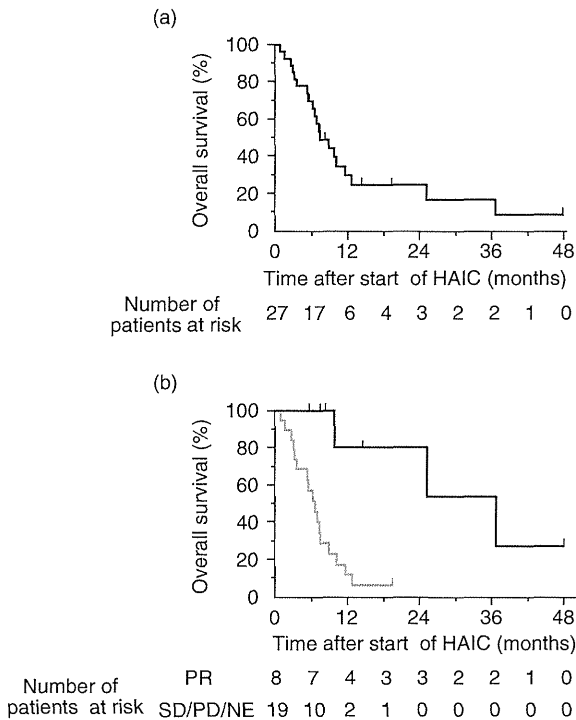

Figure 2 Kaplan–Meier plot of overall survival since commencement of hepatic arterial infusion chemotherapy (HAIC): (a) all patients and (b) according to response to HAIC. The median survival time (MST) of all patients was 7.6 months, and the MST of patients who achieved partial response (PR) were 36.7 months (black line), which was significantly better than that of the patients with stable disease (SD)/progressive disease (PD)/ not evaluable (NE), namely, 6.6 months (gray line) ($P < 0.01$).

predictive markers for the response to HAIC in this study, and further investigation is needed to examine the factors affecting the response rate of HAIC, and to select the appropriate population to receive HAIC after sorafenib therapy.

Another interesting finding of the present study was that half of our patients were categorized as Child–Pugh class B, and no correlation was observed between the response to HAIC and Child–Pugh classification. Although certain molecular targeted agents are currently being tested for sorafenib-refractory patients with HCC, the objectives in most of these trials are restricted to patients with good hepatic function. Other reports have described systemic chemotherapy by combination of gemcitabine and oxaliplatin is potentially safe for patients with Child–Pugh class B[28] and useful in sorafenib-refractory patients with HCC.[29] The results of the present study suggest that HAIC may be also considered as one of treatment procedures for patients with Child–Pugh class B after sorafenib therapy.

The present study has several limitations, including its retrospective nature, the small number of patients, the lack of controls, and single-institution subsets. A prospective trial with a larger number of patients in proper design is needed to confirm our findings.

In conclusion, HAIC has good feasibility and moderate antitumor activity and is a useful treatment option for patients with advanced HCC after failure of sorafenib therapy.

ACKNOWLEDGMENTS

NONE.

CONFLICTS OF INTEREST

NONE TO DECLARE.

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SUPPORTING INFORMATION

ADDITIONAL SUPPORTING INFORMATION may be found in the online version of this article at the publisher's web-site:

Figure S1. Kaplan–Meier plot of overall survival since commencement of hepatic arterial infusion chemotherapy according to progression pattern. Patient prognosis did not differ among intrahepatic growth (IHG) group (black line), new intrahepatic lesion (NIH) group (gray line), and new extrahepatic lesion and/or vascular invasion (NEH) group (dashed line).
Table S1. Predictive marker for response to hepatic arterial infusion chemotherapy.

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Hepatology Research

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Case Report

Sequential occurrence of acute hepatitis B among members of a high school Sumo wrestling club

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A 17-year-old male was admitted to our hospital and diagnosed with acute hepatitis B. Six weeks later, a 15-year-old male was admitted with acute hepatitis B as well. They were Sumo wrestling players in the same club. A detailed survey in the club revealed that a 28-year-old male coach was a hepatitis B surface antigen carrier with high-level viremia. The consistency of hepatitis B virus (HBV) DNA in the infected players was revealed by analyzing the complete HBV genome sequences. Sumo players are more likely to get injured, including cuts and bleeding, compared with players of other sports because of the characteristic wrestling style. Several past reports have suggested that highly viremic HBV carriers have high HBV DNA titers in both their blood and other body fluids such as sweat. In our cases, percutaneous HBV transmission through the bleeding wounds was the most probable infection route. We conclude that a universal HBV immunization program should be introduced urgently in Japan, similar to those implemented in other countries worldwide.

Key words: hepatitis B virus, horizontal transmission, Sumo, universal vaccination

INTRODUCTION

THE HORIZONTAL TRANSMISSION of hepatitis B virus (HBV) occurs in limited situations such as sexual intercourse with HBV positive partners, the transfusion of HBV-contaminated blood, and the re-use of needles and syringes used for the i.v. administration of drugs.[1-3] In addition, there are several reports of horizontal HBV transmission in elementary schools and day-care centers due to bites and scratches or exposure to blood or blood-contaminated fluids among children.[4-7] Although it is rare, HBV horizontal transmission has been reported in various sports as well, including Sumo wrestling and American football, because of contact with open wounds during training.[8,9]

In this paper, we report a sequential occurrence of acute hepatitis B in members of a high school Sumo wrestling club. After a detailed field survey, a 28-year-old male coach was determined to be a hepatitis B surface antigen (HBsAg) carrier with a high-level of viremia. This individual was identified as the source of transmission by analyzing the complete HBV genome sequences.

CASE REPORT

A 17-YEAR-OLD MALE (case 1) was admitted to our hospital with a 1-week history of jaundice and itching. He had no past medical history, except pediatric asthma, and was not taking any medications currently. There were no HBV carriers in his family. He reported no alcohol consumption, recent travel or sexual contact. He was a member of a high school Sumo wrestling club. On examination, the patient was slightly icteric with stable vital signs. Blood test results (Table 1) revealed the following: total bilirubin (T-Bil), 3.9 mg/dL; aspartate aminotransferase (AST), 1152 IU/L; alanine aminotransferase (ALT), 2856 IU/L; HBsAg, 12 229.87 IU/mL; hepatitis B e-antigen (HBeAg), 473.29 S/CO; anti-hepatitis B core (anti-HBc), 4.0 S/CO; immunoglobulin

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Table 1 Laboratory findings at the initial visit

	Case 1	Case 2	Case 3	Reference range
White blood cells ($10^3/\mu L$)	5.8	4.0	4.2	3.9–9.8
Red blood cells ($10^6/\mu L$)	5.38	5.18	5.11	4.10–5.30
Hemoglobin (g/dL)	15.8	14.7	16.4	13.5–17.6
Platelet ($10^3/\mu L$)	293	232	198	131–362
Prothrombin time (%)	100.6	89.7	106.0	72.0–130.0
APTT (s)	38.7	36.6	38.7	24.8–40.4
Total bilirubin (mg/dL)	3.9	3.2	0.9	0.3–1.2
Direct bilirubin (mg/dL)	2.5	2.0	0.1	0.0–0.2
AST (IU/L)	1152	1567	42	13–33
ALT (IU/L)	2856	2526	76	8–42
ALP (IU/L)	729	1167	237	115–359
γ-GT (IU/L)	176	170	46	10–47
Albumin (g/dL)	4.9	4.1	4.9	4.0–5.0
Immunoglobulin G (mg/dL)	1600	1730	1080	870–1700
Immunoglobulin A (mg/dL)	220	297	281	110–410
Immunoglobulin M (mg/dL)	120	107	114	33–190
HBsAg (IU/mL)	12 229.87	12 381.98	62 276.59	<0.05
HBeAg (S/CO)	473.29	553.41	1394.20	<1.00
Anti-HBe (INH%)	0.0	0.0	0.0	<50
Anti-HBc (S/CO)	4.00	7.88	7.34	<1.00
IgM anti-HBc (S/CO)	24.10	19.30	0.78	<1.00
HBV DNA (log copies/mL)	6.1	6.1	>9.1	Non-detectable
HBV genotype	C	C	C	–
Precore mutations	Wild	Wild	Wild	–
Core promoter mutations	Wild	Wild	Wild	–
HCV RNA (log IU/mL)	Non-detectable	Non-detectable	Not tested	Non-detectable
IgM anti-HAV (S/CO)	<0.40	<0.40	Not tested	<0.40
EBV-VCA IgG	0.8	3.3	Not tested	<0.5
EBV-VCA IgM	0.0	0.0	Not tested	<0.5

ALP, alkaline phosphatase; ALT, alanine aminotransferase; anti-HBc, anti-hepatitis B core; anti-HBeAg, hepatitis B e-antibody; APTT, activated partial thromboplastin time; AST, aspartate aminotransferase; EBV-VCA, Epstein–Barr virus viral capsid antigen; HBeAg, hepatitis B e-antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; IgM anti-HAV, immunoglobulin M anti-hepatitis A virus; IgM anti-HBc, immunoglobulin M anti-hepatitis B core; γ-GT, γ-glutamyl transferase.

M anti-hepatitis B core (IgM anti-HBc), 24.1 S/CO; HBV DNA, 6.1 log copies/mL; and HBV genotype, C. On the basis of these results, a diagnosis of acute hepatitis B was confirmed, although the exact route and source of infection could not be identified. The patient recovered naturally and was discharged 12 days after admission. The clinical course was uneventful, and HBsAg clearance was achieved 157 days after admission.

Six weeks after discharge of case 1, a 15-year-old male (case 2) from the same high school Sumo wrestling club was admitted to our hospital with elevated transaminases. He had no past medical history, except atopic dermatitis, and was not taking any medications currently. There were no HBV carriers in his family, except that his father was an inactive HBsAg carrier. He reported no alcohol consumption, recent travel or sexual contact. On examination, the patient was slightly icteric with stable vital signs. Blood test results (Table 1) revealed the following: T-Bil, 3.2 mg/dL; AST, 1567 IU/L; ALT, 2526 IU/L; HBsAg, 12 381.98 IU/mL; HBeAg, 553.41 S/CO; anti-HBc, 7.88 S/CO; IgM anti-HBc, 19.30 S/CO; HBV DNA, 6.1 log copies/mL; and HBV genotype, C. On the basis of these results, this individual was diagnosed with acute hepatitis B as well. However, as in the first case, we could not identify the precise route or source of infection. The patient recovered naturally and was discharged 30 days after admission. The clinical course was uneventful, and HBsAg clearance was achieved 96 days after admission.

Because acute hepatitis B was observed to occur successively in the same high school Sumo wrestling club in a relatively short time period, we suspected the presence

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of an infection route and source within the club. After obtaining informed consent from all the club members and coaches, they were tested for HBsAg and hepatitis B surface antibody (anti-HBs) by the school health service. Consequently, a 28-year-old male coach (case 3) was observed to be HBsAg and HBeAg positive with a high level of viremia. There were no HBV carriers in his family. His blood test results (Table 1) revealed the following: T-Bil, 0.9 mg/dL; AST, 42 IU/L; ALT, 76 IU/L; HBsAg, 62 276.59 IU/mL; HBeAg, 1394.20 S/CO; anti-HBc, 7.34 S/CO; IgM anti-HBc, 0.78 S/CO; HBV DNA, more than 9.1 log copies/mL; and HBV genotype, C. To identify the infection source, we performed an analysis of the complete HBV genome sequences in the two patients with acute hepatitis B as well as in the coach suspected to be the source of HBV transmission. Three isolates obtained from the two patients (cases 1 and 2) and the coach (case 3) had the same genomic length of 3215. Between case 1 and case 3, one base (nt1272) had mutated from T to G, with 99.97% (3214/3215) HBV DNA being consistent. Further, between case 2 and case 3, the HBV DNA sequence was 100% (3215/3215) consistent. Using Basic Local Alignment Search Tool (BLAST) analysis, which is a sequence similarity search program to compare a query to a database of sequences, we found that the sample from case 1 was most genetically similar to samples from cases 2 and 3 among other pooled samples. A phylogenetic tree of full-length HBV, obtained using a neighbor-joining method, revealed that the three isolates in this study (cases 1, 2 and 3) were most closely related to each other, and classified into subgenotype C2 (Fig. 1). On the basis of these results, the coach was determined to be the infection source for the successive occurrence of acute hepatitis B in this Sumo wrestling club.

DISCUSSION

ALTHOUGH SEXUAL INTERCOURSE is known as the major route for the horizontal transmission of HBV, several other routes have been reported in the past as well. Iatrogenic routes, including dental or oral surgery,[10] sharing of needles,[11] fingerstick blood-sampling devices,[12] surgical procedures[13] and acupuncture,[14] have been revealed as possible routes of horizontal HBV transmission. On the other hand, non-iatrogenic routes for horizontal HBV transmission include bites and scratches in children's day-care centers or institutions for the mentally retarded,[4–7] household contact,[15–19] tattooing,[20] sharing knives among butchers,[21] and needle pricks or scissor cuts in barbers.[22]

With regard to the field of sports, Kashiwagi *et al.* reported an acute hepatitis B outbreak in a high school Sumo wrestling club in 1982.[8] They confirmed five cases of acute hepatitis B among 10 club members within a 1-year period. Investigations identified an asymptomatic carrier who was judged to be the source of transmission for the hepatitis infection that occurred during the training sessions. Thereafter, in 2000, Tobe *et al.* reported an outbreak of acute hepatitis B in an American university football team.[9] During a period of 19 months, they confirmed five cases of acute hepatitis B among 65 team members and detected a single HBeAg carrier in the same training group. Consequently, they concluded that the carrier was the source of the hepatitis infection that occurred during the training sessions. They performed HBsAg analysis (subtype adr) and suggested that horizontal HBV transmission can occur in sports, probably because of contact with open wounds during training.

We also experienced successive occurrence of acute hepatitis B in a high school Sumo wrestling club similar to that reported by Kashiwagi *et al.*[8] We initiated an investigation in the club after confirming the diagnosis in the second patient. Sumo players wrestle on hard soil in an almost naked style, except for the Sumo belt, which is referred to as "Mawashi" in Japanese. Therefore, they are more likely to be injured and incur cuts and bleeding compared with athletes in other sports. Several recent reports have suggested that HBV carriers may exhibit high HBV DNA titers in other body fluids such as sweat, saliva, tears, nasopharyngeal fluid and urine.[23–27] In our cases, we could not determine whether the intermediate for HBV was blood or other body fluids. However, during their daily training, the players take turns wrestling with one another and continue even when injured or while bleeding from wounds. The nature of this training and our test results suggested that HBV was transmitted through cuts and bleeding wounds sustained during training. We eventually identified the carrier as the source of transmission by analyzing the complete HBV genome sequences in the infected patients. Several cases of horizontal HBV transmission have been reported previously; however, in the field of sports, this is the first report that confirmed the consistency of HBV DNA in the infected patients. Meanwhile, identification of the exact route of HBV transmission was difficult in the three patients in this outbreak. The mean incubation period for acute hepatitis B is 2–3 months after exposure but can range 1–6 months after exposure.[28] This implies that it is possible for one of the two patients with acute hepatitis B to have

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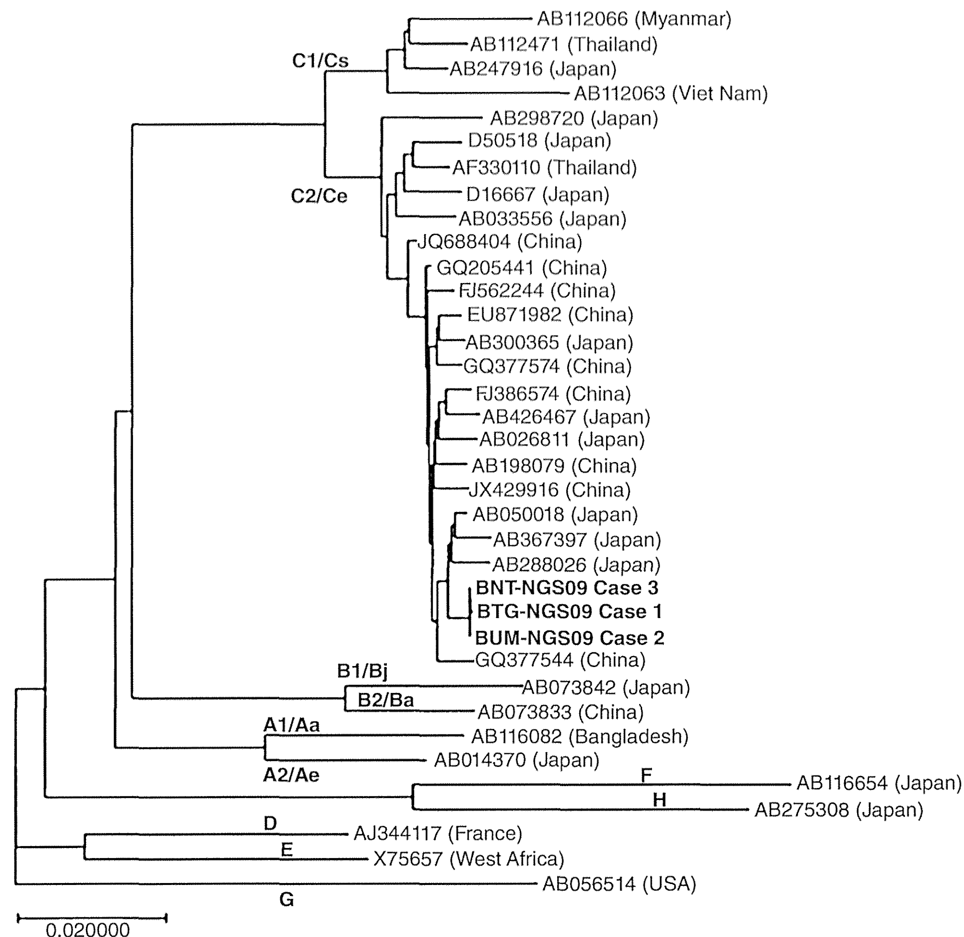

Figure 1 Phylogenetic tree of full-length hepatitis B virus by the neighbor-joining method. Isolates obtained in this study (cases 1, 2 and 3) are shown in bold.

infected the other during the incubation period. In our cases, the coach could have been the origin of transmission and could have infected at least one player, although we could not determine whether or not he infected the other player.

It is remarkable that all three reports of horizontal HBV transmission in the field of sports were from Japan. In 1992, the World Health Organization recommended that all countries should integrate hepatitis B vaccination into their national immunization programs by 1997. By the end of 2009, 177 countries had implemented a universal HBV immunization program for newborns, infants and/or adolescents. However, at the time of drafting of this manuscript (2013), Japan had not introduced this universal HBV immunization program yet. In 1986, Japan initiated a national prevention program comprising selective vaccination for newborns delivered by HBV carrier females. However, this does not aim at preventing horizontal HBV transmission but prevents vertical transmission alone.

Although the number of professional Sumo wrestling players in Japan is very few, the Japanese Ministry of Education revised the guidelines for junior high school education to include compulsory "Budo" (Japanese martial arts) education in 2008. Nowadays, all the students in Japanese junior high schools are taking martial arts classes such as Sumo and Judo. This means that they have a certain risk of exposure to HBV through body fluids or blood during the classes, even though most of them are negative for anti-HBs. In addition, recently

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8 million foreign tourists visit Japan and 18 million Japanese nationals travel abroad each year. This has resulted in the rapid development of Japan's internationalization. Consequently, HBV genotype A infections as a sexually-transmitted disease have increased in urban areas of Japan, and then spread to other areas.[29] Thus, this might have increased the risk of horizontal HBV transmission in Japan, particularly in young individuals without HBs antibodies. Therefore, there is an urgent need to prevent horizontal HBV transmission in Japan, and thus the introduction of a universal HBV immunization program is both needed and desirable.

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ORIGINAL ARTICLE

Retrospective Study

Mutations of pre-core and basal core promoter before and after hepatitis B e antigen seroconversion

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Abstract

AIM: To investigate the role of pre-core and basal core promoter (BCP) mutations before and after hepatitis B e antigen (HBeAg) seroconversion.

METHODS: The proportion of pre-core (G1896A) and basal core promoter (A1762T and G1764A) mutant viruses and serum levels of hepatitis B virus (HBV) DNA, hepatitis B surface antigen (HBsAg), and HB core-related antigen were analyzed in chronic hepatitis B patients before and after HBeAg seroconversion (n = 25), in those who were persistently HBeAg positive (n = 18), and in those who were persistently anti-HBe positive (n = 43). All patients were infected with HBV genotype C and were followed for a median of 9 years.

RESULTS: Although the pre-core mutant became predominant (24% to 65%, P = 0.022) in the HBeAg seroconversion group during follow-up, the proportion of the basal core promoter mutation did not change. Median HBV viral markers were significantly higher in patients without the mutations in an HBeAg positive status (HBV DNA: P = 0.003; HBsAg: P < 0.001; HB core-related antigen: P = 0.001). In contrast, HBV DNA (P = 0.012) and HBsAg (P = 0.041) levels were significantly higher in patients with the pre-core mutation in an anti-HBe positive status.

CONCLUSION: There is an opposite association of the pre-core mutation with viral load before and after HBeAg seroconversion in patients with HBV infection.

Key words: Seroconversion; Hepatitis B core-related antigen; Pre-core; Basal core promoter; Mutation; Hepatitis B surface antigen; Hepatitis B virus DNA

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Core tip: The exact roles of pre-core (pre-C) and basal core promoter (BCP) mutations remain unclear before and after hepatitis B e antigen (HBeAg) seroconversion.

Here, although the pre-C mutant became predominant in the HBeAg seroconversion group during follow-up, the proportion of the BCP mutation did not change. Hepatitis B virus (HBV) viral markers were significantly higher in patients without the mutations in an HBeAg positive status. HBV DNA and hepatitis B surface antigen levels were higher in patients with the pre-C mutation in an anti-HBe positive status. Taken together, the association of the pre-C mutation on viral load appears to be opposite before and after HBeAg seroconversion in patients with HBV infection.

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INTRODUCTION

Hepatitis B virus (HBV) infection is a major health concern that has an estimated 350 to 400 million carriers worldwide. Chronic infection with HBV can cause chronic hepatitis, which may eventually develop into liver cirrhosis and hepatocellular carcinoma[1-4].

In the natural history of chronic HBV infection, seroconversion from hepatitis B e antigen (HBeAg) to its antibody (anti-HBe) is usually accompanied by a decrease in HBV replication and the remission of hepatitis[5-7]. Thus, HBeAg seroconversion is a favorable sign for patients with chronic hepatitis B. However, there are some patients who persistently exhibit elevated HBV DNA levels in the serum and active liver disease, even after seroconversion[8,9].

Several mutations in the HBV genome have been reported to associate with HBeAg seroconversion. When the pre-core (pre-C) and core genes in the HBV genome are transcribed and translated in tandem, HBeAg is produced and secreted into the circulation[10,11]. The G to A mutation at nucleotide (nt) 1896 in the pre-C region (G1896A), which converts codon 28 for tryptophan to a stop codon, is associated with the loss of detectable HBeAg[12,13]. The double mutations of A1762T and G1764A in the basal core promoter (BCP) of the HBV genome have also been shown to reduce HBeAg synthesis by suppressing the transcription of pre-C mRNA[14-16]. However, the detailed mechanisms of HBeAg seroconversion, including the involvement of mutations that decrease the production of HBeAg, have not been fully clarified. Orito *et al*[17] reported that a predominance of the pre-C mutation was correlated with anti-HBe, while BCP mutations were not associated with either anti-HBe or HBeAg. We previously uncovered that the pre-C and BCP mutations were frequently seen in patients with active replication after HBeAg seroconversion, but not in those with inactive replication[18], which suggested that HBeAg seroconversion was not associated with either mutation in such patients. Since the follow-up duration of these previous reports was limited, this study analyzed the changes in pre-C and BCP mutations among patients who were followed over a longer time course. Furthermore, we assessed the mutations not only in patients who seroconverted from HBeAg to anti-HBe, but also in those whose HBeAg or anti-HBe positive status did not change during follow-up.

MATERIALS AND METHODS

Patients

Three groups of patients with chronic hepatitis B who were categorized according to HBeAg/anti-HBe positive status were enrolled between 1985 and 2000. The subjects were selected retrospectively from a database of patients who had been followed for at least two years, had not received anti-viral therapy, such as nucleos(t)ide analogues, and whose stored serum samples were available from both the start and end of follow-up. We recruited only patients with HBV genotype C since this genotype is predominant in Japan and because the clinical significance of pre-C and BCP mutations differs among genotypes. The first group consisted of 18 patients whose HBeAg was persistently positive throughout the study period. The second group contained 25 patients in whom HBeAg seroconverted to anti-HBe. The third group was made up of 43 patients whose anti-HBe was persistently positive.

Hepatitis B surface antigen (HBsAg) was confirmed to be positive on at least two occasions a minimum of 6 mo apart in all patients before the start of follow-up. Tests for hepatitis C and human immunodeficiency virus antibodies were negative in all subjects. Patients who demonstrated accompanying hepatocellular carcinoma or signs of hepatic failure at the initial follow-up were excluded from the study.

Stored serum samples were kept frozen at -20 ℃ or below until assayed. This study was approved by the Ethics Committee of Shinshu University School of Medicine.

Conventional hepatitis B viral markers

Serological markers for HBV, including HBsAg, HBeAg, and anti-HBe, were tested using commercially available enzyme immunoassay kits (Fujirebio Inc., Tokyo, Japan)[19]. HBsAg was quantified[20] using a chemiluminescence enzyme immunoassay (CLEIA)-based HISCL HBsAg assay manufactured by Sysmex Corporation (Kobe, Japan). The assay had a quantitative range of -1.5 to 3.3 log IU/mL. End titer was determined by diluting samples with normal human serum when initial results exceeded the upper limit of the assay range.

Serum HBV DNA was determined using a COBAS TaqMan HBV kit (Roche, Tokyo, Japan)[21] with a quantitative range of 2.1 to 8.9 log copies/mL. According to the manufacturer's instructions, detection of a positive signal below the quantitative range was described as a positive signal and no signal detection was considered to be a negative signal. Six HBV genotypes (A-F) were

Table 1 Clinical and virological backgrounds among 3 groups of patients classified according to changes in hepatitis B e antigen/anti-hepatitis B e

Characteristic	HBeAg/anti-HBe status			*P* value
	Continuously +/- (n = 18)	From +/- to -/+ (n = 25)	Continuously -/+ (n = 43)	
Age (yr)[1]	44 (24-63)	37 (18-53)	51 (25-77)	< 0.001
Gender (M:F)	11:7	14:11	24:19	> 0.2
Follow-up period (yr)[1]	6.3 (2.1-14.6)	10.8 (2.0-23.7)	8.5 (2.2-16.6)	0.006
Genotype C[2]	18 (100)	25 (100)	43 (100)	1
Viral markers at first follow-up				
HBV DNA (log copies/mL)[1]	8.6 (5.7-> 8.9)	6.1 (< 2.1-> 8.9)	< 2.1 (< 2.1-8.2)	< 0.001
HBsAg (log IU/mL)[1]	4.6 (1.6-5.5)	3.6 (-0.9-4.6)	2.6 (< 0.05-4.3)	< 0.001
HBcrAg (log U/mL)[1]	> 6.8 (5.5->6.8)	6.8 (3.1-> 6.8)	3.0 (< 3.0-6.8)	< 0.001
Viral markers at final follow-up				
HBV DNA (log copies/mL)[1]	7.1 (< 2.1-> 8.9)	3.3 (neg.-6.2)	< 2.1 (neg.-7.0)	< 0.001
HBsAg (log IU/mL)[1]	3.3 (1.0-5.1)	2.8 (< 0.05-2.8)	1.3 (< 0.05-4.2)	< 0.001
HBcrAg (log U/mL)[1]	6.7 (4.4-> 6.8)	< 3.0 (< 3.0-6.2)	< 3.0 (< 3.0-5.3)	< 0.001

[1]Data are expressed as the median (range); [2]Data are expressed as a positive number (%). HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; HBcrAg: Hepatitis B core-related antigen.

evaluated according to the restriction patterns of DNA fragments from the method reported by Mizokami *et al*[22]. Serum hepatitis B core-related antigen (HBcrAg) levels were measured using a CLEIA HBcrAg assay kit with a fully automated Lumipulse System analyzer (Fujirebio Inc.) as described previously[23,24]. The HBcrAg assay simultaneously measured all antigens (e, core, and p22cr) encoded by the pre-C/core genes of HBV. The immunoreactivity of pro-HBeAg at 10 fg/mL was defined as 1 U/mL. We expressed HBcrAg in terms of log U/mL with a quantitative range of 3.0 to 6.8 log U/mL.

Determination of pre-C and BCP mutations

The pre-C and BCP mutations were determined using nucleic acid samples extracted from 100 μL of serum with a DNA/RNA extraction kit (Smitest EX-R and D; Genome Science Laboratories Co., Ltd., Tokyo, Japan). The stop codon mutation in the pre-C region (A1896) was detected with an enzyme-linked mini-sequence assay kit (Smitest; Genome Science Laboratories). In principle, G1896 in wild type HBV and A1896 in the mutant were determined by mini-sequence reactions using labeled nucleotides that were complementary to either the wild type or mutant[25]. The results were expressed as percent mutation rates according to the definition by Aritomi *et al*[26] Samples were judged as positive for the pre-C mutation when the mutation rate exceeded 50% in the present study since the mutation rate was found to steadily increase to 100% once surpassing 50%[25].

The double mutation in the BCP was detected using an HBV core promoter detection kit (Smitest; Genome Science Laboratories)[25,26]. This kit detected T1762 and/or A1764 using the polymerase chain reaction (PCR) with primers specific for either wild type or mutant BCP. Results were recorded as wild, mixed, or mutant type. The pre-C and BCP mutations were tested at the start and end of follow-up with kits having manufacturer-established detection limits of 1000 copies/mL.

Full HBV genome sequencing

The nucleotide sequences of full-length HBV genomes were determined by a method reported previously[27]. Briefly, two overlapping fragments of an HBV genome were amplified by PCR, and then eight overlapping HBV DNA fragments were amplified by nested PCR. All necessary precautions to prevent cross-contamination were taken and negative controls were included in each assay. The sequencing reaction was performed according to the manufacturer's instructions (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kits, Version 3.1; Foster City, CA) with an automated ABI DNA sequencer (Model 3100, Applied Biosystems Carlsbad, CA).

Statistical analyses

The proportions of clinical factors were compared among groups using the χ^2 and Fisher's exact probability tests. Group medians were compared by means of the Mann-Whitney *U* test and Kruskal-Wallis test. The changes in proportions of the pre-C and BCP mutations between the study start and end points were compared using McNemar's test. All tests were performed using the IBM SPSS Statistics Desktop for Japan ver. 19.0 (IBM Japan Inc., Tokyo, Japan). *P* values of less than 0.05 were considered to be statistically significant.

RESULTS

Patients

The clinical and virological backgrounds of the 3 groups are summarized in Table 1. Median age was lowest in patients with seroconversion, intermediate in those with persistent HBeAg, and highest in those with persistent anti-HBe. Gender ratio was similar among the 3 groups. Following our study design, all patients had HBV ge-

notype C.

Changes in pre-C and BCP mutations

The presence of the pre-C mutation could be evaluated in 60 (98%) of 61 HBeAg positive samples and 94 (85%) of 111 HBeAg negative samples. We were able to assess the existence of the BCP mutation in 57 (93%) of 61 HBeAg positive samples and 86 (77%) of 111 HBeAg negative samples.

The changes in the proportion of the pre-C mutation between the start and end of follow-up are shown in Figure 1A. Wild type pre-C accounted for 94% of patients whose HBeAg was continuously positive at study onset and remained constant. Wild type pre-C was also predominant at the start of follow-up (76%, 19/25) in patients who experienced HBeAg seroconversion, but the mutant type had become predominant (P = 0.022) by the end of follow-up (65%, 15/23); 11 of 19 wild type pre-C patients converted to mutant type, while 2 of 6 patients with mutant type pre-C reverted to wild type. Mutant type pre-C accounted for 62% of the patients who were continuously positive for anti-HBe at study onset. Such patients with wild type pre-C at the start of follow-up tended to maintain this status (78%), although 22% of initially mutant type pre-C subjects had changed to wild type by the study end point (P = 0.687).

Of the 143 samples with determined BCP mutations, 34 (24%) were wild, 11 (8%) were mixed, and 98 (69%) were mutant types. Because few patients with mixed type BCP reverted to wild type in the present and past studies[18], samples were considered to be positive for the BCP mutation when they were either mixed or mutant type.

The changes in the proportion of the BCP mutation between the start and end of follow-up are shown in Figure 1B. Mutant type BCP accounted for 61% of patients whose HBeAg was continuously positive at study onset and remained constant. In patients who experienced HBeAg seroconversion, mutant type BCP was predominant at the start of follow-up (84%, 21/25) and remained so (80%, 16/20) until final follow-up; 3 of 4 patients with wild type BCP and 15 of 16 patients with mutant type BCP maintained their status throughout the study period. Mutant type BCP initially accounted for 82% of patients who were continuously positive for anti-HBe. Both wild (60%) and mutant (84%) types tended to remain constant until the study end point. When all points of measurement were counted for which both pre-C and BCP mutations were evaluated, the prevalence of the pre-C mutation (18%, 9/57) was significantly lower than that of the BCP mutation (82%, 42/57) in patients with persistent HBeAg (P < 0.001), as well as in subjects with persistent anti-HBe [62% (53/86) *vs* 78% (67/86), P = 0.030], albeit to a lesser degree.

Comparison of viral loads according to pre-C/BCP mutation and HBeAg/anti-HBe positive status

We next compared the serum levels of HBV DNA, HBsAg, and HBcrAg according to pre-C and BCP mutation and HBeAg and anti-HBe positive status (Figure 2). Both pre-C and BCP mutations could be evaluated in 57 (93%) of 61 HBeAg positive samples and 86 (77%) of 111 HBeAg negative samples. HBV DNA levels were significantly higher in an HBeAg positive status than in an anti-HBe positive status (P < 0.001) and significantly higher in patients without the mutations than in those with at least one mutation in an HBeAg positive status (P < 0.01). On the other hand, HBV DNA levels were significantly lower in patients without the pre-C mutation than in those with it in an anti-HBe positive status (P = 0.012).

A similar tendency to HBV DNA levels was observed for HBsAg levels. HBsAg levels were significantly higher in an HBeAg positive status than in an anti-HBe positive status (P < 0.001) and significantly higher in patients without the mutations than in those with at least one mutation in an HBeAg positive status (P < 0.001). HBsAg levels were significantly higher in patients with the pre-C mutation than in those without it irrespectively of the existence of the BCP mutation (P = 0.041).

HBcrAg levels were significantly lower with presence of pre-C and/or BCP mutations in an HBeAg positive status (P < 0.05, respectively). HBcrAg levels were uniformly low regardless of the presence of mutations in anti-HBe positive status subjects.

Full genome sequences in patients with and without appearance of the pre-C mutation

Full HBV genome sequences were determined after HBeAg seroconversion in 6 patients who seroconverted without the appearance of the pre-C mutation. All patients were positive for BCP mutations: 1 subject had T1753G and C1766T mutations, although the other mutations reported by Okamoto *et al*[14] were not identified.

DISCUSSION

Although both pre-C and BCP mutations have been associated with HBeAg seroconversion by reducing the production of HBeAg[13-15], their manifestation patterns appear to be different[17]. In the present study, the BCP mutation was already prevalent during the HBeAg positive chronic hepatitis phase and approached 80% around the time of HBeAg seroconversion. On the other hand, the pre-C mutation clearly manifested following the time of seroconversion. These results indicate that the appearance of the pre-C mutation, but not the BCP mutation, is directly associated with seroconversion. It is noteworthy that a considerable number of patients experienced HBeAg seroconversion without evidence of the pre-C G1896A mutation. Furthermore, wild type pre-C remained unchanged in almost all patients whose anti-HBe was continuously positive. Thus, two types of HBeAg seroconversion may exist for chronic HBV in terms of the appearance or absence of the G1896A pre-C mutation. We previously speculated on the possible

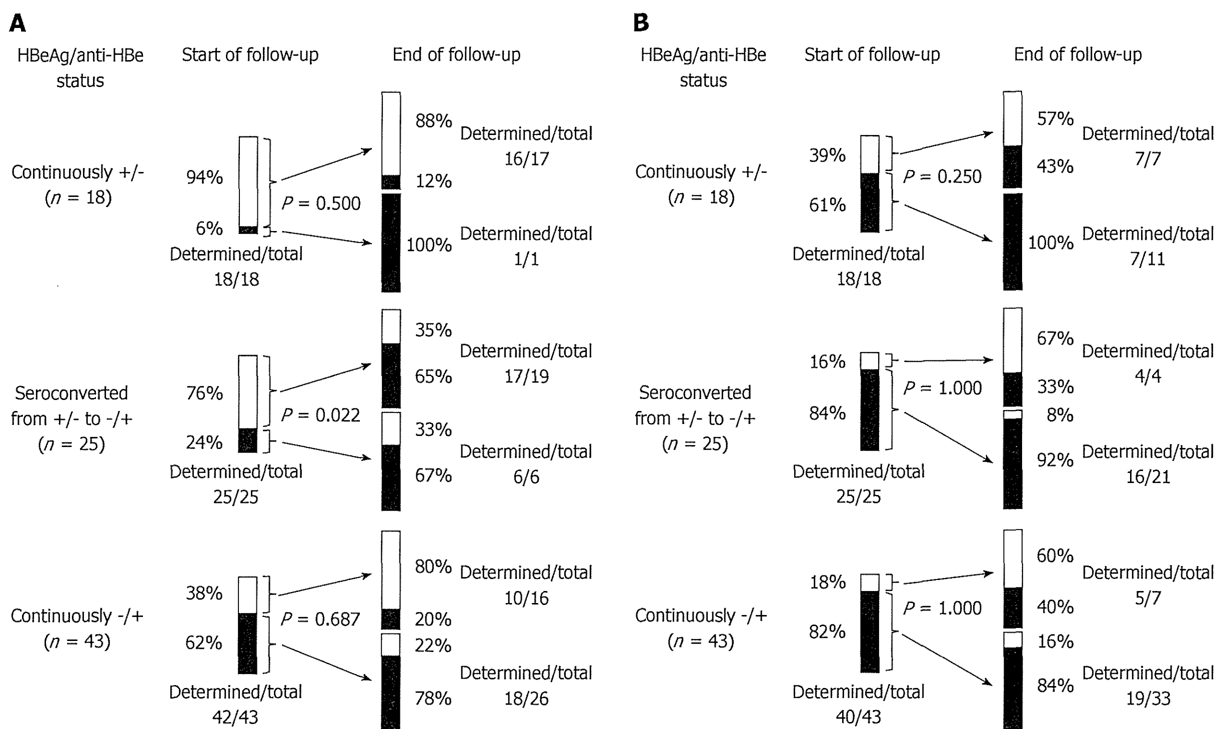

Figure 1 Comparison of changes in pre-core (A) and basal core promoter (B) mutation type among 3 groups of patients classified according to hepatitis B e antigen /anti-hepatitis B e positive status. A: A significant difference was seen in patients with hepatitis B e antigen (HBeAg) seroconversion (P = 0.022). One patient whose pre-core (pre-C) mutation was undetermined at the start of follow-up was wild type at the end point; B: Of the 3 patients whose basal core promoter (BCP) mutation was undetermined at the start of follow-up, 2 were wild type and 1 was undetermined at the end point. HBeAg: Hepatitis B e antigen.

existence of two seroconversion types in an analysis of HBV patients who experienced seroconversion[18]. Here, we were able to strengthen this notion by including patients who maintained an HBeAg or anti-HBe positive status in a study of longer duration. It should be noted that the absence of the pre-C G1896A mutation does not necessarily indicate the absence of mutations that halt HBeAg production; several patterns of mutations apart from G1896A have been associated with an HBeAg negative phenotype, such as point mutations in the ATG initiation region and deletion/insertion of nucleotides leading to premature termination[13]. Accordingly, we analyzed full genome sequences in 6 patients who seroconverted without the appearance of the pre-C mutation and uncovered T1753G and C1766T mutations in one subject[14] that might be associated with seroconversion. We observed that several patients reverted from mutant pre-C to wild type in the present report. As this important finding has not been confirmed by sequence analysis, we are planning to determine and compare entire genomic sequences using paired samples before and after HBeAg seroconversion in a future study.

We witnessed that serum HBV DNA was significantly lower in patients with the pre-C and/or BCP mutation in an HBeAg positive phase, which indicated that immune processes from the host to eliminate HBV were stronger in individuals with the mutations than in those without. This also supported the generally held belief that pre-C and BCP mutations appear as a result of host immune pressure[14]. Contrary to the HBeAg positive phase, HBV DNA was significantly higher in subjects with the pre-C mutation in an anti-HBe positive phase. Kawabe *et al*[28] have reported that patients with wild type pre-C demonstrate significantly lower viral loads and ALT levels than those with mutant pre-C among HBeAg negative patients with HBV genotype C infection. Collectively, these results imply that patients with the pre-C mutant have a higher potential to progress to hepa-titis after HBeAg seroconversion. This is consistent with the fact that HBeAg negative hepatitis is usually caused by HBeAg non-producing mutant strains of HBV. Indeed, viral replication seems to be considerably suppressed in patients with wild type HBV after achieving HBeAg seroconversion since this strain has the ability to produce HBeAg when actively replicated.

We adopted serum levels of HBsAg, HBcrAg, and HBV DNA in the present study as markers to estimate HBV replication activity. HBsAg and HBcrAg levels have been reported to reflect HBV cccDNA levels in hepatocytes[20,24,29]. HBsAg has also attracted attention as a useful predictor of treatment outcome by interferon and others[30]. Furthermore, the loss of HBsAg is an important indicator in the treatment of HBV carriers. HBcrAg assays simultaneously measure all antigens encoded by the pre-C/core genome, which include the HB core, e, and p22cr antigens, and have been reported to predict the clinical outcome of patients treated with nucleotide or nucleoside analogues[31]. HBsAg patterns according

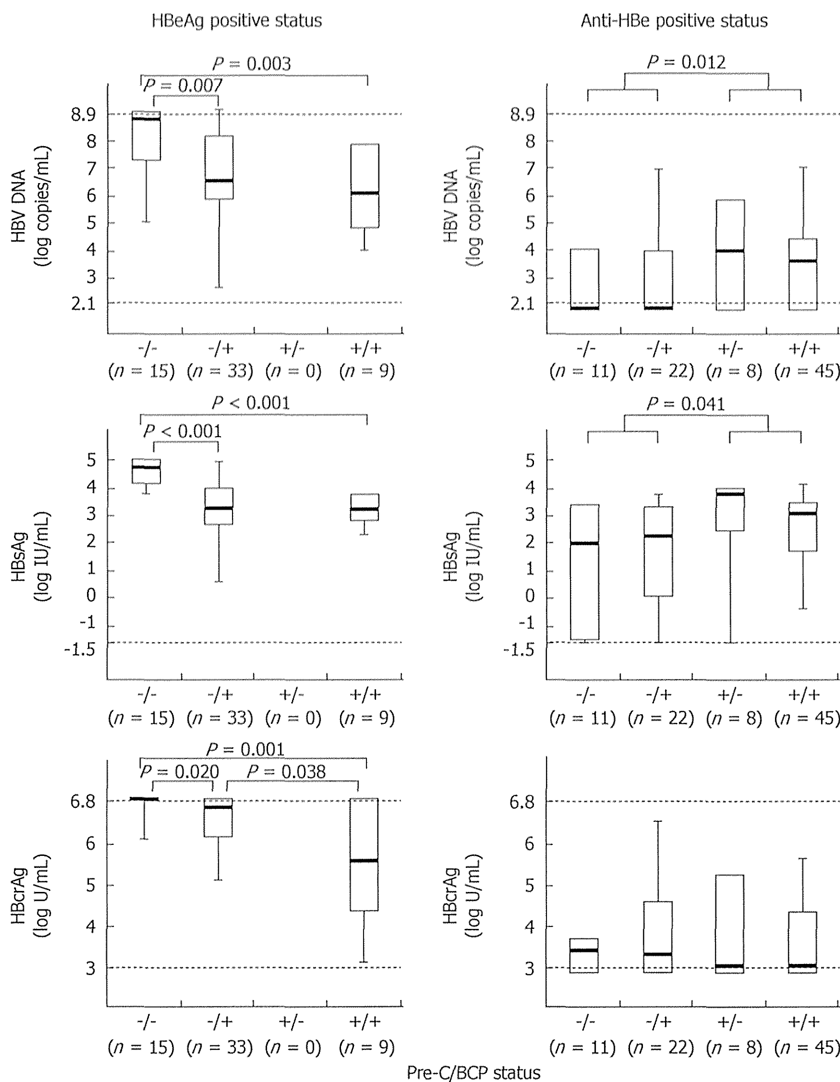

Figure 2 Comparison of serum hepatitis B virus DNA, hepatitis B surface antigen, and hepatitis core-related antigen levels among patients with wild (-/-) and mutant types of the pre-core and basal core promoter mutations. Fifty-seven of 61 samples obtained from HBeAg positive cases and 86 of 111 samples obtained from anti-HBeAg positive cases were eligible for analysis. HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen; HBsAg: Hepatitis B surface antigen; HBcrAg: Hepatitis core-related antigen; pre-C: Pre-core; BCP: Basal core promoter.

to HBeAg/anti-HBe and pre-C/BCP status were similar to HBV DNA patterns both in HBeAg and anti-HBe positive states; HBsAg was significantly lower in patients with pre-C and/or BCP mutations than in those with wild type pre-C but was significantly higher in patients with the pre-C mutation than in those without it in an anti-HBe positive state. These results confirmed that the pre-C mutation was oppositely associated with viral load in patients before and after HBeAg seroconversion. Since elevated levels of HBV DNA and HBsAg are related to a higher rate of hepatocarcinogenesis, pre-C mutation patterns appear to be clinically important, at least in the context of HBV genotype C patients. We witnessed that the patterns of HBcrAg were similar to those of HBV DNA in the HBeAg positive state but different in the anti-HBe positive state. This difference may reflect the fact that the main antigen measured by the HBcrAg assay is HBeAg.

In conclusion, our findings indicate that the association of the pre-C G1896A mutation on viral load is opposite before and after HBeAg seroconversion in patients with HBV infection in that its presence results in a higher viral load after seroconversion. These observations may shed light on the pathology and treatment of chronic hepatitis B, especially that of an anti-HBe positive status.

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COMMENTS

Background

Although pre-core (pre-C) and/or basal core promoter (BCP) mutations in the hepatitis B virus (HBV) genome have been reported to associate with hepatitis

B e antigen (HBeAg) seroconversion, the detailed mechanisms have not been fully clarified.

Research frontiers

In this study, the authors show that the association of the pre-C mutation on viral load is opposite before and after HBeAg seroconversion in patients with HBV infection in that its presence results in a higher viral load after seroconversion.

Innovations and breakthroughs

Recent reports have highlighted the importance of pre-C and BCP mutations of the HBV genome in association with HBeAg seroconversion. This study analyzed the changes in pre-C and BCP mutations in patients over a long follow-up period. The authors demonstrate that the association of the pre-C mutation on viral load is opposite before and after HBeAg seroconversion in patients with HBV infection.

Applications

This study may shed light on the pathology and treatment of chronic hepatitis B, especially that of an anti-HBe positive status.

Terminology

In the natural history of chronic HBV infection, seroconversion from HBeAg to anti-HBe is usually accompanied by a decrease in HBV replication and the remission of hepatitis. Thus, HBeAg seroconversion is a favorable sign for patients with chronic hepatitis B. However, there are some patients who persistently exhibit elevated HBV DNA levels in the serum and active liver disease, even after seroconversion.

Peer review

The authors investigated the pre-C and/or BCP mutations before and after HBeAg seroconversion. They found that the association of the pre-C mutation on viral load is opposite in patients before and after HBeAg seroconversion. It is an interesting report. However there are several concerns.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Association between *Wisteria floribunda* agglutinin-positive Mac-2 binding protein and the fibrosis stage of non-alcoholic fatty liver disease

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Abstract

Background Accurately evaluating liver fibrosis in patients with non-alcoholic fatty liver disease (NAFLD) is important for identifying those who may develop complications. The aims of this study were (1) to measure serum *Wisteria floribunda* agglutinin-positive Mac-2 binding protein (WFA^{+}-M2BP) using the glycan sugar chain-based immunoassay and (2) to compare the results with clinical assessments of fibrosis.

Methods Serum WFA^{+}-M2BP values were retrospectively evaluated in 289 patients with NAFLD who had undergone liver biopsy. Histological findings were evaluated by three blinded, experienced liver-specific pathologists.

Results For stages 0 ($n = 35$), 1 ($n = 113$), 2 ($n = 49$), 3 ($n = 41$), and 4 ($n = 51$) of liver fibrosis, the serum WFA^{+}-M2BP cutoff indexes were 0.57, 0.70, 1.02, 1.57, and 2.96, respectively. Multivariate regression analysis showed that serum WFA^{+}-M2BP values were associated with the stage of fibrosis (≥stage 2). The areas under the receiver operating characteristic curve (AUROC), sensitivity, and specificity of serum WFA^{+}-M2BP were 0.876, 85.9, and 74.6 %, respectively, for severe fibrosis (≥stage 3) and were 0.879, 74.6, and 87.0 %, respectively, for cirrhosis. When compared with six non-invasive conven-

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tional markers, serum WFA$^+$-M2BP had the greatest AUROC for diagnosing severe fibrosis and cirrhosis.
Conclusions Serum WFA$^+$-M2BP values are useful for assessing the stage of liver fibrosis in patients with NAFLD.

Keywords Mac-2 binding protein · Glycoprotein · Non-alcoholic fatty liver disease · Fibrosis marker · Cirrhosis

Introduction

Non-alcoholic fatty liver disease (NAFLD) is one of the most common liver diseases worldwide and is recognized as the hepatic manifestation of metabolic syndrome [1–3]. NAFLD can be classified as simple steatosis or non-alcoholic steatohepatitis (NASH), a progressive form of chronic liver disease (CLD), resulting in cirrhosis, hepatic failure, and hepatocellular carcinoma. Accurately evaluating liver fibrosis in NAFLD patients is important for identifying those who may progress to severe clinical conditions such as liver cirrhosis and hepatocellular carcinoma [4–7]. Liver biopsies are the gold standard for diagnosing NASH and associated liver fibrosis [8]. However, there is controversy surrounding the active use of liver biopsies for these purposes, because they have several drawbacks [9, 10]. A liver biopsy is highly costly and invasive with rare but potentially life-threatening complications [11]. In addition, sampling errors may occur, because a standard liver biopsy sample represents only 1/50,000 of the whole liver [12]. Furthermore, inter- and intra-observer variability also poses serious problems for the pathological diagnosis of NAFLD [13–17]. Accordingly, there is an urgent need for a non-invasive method for estimating the stage of liver fibrosis in NAFLD patients. Several methods using serum markers [18, 19], scoring systems [20–23], and imaging techniques, such as transient elastography [24–26], have been developed. Although each method has been reported as useful, few have been independently validated. Several problems also remain unaddressed, such as the methods' complexities, reproducibilities, and costs for routine clinical use.

Recently, we developed a new glyco-marker for liver fibrosis using the glycan sugar chain-based immunoassay. The FastLec-Hepa system was used to determine the serum values of sweet-doughnut hyperglycosylated *Wisteria floribunda* agglutinin-positive Mac-2 binding protein (WFA$^+$-M2BP) for the assessment of liver fibrosis [27–29]. Toshima et al. [30] and Yamasaki et al. [31] reported that this assay offered a feasible means of assessing liver fibrosis in patients with CLD due to the hepatitis C virus (HCV). However, the progressive patterns of fibrosis may differ for CLD due to HCV and CLD due to NAFLD. Indeed, liver specimens from NAFLD patients show pericellular fibrosis around the central vein in the early stages, with gradual progression to fibrosis when central veins become connected to surrounding lobules. In contrast, central vein involvement in patients with CLD due to HCV is generally preceded by portal tract damage with pathological changes to the portal vein.

We investigated the clinical usefulness of serum WFA$^+$-M2BP values in patients with well-characterized NAFLD. First, we confirmed the efficacy of serum WFA$^+$-M2BP values for assessing the stage of fibrosis. Second, we compared the diagnostic performances of serum WFA$^+$-M2BP and other non-invasive fibrosis markers and tests that are used to estimate the stage of liver fibrosis.

Methods

Patients

We retrospectively reviewed 325 NAFLD patients who underwent liver biopsy at Ehime University Hospital (Ehime, Japan), Ikeda Municipal Hospital (Osaka, Japan), Kawasaki Medical School Hospital (Okayama, Japan), or Sapporo Kosei General Hospital (Hokkaido, Japan). The exclusion criteria were as follows: a history of other liver diseases, including hepatitis B virus or HCV infection; administration of drugs that influence the activity of the disease, such as tamoxifen or a glucocorticoid; or a history of alcohol abuse (defined as $\geq$20 g of alcohol daily). Written informed consent was obtained from all patients who participated. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected by each institutional review committee's a priori approval of this study.

Histological evaluation

Each NAFLD patient received a liver biopsy under laparoscopy or ultrasonography between July 2003 and September 2013. The biopsied liver samples were fixed in formalin and were embedded in paraffin according to the standard procedure at each institution. Slices (4 μm thick) were stained with hematoxylin and eosin (H&E), Azan-Mallory, silver, and Elastica van Gieson at Keio University. Liver samples <15 mm long were excluded, because the detection of liver fibrosis may be affected by sampling errors with such samples. A minimum of six portal tracts in the specimen was required for diagnosis. All liver samples were independently evaluated by three experienced liver-specialized pathologists (M.S., G.Y., and M.K.) who were blinded to the clinical data, and all evaluations were validated through discussion. The liver fibrosis stages were

assessed according to Brunt's criteria [32]. Significant and severe fibrosis was defined as $\geq$stage 2 and $\geq$stage 3, respectively. Thirty-six patients were excluded because of clinical and/or histological reasons; thus, 289 patients were included in the final analysis.

Clinical and biochemical data

Relevant clinical data were recorded, including the patient's age, sex, weight, and height. Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared. Venous blood samples were obtained in the morning after overnight fasting, either immediately before or no more than 2 months after liver biopsy. The blood samples were stored at −80 °C until analysis.

The biochemical variables were measured using a conventional automated analyzer at the respective hospitals. We analyzed the serum levels for the following: platelet count, prothrombin time, bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltranspeptidase, albumin, cholesterol, triglyceride, fasting plasma glucose (FPG), ferritin, and hyaluronic acid. The AST-to-platelet ratio index (APRI) was calculated as follows: [AST (U/L)/UNL $\times$ 100]/platelet count. In this equation, UNL is the upper limit of the normal AST [33]. The FIB-4 index was calculated as follows: age (years) $\times$ AST (U/L)/platelet count ($\times 10^9$/L) $\times$ $\sqrt{\mathrm{ALT}}$ (U/L) [20]. The NAFLD fibrosis score was calculated as follows: $-1.675 + 0.037 \times$ age (years) $+ 0.094 \times$ BMI (kg/m^2) $+ 1.13 \times$ impaired fasting glycemia or diabetes (yes = 1; no = 0) $+ 0.99 \times$ AST/ALT ratio $- 0.013 \times$ platelet ($\times 10^9$/L) $- 0.66 \times$ albumin (g/dL) [21].

Serum *Wisteria floribunda* agglutinin-positive Mac-2 binding protein value

The WFA$^+$-M2BP value in sera was measured by a WFA-antibody immunoassay using a chemiluminescence enzyme immunoassay machine (HISCL-2000i; Sysmex, Kobe, Japan), as previously reported [27, 28, 30, 31]. The measured values of WFA$^+$-M2BP using the conjugated WFA were indexed with the obtained values using the following equation: cutoff index (COI) = ([WFA$^+$-M2BP]$_{\mathrm{sample}}$ − [WFA$^+$-M2BP]$_{\mathrm{NC}}$) ÷ ([WFA$^+$-M2BP]$_{\mathrm{PC}}$) − [WFA$^+$-M2BP]$_{\mathrm{NC}}$). In this equation, [WFA$^+$-M2BP]$_{\mathrm{X}}$ denotes the [WFA + -M2BP] count of the serum sample (x = sample), positive control (x = PC), or negative control (x = NC).

Statistical analysis

Quantitative values are presented as mean ± standard deviation, unless otherwise noted. The Steel–Dwass test was used for multiple comparisons of continuous variables among the different groups. Univariate and multivariate analyses were performed using a logistic regression model. Each cutoff value was determined from the receiver operating characteristic (ROC) curve analyses. The diagnostic performances of the markers were expressed as the diagnostic specificity, sensitivity, positive predictive value, negative predictive value, and area under the ROC (AUROC) curve. p values <0.05 were considered statistically significant. All statistical analyses were performed using JMP, version 11 software (SAS Institute, Tokyo, Japan).

Results

Cross-sectional association between *Wisteria floribunda* agglutinin-positive Mac-2 binding protein values and the fibrosis stage

The patients' characteristics are summarized in Table 1. The mean age of the 289 patients (159 men and 130 women) was 54.8 ± 14.6 years old. Figure 1 shows the serum WFA$^+$-M2BP values for each fibrosis stage. The serum WFA$^+$-M2BP values measured by glycan-based immunoassay ranged from 0.12 to 11.06 (COI). The

Table 1 Patients' clinical characteristics and laboratory data

Features	Total (n = 289)
Male/female	159/130
Age (years)	54.8 ± 14.6
Body mass index (kg/m^2)	27.6 ± 4.7
Platelet count (10^9/l)	18.9 ± 6.8
Prothrombin time (%)	99.3 ± 16.7
Bilirubin (mg/dl)	0.97 ± 0.6
AST (U/l)	61.4 ± 48.9
ALT (U/l)	85.5 ± 68.9
GGT (U/l)	92.3 ± 89.9
Albumin (g/dl)	4.2 ± 0.4
Cholesterol (mg/dl)	195.4 ± 41.1
Triglyceride (mg/dl)	144.4 ± 77.2
FPG (mg/dl)	115.2 ± 38.4
Ferritin (ng/ml)	261.2 ± 258.5
WFA$^+$-M2BP (COI)	1.26 ± 1.44
Fibrosis stage (0/1/2/3/4)	35/113/49/41/51

Values are expressed as mean ± standard deviation

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *COI* cutoff index, *GGT* gamma-glutamyl transpeptidase, *FPG* fasting plasma glucose, WFA$^+$-*M2BP Wisteria floribunda* agglutinin-positive Mac-2 binding protein

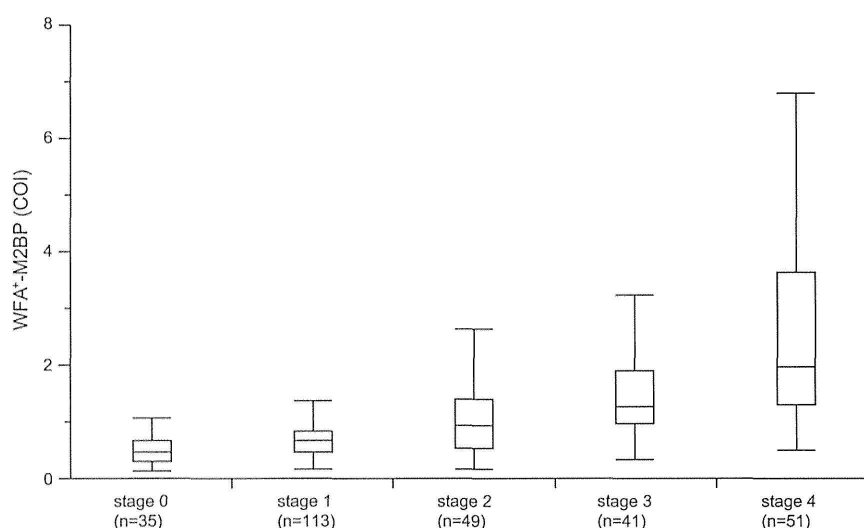

Fig. 1 The serum *Wisteria floribunda* agglutinin-positive Mac-2 binding protein (WFA$^+$-M2BP) values for each fibrosis stage. The *top* and *bottom* of each *box* represent the first and third quartiles, respectively, with the height of the box representing the interquartile range, covering 50 % of the values. The *line* across each *box* represents the median. The *whiskers* show the highest and lowest values. All pairs of groups are significantly different, as assessed using the Steel–Dwass test ($p < 0.01$). *COI* cutoff index

Table 2 Variables associated with the fibrosis stage according to multivariate regression analyses

	Stage 0 vs. stages 1–4		Stages 0–1 vs. stages 2–4		Stages 0–2 vs. stages 3–4		Stages 0–3 vs. stage 4	
	Odds ratio (95 % CI)	*p* value	Odds ratio (95 % CI)	*p* value	Odds ratio (95 % CI)	*p* value	Odds ratio (95 % CI)	*p* value
Age (years)			1.049 (1.014–1.087)	0.006				
BMI (kg/m^2)	1.228 (1.089–1.412)	0.002						
Platelet count (10^9/L)					0.864 (0.787–0.941)	0.001	0.895 (0.814–0.978)	0.017
Prothrombin time (%)	0.948 (0.914–0.982)	0.004	0.957 (0.925–0.986)	0.007			0.963 (0.927–0.993)	0.028
AST (U/l)	1.078 (1.023–1.144)	0.008	1.036 (1.022–1.052)	<0.001				
FPG (mg/dl)			1.013 (1.004–1.024)	0.007	1.014 (1.004–1.024)	0.004	1.012 (1.002–1.022)	0.013
WFA$^+$-M2BP (COI)			5.875 (2.339–16.369)	<0.001	8.471 (3.562–22.725)	<0.001	2.390 (1.463–4.423)	0.002

CI confidence interval, *BMI* body mass index, *AST* aspartate aminotransferase, *FPG* fasting plasma glucose, WFA$^+$-*M2BP Wisteria floribunda* agglutinin-positive Mac-2 binding protein, *COI* cutoff index

WFA$^+$-M2BP value in patients with stages 0 ($n = 35$), 1 ($n = 113$), 2 ($n = 49$), 3 ($n = 41$), and 4 ($n = 51$) of fibrosis had COIs of 0.57, 0.70, 1.02, 1.57, and 2.96, respectively, demonstrating a stepwise increase with an increasing severity of liver fibrosis (Fig. 1). All pairs of groups differed significantly according to the Steel–Dwass test (stage 0 vs. stage 1, $p = 0.012$; stage 0 vs. stage 2, $p < 0.001$; stage 0 vs. stage 3, $p < 0.001$; stage 0 vs. stage 4, $p < 0.001$; stage 1 vs. stage 2, $p = 0.002$; stage 1 vs. stage 3, $p < 0.001$; stage 1 vs. stage 4, $p < 0.001$; stage 2 vs. stage 3, $p = 0.014$; stage 2 vs. stage 4, $p < 0.001$; and stage 3 vs. stage 4, $p = 0.008$).

Comparisons of variables associated with the diagnosis of the fibrosis stage

The variables associated with each stage of liver fibrosis were assessed by univariate and multivariate analyses (Tables S1, 2).

Variables associated with the presence of fibrosis (≥stage 1)

According to univariate analysis, eight variables (age, BMI, platelet count, prothrombin time, AST, ALT,