of patients with fulminant hepatitis (FH) and late-onset hepatic failure (LOHF)

	FH			LOHF ($n = 28$)
	Total ($n = 460$)	Acute type ($n = 227$)	Subacute type ($n = 233$)	
(a) Symptoms at diagnosis				
Fever†	13.0 (42/322)	17.5 (28/160)	8.6 (14/162)*	0 (0/23)*
Icterus	96.8 (427/441)	95.0 (208/219)	98.6 (219/222)*	96.4 (27/28)
Ascites	57.2 (237/414)	45.2 (88/204)	71.0 (149/210)**	81.5 (22/27)**
Convulsion	5.2 (22/422)	6.7 (14/210)	3.8 (8/212)	0 (0/27)
Tachycardia‡	36.7 (117/319)	39.5 (62/157)	34.0 (55/162)	47.8 (11/23)
Tachypnea§	34.5 (87/252)	39.1 (52/133)	29.4 (35/119)	31.6 (6/19)
Flapping tremor	79.0 (309/391)	75.8 (144/190)	82.1 (165/201)	80.8 (21/26)
Hepatic fetor	46.6 (146/313)	49.0 (73/149)	44.5 (73/164)	42.1 (8/19)
Pretibial edema	35.5 (127/358)	24.1 (42/174)	46.2 (85/184)**	75.0 (15/20)**,***
(b) Imaging findings				
Liver atrophy¶	58.8 (255/434)	45.6 (98/215)	71.7 (157/219)**	92.6 (25/27)**,***
(c) Complications				
Infection	34.8 (149/428)	32.9 (68/207)	36.7 (81/221)	51.9 (14/27)
Brain edema	18.5 (71/384)	24.1 (46/191)	13.0 (25/193)**	22.7 (5/22)
Gastrointestinal bleeding	13.2 (59/446)	11.0 (24/219)	15.4 (35/227)	20.0 (5/25)
Renal failure	38.9 (177/455)	40.9 (92/225)	37.0 (85/230)	39.3 (11/28)
DIC	34.6 (150/433)	35.7 (76/213)	33.6 (74/220)	53.8 (14/26)
Congestive heart failure	7.3 (31/427)	8.9 (19/214)	5.6 (12/213)	12.0 (3/25)

*$P < 0.05$, **$P < 0.01$ vs acute type, ***$P < 0.05$ vs subacute type.
†Temperature: >38°C or <36°C.
‡Heart rate: >90 beats/min.
§Respiratory rate: >20 breaths/min or $PaCO_2$: <32 Torr.
† ‡ § Cases between 2005 and 2009.
¶Liver atrophy detected by ultrasound and/or computed tomography imaging.
Data in parentheses indicate patient numbers.
DIC, disseminated intravascular coagulation.

reactivation, rituximab plus steroid combination chemotherapy was administrated to 35% of patients in inactive carriers and to 59% of those with transiently infected patients. HCV and HEV infection were less frequently found. In the survey, Epstein–Barr virus, herpes simplex virus and human herpes virus type-6 were found as other causes of viral hepatitis.

Autoimmune hepatitis was frequently observed in patients with the subacute type of FH and LOHF. Drug allergy-induced liver injury was observed in approximately 10–20% of patients irrespective of disease types. Anti-tuberculosis agents, non-steroidal anti-inflammatory drugs, anticancer agents, drugs for metabolic syndrome, and various herbal and natural remedies were the probable causative agents for this liver injury in the survey. Notably, the etiology was indeterminate in approximately 40% of patients with the subacute type of FH.

Therapies

For artificial liver support, plasma exchange and HDF were performed in most patients with FH (Table 4). Conventional HDF and continuous HDF (CHDF) were performed in 22.5% and 51.8% of patients with FH, respectively. A more powerful method, high-flow HDF (HF-HDF), high-flow CHDF (HF-CHDF) and on-line HDF were performed in 2.6%, 11.7% and 1.8% of the patients, respectively. The nucleoside analogs lamivudine and entecavir were used in approximately a quarter of patients with FH. Entecavir were used more frequently than lamivudine since 2007. Glucocorticosteroid, mainly as steroid pulse therapy, were administrated in more than 70% of patients with FH and LOHF. Anticoagulation therapy were performed in approximately 40–50% of patients with FH and LOHF. Glucagon/insulin, branched-chain amino acid-rich solution,

Table 3 Causes of fulminant hepatitis (FH) and late-onset hepatic failure (LOHF)

	FH			LOHF ($n = 28$)
	Total ($n = 460$)	Acute type ($n = 227$)	Subacute type ($n = 233$)	
Viral infection	46.1 (212)	62.6 (142)	30.0 (70)	32.1 (9)
HAV	3.0 (14)	5.7 (13)	0.4 (1)	0 (0)
HBV	40.2 (185)	54.2 (123)	26.6 (62)	32.1 (9)
(1) Transient infection	19.6 (90)	35.2 (80)	4.3 (10)	3.6 (1)
(2) Acute exacerbation in HBV carrier	14.1 (65)	7.9 (18)	20.2 (47)	25.0 (7)
(i) Inactive carrier, without drug exposure	7.4 (34)	6.2 (14)	8.6 (20)	3.6 (1)
(ii) Reactivation in inactive carrier†	3.3 (15)	1.8 (4)	4.7 (11)	17.9 (5)
(iii) Reactivation in transiently infected patient‡	3.5 (16)	0 (0)	6.9 (16)	3.6 (1)
(3) Indeterminate infection patterns	6.5 (30)	11.0 (25)	2.1 (5)	3.6 (1)
HCV	1.1 (5)	0.9 (2)	1.3 (3)	0 (0)
HEV	0.9 (4)	0.9 (2)	0.9 (2)	0 (0)
Other viruses	0.9 (4)	0.9 (2)	0.9 (2)	0 (0)
Autoimmune hepatitis	8.3 (38)	2.2 (5)	14.2 (33)	32.1 (9)
Drug allergy-induced liver injury	14.6 (67)	13.7 (31)	15.5 (36)	17.9 (5)
Indeterminate§	29.6 (136)	19.4 (44)	39.5 (92)	17.9 (5)
Unclassified¶	1.5 (7)	2.2 (5)	0.9 (2)	0 (0)

†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.

cyclosporin A and prostaglandin E_1 therapy were administrated less frequently compared with the previous survey.

Liver transplantation was performed in 23.5% and 17.9% of patients with FH and LOHF, respectively. Two patients received deceased-donor LT and 111 patients received living-donor LT. The frequency of LT was significantly greater in the subacute type than in the acute type of FH.

Prognosis

The prognosis of patients with FH and LOHF differed depending on the etiology (Table 5). Prognosis was good in patients with HAV infection. The prognosis was fair in patients with transient HBV infection. In contrast, the prognosis was poor in acute exacerbation in HBV carriers. The prognosis was extremely poor in patients with HBV reactivation, either from inactive carriers or transiently infected patients. Patients with the subacute type of FH and LOHF caused by autoimmune hepatitis, drug allergy-induced liver injury and indeterminate etiology also showed a poor prognosis.

The clinical features of the patients appeared to be associated with the prognosis. In the acute type of FH with no LT, the frequency of patients with SIRS (tachycardia or tachypnea) was greater in patients who died than in surviving patients ($P < 0.05$). Liver atrophy on ultrasound and/or computed tomography imaging was an important factor in predicting the prognosis of FH and LOHF with no LT. The frequencies were 25.0% and 64.5% in patients with the acute type ($P < 0.01$) and 55.6% and 78.1% in those with the subacute type of FH in surviving patients and those who died, respectively ($P < 0.05$).

Prognosis also appeared to be affected by complications. Any of the complications significantly decreased survival rate (data not shown). Furthermore, the number of these complications affected the prognosis. The survival rate of patients with the acute type of FH was greater than 80% in those with no complications, while it was less than 30% in those with two or more complications. The survival rate of patients with the subacute type of FH was decreased in proportion to the number of complications.

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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Japan College of Rheumatology
MODERN RHEUMATOLOGY

http://informahealthcare.com/mor
ISSN 1439-7595 (print), 1439-7609 (online)

Mod Rheumatol, 2014; 24(1): 1–7

DOI 10.3109/14397595.2013.852834

informa
healthcare

REVIEW ARTICLE

A proposal for management of rheumatic disease patients with hepatitis B virus infection receiving immunosuppressive therapy

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Received: 5 November 2012 / Accepted: 30 November 2012

Abstract Reactivation of hepatitis B virus (HBV) and de novo HBV hepatitis in patients with rheumatic diseases given intensive and long-term immunosuppressive therapy with or without biological disease-modifying antirheumatic drugs is of great concern, especially in regions where the virus is endemic, including Japan. To ascertain a better benefit–risk balance for immunosuppressive therapy for patients with rheumatic diseases, the Japan College of Rheumatology developed this proposal. All patients with rheumatic diseases commencing immunosuppressive therapy should be screened for hepatitis B surface antigen (HBsAg); those who are negative for HBsAg should be screened for hepatitis B core antibody (HBcAb) and hepatitis B surface antibody (HBsAb) as well. HBV carriers and serum HBV DNA positive patients with resolved infection should receive nucleoside analog as soon as possible, prior to commencing immunosuppressive therapy. For serum HBV DNA negative patients with resolved infection, careful monthly monitoring using serum levels of aspartate and alanine aminotransferases and HBV DNA is recommended during and at least 12 months after withdrawal of immunosuppressive therapy. If serum HBV DNA becomes positive, patients should receive nucleoside analog treatment as soon as possible, while ongoing immunosuppressive therapy should be continued to avoid severe or fulminant hepatitis development. To facilitate proper management of patients with HBV infection, collaboration between rheumatologists and hepatologists is strongly encouraged.

Keywords Hepatitis B virus · Reactivation · Rheumatic diseases · Immunosuppressive therapy · Glucocorticoid

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Published online: 16 December 2012

Introduction

Epidemiological data have indicated that about 350 million people worldwide (6 % of the world population) are infected with hepatitis B virus (HBV) and that 200 million of those live in Asian countries [1, 2]. Previous studies estimated that the number of HBV carriers who are positive for hepatitis B surface antigen (HBsAg) in Japan is 1.0–1.5 million and that 23.2 % of the total Japanese population has been previously infected with HBV [3, 4]. Once hepatocytes are infected with HBV, replication-competent covalently closed circular DNA (cccDNA) is formed in the nuclei of the infected hepatocytes during the viral

replication process. The cccDNA serves as the main template for transcription of viral pregenome RNA, as well as messenger RNA (mRNA), and persists permanently in the cells [5]. Hence, HBV carriers and patients with resolved HBV infection [HBsAg negative and hepatitis B core antibody (HBcAb) and/or hepatitis B surface antibody (HBsAb) positive] are equivalent in terms of the presence of replication-competent HBV genome in their hepatocytes. Chemotherapy- or immunosuppressive therapy-associated immunosuppressed status may increase the risk for reactivation of HBV, both in patients who are HBV carriers and in patients with resolved HBV infection. Hepatitis following viral reactivation in patients with resolved HBV infection is called "de novo HBV hepatitis" and often leads a fatal and fulminant course, especially in patients with malignant lymphoma given chemotherapy containing rituximab, i.e., anti-CD20 chimeric antibody, and corticosteroids [6, 7].

Recent advances in treatment for rheumatoid arthritis (RA) have improved outcomes for patients. In Japan, six biological disease-modifying antirheumatic drugs (DMARDs) have been approved for RA since 2003, and the maximum approved dosage of methotrexate for RA was increased to 16 mg/week in February 2011. Together with these changes in medications, goal-oriented early aggressive therapy has been introduced in clinical practice, aiming at remission and maintenance of remission of the disease [8]. Similar therapeutic strategies have also been introduced for other rheumatic diseases, such as systemic lupus erythematosus and systemic vasculitides [9, 10]. As a result, patients with rheumatic diseases receive intensive remission-induction treatment with long-term maintenance therapy using corticosteroids, immunosuppressants, and/or biological DMARDs, which have potential risk for reactivation of HBV and de novo HBV hepatitis. During the past few years, several investigators reported reactivation of HBV in patients with rheumatic diseases given biological DMARDs, especially tumor necrosis factor inhibitors [11–16]. Development of HBV reactivation and fatal fulminant hepatitis was also reported in patients with rheumatoid arthritis given low-dose methotrexate [17–19]. These data strongly suggest that appropriate screening for HBV infection and monitoring for reactivation in HBV-infected patients are mandatory in rheumatology clinical practice (Fig. 1).

In this proposal we summarize epidemiological data on reactivation of HBV in Japan and in patients with rheumatic diseases. Based on the latest evidence and expert opinions, we indicate methods of proper management for HBV-infected patients with rheumatic diseases who will receive immunosuppressive therapy. The diagnosis of HBV infection and prophylaxis of reactivation are in accordance with the "Guidelines for prevention of immunosuppressive therapy or chemotherapy-induced reactivation of hepatitis B virus infection" that were jointly developed by the Intractable Hepatobiliary Disease Study Group of Japan and the Study Group for the Standard Antiviral Therapy for Viral Hepatitis in the Health and Labour Sciences Research [20, 21]. This proposal is subject to changes as advances occur in research in this and related medical fields. This proposal was originally published in Japanese on the website of the Japan College of Rheumatology on September 6, 2011, and revised on October 17, 2011 and September 5, 2012.

Fulminant hepatitis and late-onset hepatic failure in Japan

Since 1998, the Intractable Hepatobiliary Diseases Study Group of Japan has conducted an ongoing nationwide annual survey for fulminant hepatitis and late-onset hepatic failure (LOHF). By 2009, 1,186 patients with these hepatic disorders [19–21], 39 % of which were HBV related, had been accumulated. In Japan, HBV-related acute liver failure is classified into transient infection, acute exacerbation in HBV carrier, and indeterminate infection patterns; de novo HBV hepatitis due to viral reactivation in patients with resolved HBV infection is classified as one of the subgroups of acute exacerbation in HBV carriers [22]. According to this classification, the causes of HBV-related fulminant hepatitis and LOHF in Japan are transient infection (55 %), acute exacerbation in HBV carrier including reactivation in patients with resolved infection (35 %), and indeterminate infection pattern (10 %) [19–21]. The percentage of HBV carriers who developed fulminant hepatitis or LOHF gradually decreased from 1998 to 2004, but increased again in and after 2005 due to the increased number of patients with viral reactivation in resolved HBV infection [23–25]. Of 488 patients who developed fulminant hepatitis or LOHF during 2004 and 2009, 194 (40 %) were HBV related; causes of these infections were transient infection in 91 (47 %), acute exacerbation in HBV carrier including reactivation in patients with resolved infection in 72 (37 %), and indeterminate infection pattern in 31 (16 %). Among the 72 patients classified into acute exacerbation in HBV carrier, the investigators identified 17 patients with reactivation of HBV in patients with resolved infection; these patients had been initially classified as HBV carriers showing acute hepatitis exacerbation. Thirteen of these 17 patients were treated with rituximab-containing regimens, but some received other chemotherapy or immunosuppressive therapy. All of these patients died, pointing to an extremely unfavorable prognosis [21]. Although the "Guidelines for prevention of immunosuppressive therapy or chemotherapy-induced reactivation of hepatitis B virus infection" were first published in 2009 [26], more recent data from the Intractable Hepatobiliary Diseases Study Group of Japan

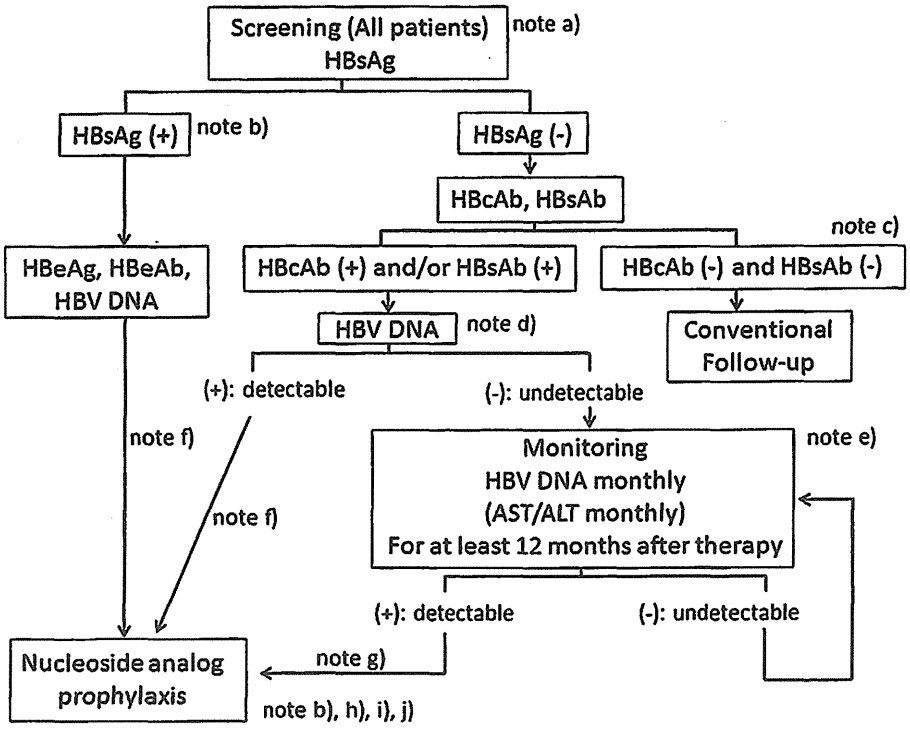

Fig. 1 Algorithm for screening and management of hepatitis B virus infection in patients with rheumatic diseases. All patients with rheumatic diseases who start immunosuppressive therapy should be screened for hepatitis B virus (HBV) infection using this algorithm. HBV carriers or patients with resolved HBV infection should be managed accordingly. Notes: *a* All patients with rheumatic diseases commencing immunosuppressive therapy should be screened for HBsAg. Those who are negative for HBsAg should be screened for HBcAb and HBsAb as well to identify patients with resolved infection. Chemiluminescent immunoassay/chemiluminescent enzyme immunoassay (CLIA/CLEIA) is highly recommended to measure HBsAg, HBcAb, and HBsAb. *b* HBsAg positive patients are subject to consultation with a hepatologist. Consultation with a hepatologist is desirable in all patients subject to administration of nucleoside analog. *c* Detection of serum HBV DNA is desirable in those patients who have previously received immunosuppressive therapy and have no results of HBcAb and HBsAb before the start of the therapy. *d* Detection by real-time polymerase chain reaction (PCR) method (Taq-Man™ PCR method) is recommended. *e* Patients receiving rituximab plus corticosteroid combination therapy for malignant lymphoma or patients receiving hematopoietic stem cell transplantation are at particular risk for HBV reactivation and deserve careful attention. *f* Prophylactic nucleoside analogs should be started as soon as possible before starting immunosuppressive therapy. *g* Nucleoside analogs should be administered immediately when HBV DNA becomes positive during and after immunosuppressive therapy. *h* Entecavir is recommended as the nucleoside analog. HBV DNA is monitored monthly during administration of nucleoside analogs. *i* Criteria for discontinuation of nucleoside analog treatment are described in the text. *j* Patients should be closely observed for 12 months after treatment with nucleoside analogs as described in the text. Nucleoside analog should be readministered immediately when HBV DNA becomes positive during observation. *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *HBcAb* hepatitis B core antibody, *HBsAg* hepatitis B surface antigen, *HBsAb* hepatitis B surface antibody, *HBeAg* hepatitis B envelope antigen, *HBeAb* hepatitis B envelope antibody. Adapted and modified from Oketani et al. [21]

shows that an even larger number of patients developed reactivation from a resolved HBV infection status [24], indicating a necessity for broadened publicity of the guidelines among physicians of related specialties.

Reactivation of HBV in patients with rheumatic diseases

The Health and Labour Sciences Research Group for "Clarification of current status for reactivation of hepatitis B virus associated with immunosuppressants and antineoplastics and establishment of the preventive measures" started a registry in 2009 for HBV-infected patients with solid cancers, hematopoietic malignancies, renal diseases, and rheumatic diseases [27, 28]. Rheumatic disease patients eligible for this study are those who are (1) positive for HBsAg, HBcAb or HBsAb, and (2) treated with corticosteroids (prednisolone equivalent dose ≥0.5 mg/kg body weight/day), immunosuppressive drugs or biological DMARDs approved in Japan, including infliximab, etanercept, adalimumab, tocilizumab, abatacept, and golimumab. As of March 2012, 127 patients from 19 medical institutions were enrolled in this study and were followed up according to the study protocol. An interim analysis of this prospective observation study found 11 of the 127 patients were HBV carriers; the remaining patients had resolved HBV infection. By the end of March 2012, nine patients with resolved

infection became positive for serum HBV DNA, two patients before and seven patients after commencing immunosuppressive therapy. Overall, 7.8 % of the 116 patients with resolved HBV infection had viral reactivation. All of these patients were successfully treated according to the guidelines developed by the Intractable Hepatobiliary Diseases Study Group of Japan [26], and none of them developed hepatitis.

Screening for HBV infection

Patients who should be screened for HBV infection

According to this proposal, all patients with rheumatic diseases who commence immunosuppressive therapy in clinical practice should be screened for HBV infection. At present, immunosuppressive therapy in this proposal includes moderate or high doses of corticosteroids, biological DMARDs, synthetic DMARDs with immunosuppressive potential, (e.g., methotrexate, tacrolimus, leflunomide, and mizoribine), and immunosuppressants (e.g., azathioprine, cyclophosphamide, cyclosporine A, and mycophenolate mofetil). Other immunosuppressants will be added to this list following their approval by the Japanese Ministry of Health, Labour, and Welfare.

Recommended methods for screening

All rheumatic disease patients commencing immunosuppressive therapy should be screened for HBsAg. Those negative for HBsAg should be screened for HBcAb and HBsAb as well. Among various methods currently available for measurement of these HBV-associated antigens and antibodies, chemiluminescent immunoassay/chemiluminescent enzyme immunoassay (CLIA/CLEIA) is highly recommended because of its sensitivity and specificity. An assay system for HBsAg with even higher sensitivity is under development; application of such an assay system for clinical practice should be considered in the future. Rheumatologists are encouraged to consult hepatologists regarding HBV carriers and patients with resolved HBV infection with rheumatic diseases prior to commencing immunosuppressive therapy. Patients positive for HBsAb alone due to previous HBV vaccination are not subject to the following management.

Management of high-risk patients with rheumatic diseases

Management of HBV carriers

Hepatitis B envelope antigen (HBeAg), anti-HBe antibody, and serum HBV DNA should be measured for HBV carriers. The real-time polymerase chain reaction (PCR) method (Taq-Man™ PCR method) is highly recommended for quantification of HBV DNA in sera because of its high sensitivity and specificity. Analyses for genotype of HBV and precore and core promoter gene mutation may also be indicated.

HBV carriers should receive nucleoside analog as soon as possible prior to commencing immunosuppressive therapy and should be concurrently followed up by both rheumatologists and hepatologists. Entecavir hydrate, lamivudine, and adefovir pivoxil are currently approved nucleoside analogs in Japan. We recommend 0.5 mg of entecavir hydrate, once a day at fasting as a first choice because emergence of entecavir hydrate-resistant HBV variants has been reported at a very low rate [29–33]. Nucleoside analog treatment should be continued during and at least 12 months after withdrawal of immunosuppressive therapy with careful monitoring of patients using alanine aminotransferase, HBeAg, HBeAb, and serum HBV DNA [21, 34, 35]. If copy numbers of serum HBV DNA do not significantly decrease with nucleoside analog treatment, resistance to the drug is suspected and consultation with hepatologists is needed.

Discontinuation of nucleoside analog treatment is based on the status of serum viral markers: negative for HBeAg and positive for HBeAb, and low levels of HBV DNA, HBV core-related antigen, and HBsAg [36]. Consultation with hepatologists is recommended before discontinuing nucleoside analog treatment and for monitoring patients afterwards. Patients who discontinue nucleoside analog treatment should be strictly followed up for at least 12 months and restarted on the drug if serum HBV DNA levels increase.

Management of patients with resolved HBV infection

Serum HBV DNA should be measured using the Taq-Man™ PCR method for patients with resolved HBV infection. If serum HBV DNA of a patient is positive (i.e., detectable with agarose gel electrophoresis or equal to or more than 2.1 log copies/ml), the patient should receive nucleoside analog treatment as soon as possible before commencing immunosuppressive therapy, as described for HBV carriers. Duration, monitoring, and discontinuation of nucleoside analog treatment for these patients are the same as those for HBV carriers. It should be mentioned that reactivation of HBV cannot be predicted by HBsAb titers at baseline or changes over time [21].

If serum HBV DNA levels in a patient are <2.1 log copies/ml and undetectable with agarose gel electrophoresis, careful monthly monitoring of patients using serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and HBV DNA is recommended during and for at least 12 months after withdrawal of immunosuppressive therapy. The median lag period

between elevation of serum HBV DNA and alanine aminotransferase levels was 18.5 weeks (range 12–28 weeks) [37]; starting nucleoside analog after the onset of hepatitis could not prevent progression to fatal hepatitis [21]. Therefore, if serum HBV DNA of a patient becomes positive, the patient should receive nucleoside analog treatment as soon as possible, as described above. Duration, monitoring, and discontinuation of nucleoside analog treatment for these patients are the same as those for HBV carriers. Ongoing immunosuppressive therapy should be continued to avoid restoration of host immunity against HBV, which may result in an immunological attack on infected hepatocytes and cause hepatitis.

Differential diagnosis for patients with abnormal hepatic function test

If a patient with a rheumatic disease shows abnormal results of hepatic function tests during or after immunosuppressive treatment, major differential diagnoses include, in addition to reactivation of HBV, drug-induced liver disease, hepatic involvement of rheumatic diseases, alcoholic or nonalcoholic fatty liver disease, autoimmune liver diseases (e.g., autoimmune hepatitis and primary biliary cirrhosis), diseases of the bile duct and pancreas, acute hepatitis due to hepatitis A, B, C or E virus, acute hepatitis due to other viruses (e.g., *Epstein–Barr virus, cytomegalovirus, herpes virus, adenovirus, coxsackie virus, rubeola virus, rubella virus, human immunodeficiency virus*, and *parvovirus*), abnormal thyroid function, and other hepatic diseases, including malignancy.

Points to consider for patients with rheumatic disease developing HBV reactivation or de novo hepatitis

Reactivation of HBV or de novo HBV hepatitis in rheumatic disease patients without previous screening and monitoring for HBV

If reactivation of HBV or de novo HBV hepatitis develops in a patient with rheumatic disease who had not been screened or appropriately monitored for HBV infection, the patient should receive nucleoside analog as soon as possible and hepatologists should be consulted.

Discontinuation and reintroduction of immunosuppressive therapy after reactivation of HBV or de novo HBV hepatitis

Discontinuation of immunosuppressive therapy in rheumatic disease patients with HBV reactivation or de novo HBV hepatitis should be carefully discussed with hepatologists because abrupt withdrawal of the therapy may induce severe or fulminant hepatitis. Based on currently available evidence and expert opinions, we recommend continuation of immunosuppressive therapy together with nucleoside analog treatment. Prospective observational studies are being implemented to address this issue in Japan [28]. For a patient who has successfully discontinued immunosuppressive therapy, benefit–risk balance should be carefully assessed before restarting immunosuppressive therapy for rheumatic diseases.

Collaboration with board-certified hepatologists

In-house and regional collaborations between rheumatologists and hepatologists are encouraged and required to facilitate prompt and proper management of HBV carriers and rheumatic disease patients with resolved HBV infection. Lists of board-certified rheumatologists and board-certified hepatologists are available on the websites of the Japan College of Rheumatology (http://pro.ryumachi-net.com/index.php?option=com_content&view=article&id=49&Itemid=57) and the Japan Society of Hepatology (http://www.jsh.or.jp/specialist/list.html).

Summary

Reactivation of HBV and subsequent de novo HBV hepatitis are preventable serious adverse events associated with immunosuppressive therapy for patients with rheumatic diseases. Before starting immunosuppressive therapy, it is highly recommended that all patients be thoroughly screened for current and resolved HBV infection according to the procedures described in this proposal. HBV carriers and patients with resolved HBV infection who are positive for serum HBV DNA should be treated with nucleoside analog prior to commencing immunosuppressive therapy. Close monitoring for reactivation of HBV is necessary for prompt intervention with nucleoside analog to mitigate subsequent hepatitis. Collaboration with hepatologists is encouraged and required to facilitate these management processes for patients with rheumatic diseases infected with HBV.

Acknowledgments This work was supported by a Health and Labour Sciences Research grant (H23-meneki-sitei-016 to M. Harigai and N. Miyasaka), a grant-in-aid for scientific research from the Japan Society for the Promotion of Science (no. 23590171 to M. Harigai and N. Miyasaka), and a grant from the Japanese Ministry of Education, Global Center of Excellence (GCOE) Program, International Research Center for Molecular Science in Tooth and Bone Diseases (to N. Miyasaka). This work was supported by a Health and Labour

Sciences Research grant, Research on Hepatitis and BSE (H-23-kanen-ippan-002 and H24-kanen-ippan-004 to S. Mochida), and Research on Measures for Intractable Diseases (H-23-nanchi-ippann-025 to S. Mochida) from the Ministry of Health, Labour, and Welfare of Japan.

Conflict of interest M.H. has received research grants, speaking fees or honoraria from Abbott, Astellas Pharma Inc., Bristol Myers Squibb, Chugai Pharmaceutical, Eisai Pharmaceutical, Janssen Pharmaceutical, Mitsubishi Tanabe Pharma Corporation, Santen Pharmaceutical, Takeda Pharmaceutical, UCB Japan, and Pfizer and received consultant fees from Abbott, Bristol Myers Squibb, Chugai Pharmaceutical, and Janssen Pharmaceutical. S.M. has received research grants, speaking fees or honoraria from Ajinomoto Pharmaceuticals Co., Ltd., Astellas Pharma Inc., Bayer Yakuhin Ltd., Bristol Myers Squibb, Chugai Pharmaceutical, Dainippon Sumitomo Pharma, Eisai Pharmaceutical, GlaxoSmithKline K.K., Mitsubishi Tanabe Pharma Corporation, MSD K.K., Otsuka Pharmaceutical Co., Ltd., and Toray Medical Co., Ltd. T.M. has received research grants from Abbott, Bristol Myers Squibb, Chugai Pharmaceutical, Eisai Pharmaceutical, Mitsubishi Tanabe Pharma Corporation, Takeda Pharmaceutical, Astellas Pharmaceutical, and Pfizer and received lecture fees from Chugai Pharmaceutical, Mitsubishi Tanabe Pharma Corporation, and Takeda Pharmaceutical. T.K. has received consultancies, speaking fees, and honoraria from Abbott, Astellas Pharma Inc., Bristol Myers Squibb, Chugai Pharmaceutical, Daiichi Sankyo Pharmaceutical, Eisai Pharmaceutical, Mitsubishi Tanabe Pharma Corporation, Santen Pharmaceutical, Takeda Pharmaceutical, Teijin Pharmaceutical, Pfizer, and Otsuka Pharmaceutical. N.M. has received research grants from Abbott, Astellas Pharmaceutical, Banyu Pharmaceutical, Chugai Pharmaceutical, Daiichi Sankyo Pharmaceutical, Eisai Pharmaceutical, Janssen Pharmaceutical, Mitsubishi Tanabe Pharma Corporation, Takeda Pharmaceutical, and Teijin Pharmaceutical.

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Hepatol Int (2013) 7:683–692
DOI 10.1007/s12072-012-9404-1

ORIGINAL ARTICLE

SNPs in the promoter region of the osteopontin gene as a possible host factor for sex difference in hepatocellular carcinoma development in patients with HCV

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Received: 1 May 2012 / Accepted: 18 September 2012 / Published online: 6 October 2012

Abstract
Aims Four single nucleotide polymorphisms (SNPs) exist in the promoter region of the osteopontin (*OPN*) gene, namely, the SNPs at nucleotide (nt) -155, -616, and -1748 showing linkage disequilibrium to each other, and an independent SNP at nt -443. The significance of these SNPs in the risk of hepatocellular carcinoma (HCC) development was examined in patients with hepatitis C virus (HCV).
Methods The SNPs at nt -155 and nt -443 were analyzed in 120 patients with HCC. The promoter activity was measured in HepG2 cells by the dual-luciferase reporter assay. The electrophoretic mobility shift assay was performed using nuclear extracts from the cells.
Results Peripheral platelet counts at the time of HCC detection were greater in women with homozygous deletion at nt -155 and C/C or C/T at nt -443 than in those showing other allelic combinations, while no such difference was observed in men. The promoter activity was greater in oligonucleotides with deletions at nt -155 and C at nt -443 than in those with other haplotypes. The mobility shift assay showed double and single complexes with oligonucleotides around nt -155 and nt -443, respectively. Binding activities were greater in deletion than in G in the case of the retarded complex in the former assay and in T than in C in the latter assay. The other complex in the former assay included SRY, showing an equivalent binding activity to oligonucleotides with both alleles.
Conclusion *OPN* promoter SNPs may play a role in the sexual difference in the risk of HCC development through the regulation of *OPN* expression in patients with HCV.

Keywords Osteopontin · Hepatocellular carcinoma · Sex difference · SNPs · SRY

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Introduction

Osteopontin (*OPN*) is an extracellular matrix protein with an RGD motif, which has been shown to act as a cytokine essential for the initiation of Th1 immune responses in mice [1]. *OPN* is physiologically expressed in the kidney and bone, but not in the liver; however, under disease conditions, *OPN* expression is found in various organs, including the liver. We previously demonstrated *OPN* expression in activated Kupffer cells and stellate cells, and showed that *OPN* contributed to the migration of macrophages into the necrotic areas in injured rat liver [2, 3]. *OPN* expression has been reported in various cancers, including cancer of the ovary [4], prostate [5], pancreas [6], stomach [7], colon [8], and liver [9–11]. The protein has been shown to play an important role in cancer growth, invasion and metastasis, and angiogenesis, as well as in inhibiting apoptosis [12, 13]. Similar observations have been reported in the case of hepatocellular carcinoma (HCC); *OPN* contributes to the progression and metastasis of HCC, and its expression may be a useful marker to predict the prognosis of patients with HCC [9, 14, 15].

In a previous study, we detected four SNPs in the promoter region of the *OPN* gene; SNPs at nt -155, nt -616, and nt -1,748, which showed linkage disequilibrium to each other, and an independent SNP at nt -443. We also demonstrated that SNP at nt -443 is a marker of the activity of hepatitis in patients with hepatitis C virus (HCV) infection [16]. On the other hand, the SNPs at nt -155 and nt -443 have been reported to be associated with susceptibility to oral carcinoma [17], systemic lupus erythematosus [18], pseudoxanthoma elasticum [19], and glioma [20]. Moreover, these SNPs have also been shown to regulate the promoter activity of *OPN* [21–23] and the expression of *OPN* in tumor tissues [17].

Sexual differences have been reported to exist in the risk of development and rate of progression of liver diseases, including HCC [24]. It has been reported that among patients with persistent HCV infection, liver fibrosis progresses more rapidly in men than in women [25–28]. Thus, men with HCV infection generally tend to develop HCC earlier in life than women with the infection [24, 29]. Men predominance among patients with HCC is observed in both high- and low-risk areas, and regardless of ethnic and geographic diversities [30, 31]. In a retrospective study conducted in cirrhotic patients with HCV infection in Japan, men were found to be at a higher risk of development of HCC than women, even after adjustments for age, presence of antibodies against hepatitis B virus, and alcohol consumption [32]. Moreover, women patients with HCC have been reported to show a better survival rate than men patients [29, 33–35], probably due to the differences in the pathological features of HCC between men and women; encapsulation is more frequent in women than in men, while in contrast, vessel invasion is more frequent in men [33]. These sexual differences may also explain the lower recurrence rate of HCC in women patients after surgical resection [34]. Thus, we considered that the genetic background responsible for such sexual differences in the characteristics of HCC needs to be elucidated.

As genetic factors potentially associated with sexual differences in the features of HCC, we paid attention to SNPs in the promoter region of *OPN*, since our preliminary computer search revealed that an oligonucleotide around nt -155 had a nucleotide sequence motif that could bind to the sex-determining region Y gene product (SRY), a transcriptional factor encoded on the Y-chromosome [36]. Thus, in the present paper, we analyzed the SNPs in the promoter region of *OPN* in HCC patients with HCV infection, and evaluated the significance of the SNPs in the regulation of *OPN* expression in relation to sexual differences in the features of HCC, through the in vitro promoter assay and electrophoretic mobility shift assay.

Patients and methods

Patients

The subjects were 296 Japanese patients (165 men and 131 women) with persistent HCV infection who underwent medical examination at the outpatient clinic at Saitama Medical University Hospital between March 2005 and May 2007. All the patients were seropositive for HCV-RNA and negative for hepatitis B virus surface antigen. The presence of HCC was evaluated both retrospectively and prospectively in these patients until March 2012 through ultrasound examination, and clinical data at the detection of HCC were obtained from the patients. Ultrasonography had been performed between every 3 and 12 months depending on the possible stage of the chronic liver disease as assessed by blood chemistry examinations, and the diagnosis of HCC was confirmed when the hepatic tumor was detected on ultrasonography and consistent findings were observed on histological examination of biopsy specimens and/or on at least two of the following imaging modalities; ultrasonography with the contrast medium, contrast-enhanced computed tomography (CT), gadolinium-enhanced magnetic resonance imaging (MRI), and hepatic arteriography.

SNPs in the promoter region of *OPN* were measured in all patients. Written informed consent was obtained from all the patients before their enrollment in the study, which was conducted with the approval of the Institutional Review Board of Saitama Medical University.

In patients with HCC, peripheral blood platelet, serum levels of albumin, ALT, AST, alkaline phosphatase, and total bilirubin at the time that they were first diagnosed as HCC, were retrospectively examined.

Analysis of SNPs in the promoter region of OPN

Genomic DNA was extracted from the peripheral blood mononuclear cells. SNPs in the promoter region of *OPN* at nt -155 and nt -443 were determined by the Invader assay. The Invader assay was performed according to a previously described method [37], with minor modifications. Primer probes and the Invader oligonucleotide for each SNP were designed with the Invader® Creator software to have theoretical annealing temperatures of 63 and 77 °C, respectively, as shown in Table 1. The reactions were performed using 384-well Invader assay FRET detection plates (Third Wave Technologies, Inc., Madison, WI, USA), in which Cleavase® XI enzyme, both F (FAM) dye and R (Redmond Red™) dye (Epoch Biosciences, Redmond, WA, USA) FRET cassettes, and reaction buffer were dried on each well. Three microliters of mixtures consisting of the appropriate primary probe, Invader oligonucleotide, and

Table 1 Probes for invader assay of the SNPs in the promoter region of the osteopontin gene

SNPs	Types of probes	Probe sequences
nt -155	Primary probe-1	acggacgcggagCCAAAAACGCACACAC
	Primary probe-2	cgcgccgaggCAAAAACGCACACACAC
	Invader probe	CCACACTTCCCCCTCTGGTTTTGTGGTTAAAACAAAAAAAAAT
nt -443	Primary probe-1	cgcgccgaggAAACTTGCCTCTGTCC
	Primary probe-2	acggacgcggagGAACTTGCCTCTGTCC
	Invader probe	GAAGGCTATTGTTCAAGCCTGCAAGGAGTTCAGAT

The small letters indicate the flap sequences of the primary probes

$MgCl_2$ were added to the wells, followed by the addition of 3 μl of the heat-denatured genomic DNA (≥10 ng/μl), and overlaid with 6 μl of mineral oil (Sigma Chemical Co., St. Louis, MO, USA). The plates were incubated at 63 °C for 4 h in the DNA thermocycler (RTC-200; MJ Research, Watertown, MA, USA), and then kept at 4 °C until the fluorescence measurement. The fluorescence intensities were measured on a Cytofi® 4000 fluorescence plate reader (Applied Biosystems, Foster City, CA, USA) at an excitation wavelength/bandwidth of 485/20 nm and emission wavelength/bandwidth of 530/25 nm for F dye detection, and at corresponding values of 560/20 nm and 620/40 nm for R dye detection.

Plasmid constructions

The fragments of *OPN* from nt -1 to nt -658 were constructed as follows: the genomic DNA of each patient having different haplotypes at nt -155, nt -443, and nt -616 of *OPN* was amplified in a 50 μl solution of Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA, USA) using 5′-CGGAGCTC CACGGTCTGGCTCCTGAAGCAG-3′ as the sense primer, and 5′-CGAGATCTGACAACCAAGCCCTCCCAGAAT T-3′ as the antisense primer. The polymerase chain reaction (PCR) consisted of 30 cycles of 30 s at 95 °C, 30 s at 65 °C, and 1 min at 72 °C, and final extension at 72 °C for 10 min. Then, the PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen, Inc., Valencia, CA, USA). The fragments were cloned into the pCR II vector using the TA cloning kit (Invitrogen, Carlsbad, LA, USA). Then, the cloned fragments were confirmed by vector-specific PCR using M13 primers and by sequencing using the DTCS kit (Beckman Coulter, Miami, FL, USA) and CEQ 2000 DNA Analysis System (Beckman Coulter). The fragments were cut out from the pCR II vector by Sac I and Bgl II, and cloned into the pGL3 vector using the DNA ligation kit (Takara Bio INC, Kyoto, Japan) and *E. coli* DH5α competent cells (Takara). The cloned fragments were further confirmed by vector-specific PCR performed using the Bgl II primer.

Transient transfection and dual-luciferase reporter assay

Transfections were carried out in HepG2 cells (human hepatoblastoma cell line) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. The cells were plated at a density of 2×10^5 cells per well in six-well plates and cultured in Dulbecco's Modified Eagle Medium (DMEM) for 16 h before transfection. One microgram of the respective haplotype plasmids and 0.2 μg of the Renilla luciferase control construct, pRL-TK (Toyo B-Net, Tokyo, Japan), in 100 μl of OPTI-MEM® (Invitrogen) were mixed with 10 μl of Lipofectamine 2000 in 100 μl of OPTI-MEM®. The mixture was incubated for 30 min at room temperature and diluted into 800 μl of OPTI-MEM®. The cells were incubated in the transfection mixture for 5 h and then in DMEM containing 10 % fetal calf serum for 60 h. Then, 1 μM of dexamethasone or vehicle was added to the medium 18 h before harvesting. Lysates of these cells were prepared and the luciferase activities were measured using the dual-luciferase reporter assay system (Promega, Madison, WI, USA) and Fluoroskan Ascent FL (DC Phama Biomedical, Osaka, Japan).

Computer searches for transcription factors

Transcription factors possibly binding to oligonucleotides around nt -155 and nt -443 in the promoter region of *OPN* were evaluated using the transcriptional factor analysis tool, TFSEARCH (version 1.3).

Electrophoretic mobility-shift assay

Four duplex target oligonucleotide probes containing SNPs at nt -155 or nt -443 were constructed, as shown in Table 2. The probes were end-labeled using the Biotin 3′ End DNA Labeling Kit (Pierce Biotechnology Inc., Irvine, CA, USA). The electrophoretic mobility-shift assay (EMSA) was performed with nuclear extract proteins of the HepG2 cells stimulated by IL-6 (Abcam plc, Cambridge, UK) and oligonucleotides, as follows. Labeled oligonucleotide probes (20 fmol) were incubated with 5 μg of the nuclear extract for 20 min at room temperature using LightShift Chemiluminescent EMSA Kit (Pierce Biotechnology). In the specific competition binding assay, unlabeled oligonucleotide containing the same allele was added at a fivefold molar excess. A specific cross-competition binding assay was performed for the alleles at nt -155 and nt -443, respectively, with 6.25-, 12.5-, 25-, 50-, or 100-fold molar

Table 2 Oligonucleotide probes used for the electrophoretic mobility-shift assay

SNPs	Types of probes	Probe sequences
nt -155(del*)	Sense	5′ TGTGCGTTTTTGTTTTTTTTTGTT 3′
	antisense	5′ AAAAAAAACAAAAACGCACACACA 3′
nt -155(G)	Sense	5′ TGTGCGTTTTTGGTTTTTTTTTGTT 3′
	Antisense	5′ AAAAAAAACCAAAAACGCACACACA 3′
nt -443(T)	Sense	5′ AGGCAAGTTTTCTCAACTCCTTGC 3′
	Antisense	5′ GGAGTTCAGAAAACTTGCCTCTGT 3′
nt -443(C)	Sense	5′ AGGCAAGTTCTCTCAACTCCTTGC 3′
	Antisense	5′ GGAGTTCAGAGAACTTGCCTCTGT 3′

*del** deletion mutation

excess, and 3.75-, 7.5-, 15-, 30-, or 60-fold molar excess of the unlabeled oligonucleotide containing another allele. In the super-shift assays, 1 μl of anti-human SRY (H-60) rabbit polyclonal IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or anti-FoxD3 antibody (Santa Cruz Biotechnology) and 5 μg of nuclear extracts were incubated for 30 min at 4 °C, followed by incubation with 20 fmol of labeled oligonucleotide probes with deletion mutation at nt -155 for 20 min at room temperature, using the LightShift Chemiluminescent EMSA Kit. The resultant binding products were separated by 4 % nondenaturing polyacrylamide gel electrophoresis, and the products were transferred onto a nylon membrane. Following UV cross-linking, biotin-labeled oligonucleotide complexes were detected using the Thermo Scientific Chemiluminescent Nucleic Acid Detection Module (Thermo Fisher Scientific, Rockford, IL, USA).

Western blot analysis

HepG2 cells were sonicated in the Laemmli Sample Buffer (BIO RAD Laboratories Inc., Hercules, CA, USA). The extracted proteins were electrophoresed on e-PAGEL® (ATTO Corp., Tokyo, Japan) and transferred onto a polyvinylidene difluoride membrane. After incubation with the 5 % ECL blocking agent (GE Healthcare UK Ltd., Buckinghamshire, UK), the membranes were exposed to anti-human SRY (H-60) rabbit polyclonal IgG (1:40,000) for 1 h at room temperature. Membranes were then washed and exposed to horse radish peroxidase-conjugated anti-IgG as a secondary antibody (1:25,000), and the antigen–antibody complex was visualized using Typhoon 9410 (GE Healthcare UK Ltd.).

Reverse transcription-polymerase chain reaction

RNA was isolated from the HepG2 cells using the Qiagen RNeasy Mini Kit (Qiagen); 1.3 μg of the RNA was reverse transcribed using the Qiagen OneStep reverse transcription-polymerase chain reaction (RT-PCR) kit, in accordance with the manufacturer's protocol (Qiagen). The amplification of SRY long cDNA (612 bp) was achieved using the primers SRY-f ATGCAATCATATGCTTCT GCTATG and SRY-r CAGCTTTGTCCAGTGGC TGTAGCG. For the amplification of the SRY short cDNA (270 bp), the primers SRY-fs CAGTGTGAAACGG GAGAAAACAGT and SRY-rs CTTCCGACGAGGTC GATACTTATA were used. PCR amplifications were achieved by 40 cycles of 30 s at 94 °C, at 55 °C for 30 s, and at 72 °C for 1 min, with a final extension step at 72 °C for 10 min. The resultant samples were electrophoresed on a 3 % agarose gel.

Statistical analysis

Statistical analysis was performed using the unpaired t test or Fisher's exact test. Values of $p < 0.05$ were considered to be statistically significant.

Hardy–Weinberg equilibrium (HWE) tests were performed in the frequencies of SNPs at nt -155 and nt -443 observed in patients, using the standard Chi-square statistic.

Results

Demographic and clinical features of the patients with HCC

Among 120 Japanese patients with HCC, the age (years; mean ± SD) at the diagnosis was significantly higher in women than in men (Table 3). The peripheral blood platelet counts at HCC diagnosis were significantly lower in women than in men, while the serum levels of albumin, total bilirubin, and ALT, AST/ALT ratios, ratios of patients between chronic hepatitis and cirrhosis, Child–Pugh class and HCC stage did not differ between the men and women patients (Table 3).

SNPs in the promoter region of OPN in patients with and without HCC

There were no differences in the frequencies of alleles at either nt -155 or nt -443 in the promoter region of *OPN*

Table 3 Demographic and clinical features of patients with hepatocellular carcinoma classified according to the alleles associated with the SNPs at nt -155 and nt -443 in the promoter region of the osteopontin gene

SNPs	nt -155	Deletion/deletion		Deletion/G or G/G		Total
	nt -443	C/C or C/T	T/T	C/C or C/T	T/T	
	Men:women[a]	29:15	12:8	19:10	18:9	
Men	Age (year)[b]	68.0 ± 6.5	62.6 ± 9.8	64.2 ± 7.1	69.9 ± 6.0	66.7 ± 7.5
	Serum ALT level (IU/l)[b]	61.7 ± 43.6	74.6 ± 36.5	58.1 ± 24.1	54.9 ± 24.9	54.1 ± 29.5
	Serum AST level (IU/l)[b]	68.5 ± 38.1	90.3 ± 57.1	88.1 ± 49.1	60.9 ± 15.5	74.9 ± 41.9
	AST/ALT ratio[b]	1.27 ± 0.451.	1.28 ± 0.800.	1.59 ± 0.72	1.20 ± 0.39	1.33 ± 0.58
	Serum billirubin level (mg/dl)[b]	21 ± 1.16	93 ± 0.42	1.35 ± 1.38	0.89 ± 0.41	1.13 ± 1.01
	Serum albmin level (g/dl)[b]	3.67 ± 0.66	3.73 ± 0.59	3.34 ± 0.82	3.74 ± 0.80	3.61 ± 0.73
	Serum ALP level (IU/l)[b]	345.9 ± 121.3	365.0 ± 154.2	384.6 ± 137.4	337.6 ± 109.9	354.4 ± 125.1
	Platelet count ($\times 10^4/mm^3$)[b]	11.5 ± 4.3	10.8 ± 5.8	11.7 ± 5.8	11.0 ± 3.5	11.3 ± 4.7
	BMI[b]	22.7 ± 3.3	23.3 ± 3.1	22.3 ± 3.0	21.5 ± 2.8	22.9 ± 2.8
	CH:Child-Pugh class (A:B:C)[a]	13:10:2:4	6:2:3:1	7:4:5:3	7:6:3:2	33:22:13:10
	HCC stage (I:II:III:IV)[a]	2:15:9:3	3:6:2:1	4:6:7:2	4:7:6:1	13:34:24:7
Women	Age[b]	73.2 ± 5.8	71.9 ± 3.8	69.8 ± 6.9	65.7 ± 7.7	70.4 ± 6.5*
	Serum ALT level (IU/l)[b]	51.4 ± 25.2	61.6 ± 27.8	58.7 ± 36.4	49.2 ± 12.0	53.6 ± 26.5
	Serum AST level (IU/l)[b]	63.5 ± 30.3	82.9 ± 21.3	75.7 ± 47.7	62.3 ± 17.9	70.3 ± 32.0
	AST/ALT ratio[b]	1.39 ± 0.46	1.48 ± 0.43	1.35 ± 0.32	1.32 ± 0.36	1.39 ± 0.39
	Serum billirubin level (mg/dl)[b]	0.9 ± 0.6	0.9 ± 0.2	1.2 ± 1.1	1.1 ± 0.5	1.0 ± 0.7
	Serum albmin level (g/dl)[b]	3.59 ± 0.59	3.59 ± 0.47	3.67 ± 0.48	3.48 ± 0.91	3.58 ± 0.60
	Serum ALP level (IU/l)[b]	352.4 ± 118.9	401.4 ± 111.0	374.0 ± 65.5	269.5 ± 53.7	356.3 ± 98.7
	Platelet count ($\times 10^4/mm^3$)[b]	11.8 ± 4.6#	7.5 ± 2.3	8.3 ± 3.6	7.9 ± 3.6	9.0 ± 4.0*
	BMI[b]	21.5 ± 3.4	22.4 ± 2.2	22.7 ± 3.0	26.9 ± 3.6	24.8 ± 4.2
	CH: Child-Pugh class (A:B:C)[a]	5:8:2:0	1:4:3:0	0:8:1:1	1:2:6:0	7:22:12:1
	HCC stage (I:II:III:IV)[a]	5:2:5:1	4:3:0:1	4:6:0:0	4:3:2:0	18:15:7:2

CH chronic hepatitis

* $p < 0.05$ versus men

# $p < 0.05$ versus other alleles

[a] Values denote the numbers of patients

[b] Values indicate mean ± SD

between the patients with and without HCC (Table 4). The frequencies of each allele for both SNPs were also equivalent between men and women; similar results were obtained in both those with and without HCC (Tables 3, 4). According to the Chi-square test, each polymorphism frequency was in line with HWE ($p > 0.05$; Table 4).

The demographic and clinical features of the HCC patients were evaluated depending on the alleles of the promoter region of *OPN* (Table 3). Age, BMI, serum albumin, bilirubin and ALT levels, AST/ALT ratio, Child–Pugh class at the detection of HCC, and the HCC stage distribution did not differ among those with different combinations of alleles at nt -155 and nt -443 in either the men or the women patients. However, the peripheral blood platelet counts at the detection of HCC were significantly higher in the women patients with homozygous deletion mutation at nt -155 and allele C/C or C/T at nt -443 than in those with other combinations of both alleles (Table 3). On the other hand, no such differences were observed in the men patients with HCC.

Promoter activity and SNPs in the promoter region of the OPN gene

There were three haplotypes of cDNA fragments from nt -1 to nt -658 of *OPN* in DNA extracted from Japanese patients with persistent HCV infection; alleles at nt -155, nt -443, and nt -616, respectively, were deletion mutation, C and G in haplotype-I, deletion mutation, T and G in haplotype-II, and G, T, and T in haplotype-III. The promoter activities of the fragments following transfection into HepG2 cells were greater in the case of haplotype-I than in that of haplotype-II, irrespective of the addition of dexamethasone to the culture medium, while a minimal promoter activity was observed for haplotype-III (Fig. 1).

Table 4 Frequencies of SNPs at nt -155 and nt -443 in the promoter region of the osteopontin gene

SNP at nt -155	No. of patients	Alleles			HWE test	
		deletion/deletion	Deletion/G	G/G	X^2	p
With HCC	120	64 (53)	44 (37)	12 (10)	0.562	0.755
Men	78	41	26	11	1.862	0.394
Women	42	23	18	1	1.206	0.547
Without HCC	176	98 (56)	66 (38)	12 (7)	0.013	0.994
Men	87	49	32	6	0.078	0.998
Women	89	49	34	6	0.005	0.962
SNP at nt -443	Total	Alleles			HWE test	
		C/C	C/T	T/T	X^2	p
With HCC	120	18 (15)	55 (46)	47 (39)	0.067	0.967
Men	78	12	36	30	0.013	0.993
Women	42	6	19	17	0.020	0.990
Without HCC	176	30 (17)	91 (52)	55 (31)	0.187	0.911
Men	87	15	45	27	0.162	0.922
Women	89	15	46	28	0.138	0.934

Values indicate the numbers of patients and the values within parentheses indicate the frequencies of each allele (%)

HWE Hardy–Weinberg equilibrium

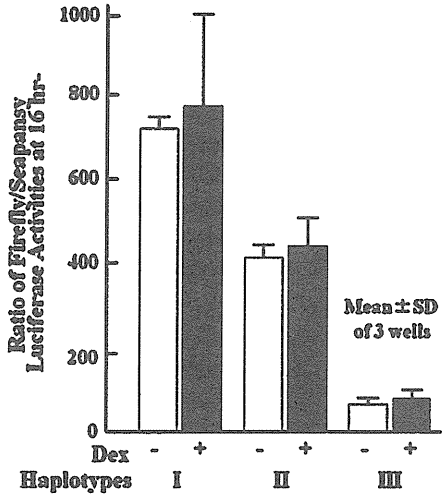

Fig. 1 Dual-luciferase reporter assay for the promoter region of the osteopontin gene in HepG2 cells. Alleles of cDNA fragments at nt -155, -443, and -616 were deletion mutation, C and G in haplotype-I, deletion mutation, T and G in haplotype-II, and G, T, and T in haplotype-III. The experiments were repeatedly done three times, and similar results were obtained in each experiment. The representative results from one of the experiments are shown. Data are expressed as mean ± SD (*error bar*) of three wells. The promoter activity of the haplotype-1 fragment was greater than that of the haplotype-II fragment, both with and without dexamethasone addition, while the haplotype-III fragment showed minimal promoter activity. The results of the assay were confirmed three times

Computer searches for possible transcription factors

According to the computer program, SRY has the ability to bind to the promoter region of *OPN* around nt -155, irrespective of the allele of the SNP. Forkhead box D3 (FoxD3) can also bind to a similar motif, but only when the allele is a deletion mutation. On the other hand, chicken homeobox transcription factor (CdxA) can bind to the promoter region of *OPN* around nt -443 in the presence of allele T, but not in that of allele C.

Electrophoretic mobility-shift assay

The oligonucleotides around nt -155 formed two complex bands in nuclear extracts from HepG2 cells (Fig. 2a). In the cross-competition binding assay, the retarded complex showed a greater binding activity for the oligonucleotides with deletion mutation than for those with allele G (Fig. 2b). However, the other complex showed the same binding activity to oligonucleotides, irrespective of the allele of nt -155 (data not shown) and the band disappeared following the addition of the anti-SRY antibody (Fig. 2c). On the other hand, no effect was observed in the retarded complex band by the addition of anti-FoxD3 antibody (data not shown).