Table 1. Laboratory Data (August 2008)

WBC	5,580	/μL	HBsAg	(+) >2^{12}	
RBC	405	10^4/μL	Anti-HBs	(-)	
Hb	12.3	g/dL	HBeAg	(+) 1497.3	S/CO
Ht	36.2	%	Anti-HBe	(-) 0.1	%
PLT	23.8	10^4/μL	HBV-DNA	>7.6	LC/mL
T.Bil	0.2	mg/dL	AFP	2.1	ng/mL
D.Bil	0.1	mg/dL			
AST	32	IU/L	RPR	(-)	
ALT	44	IU/L	TPHA	(-)	
ALP	154	IU/L	HIV	(-)	
LDH	135	IU/L	HTLV-1	(-)	
ChE	327	IU/L	C. trachomatis antigen	(-)	
T.P.	6.8	g/dL	gonococcal DNA	(-)	
Alb	3.9	g/dL			
T.Chol	232	mg/dL			
Glucose	137	mg/dL			
BUN	6.4	mg/dL			
Cr	0.55	mg/dL			
Na	136	mEq/L			
K	3.5	mEq/L			
Cl	107	mEq/L			

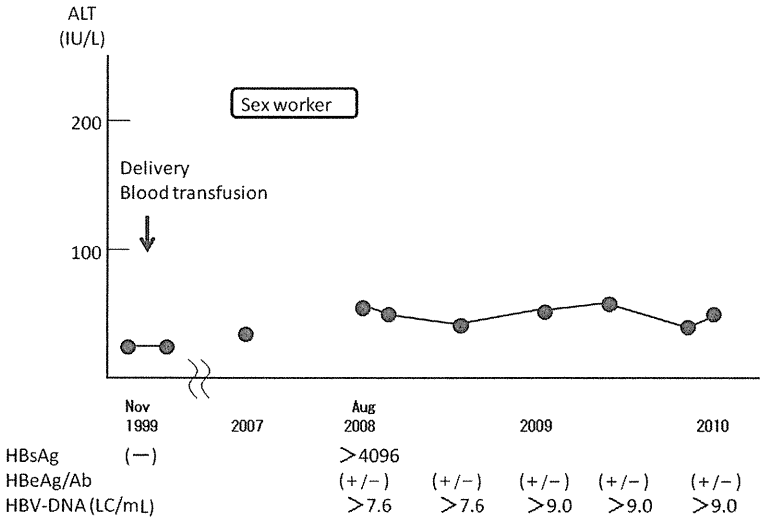

Figure 1. Clinical course.

was positive for hepatitis B virus surface antigen (HBsAg). The laboratory test results are shown in Table 1. HBeAg was positive, anti-HBe was negative, and HBV-DNA, tested by amplicor monitor assay, was >7.6 log copies/mL. She was diagnosed as an HBeAg-positive, asymptomatic HBV carrier (immune-tolerant phase) and was followed up thereafter. Except for mild transient elevation, her ALT level was within normal range. Her HBeAg findings were persistently positive (Fig. 1). She was positive for anti-HBc with a high titer in 2010 (cut-off index; 13.2 by CLIA method). HIV and HTLV-1 were re-examined in 2010, and both were negative. It has been proven in 2010, by careful history taking followed by a search of the patient's records of her pregnancy in 1999, that she had been negative for HBsAg at the time of pregnancy. She had a history of blood transfusion because of bleeding at delivery, although symptoms of acute hepatitis did not emerge after blood transfusion. She has no brothers and sisters. Both of her parents had no history of hepatitis B. Her daughter, who was born in 1999, was examined for HBV markers in 2010. She was negative for HBsAg, anti-HBs, and anti-HBc.

A complete HBV-DNA sequence obtained from the patient's serum in 2010 revealed that genomic length was 3,215 bases, the genotype was C, and deduced HBsAg subtype was *adr* (DDBJ accession number AB640730). The nucleotide sequences at nt 1762, nt 1764, and nt 1896 were A, G, and G, respectively, therefore, core promoter mutation and precore mutation at these positions were not found. Phylogenetic tree including the strain in the present case is shown in Fig. 2.

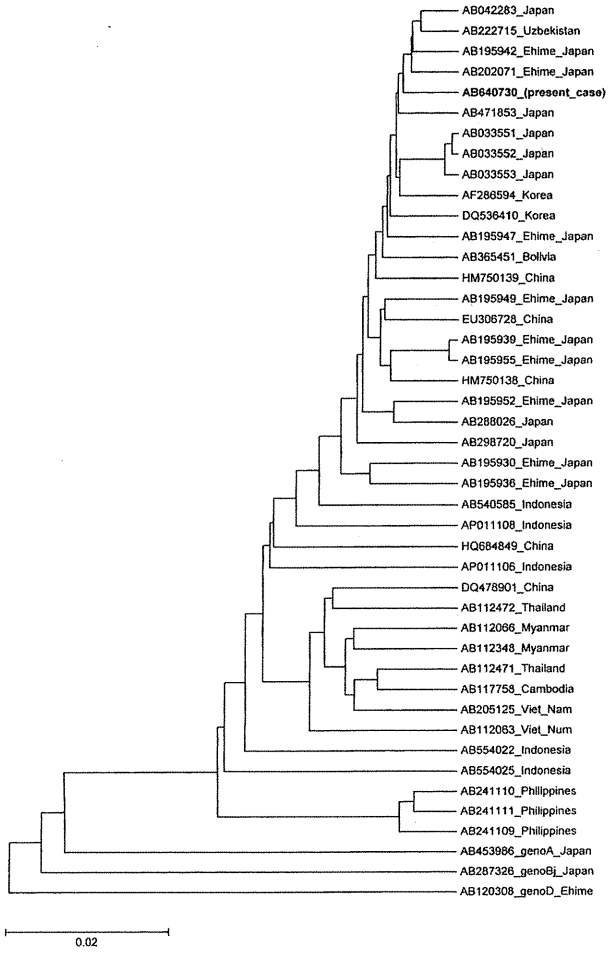

Figure 2. Phylogenetic tree based on complete sequence of HBV. All strains in the figure are genotype C, except 3 strains (AB453986, genotype A; AB287326, genotype B; AB120308, genotype D) in the lower area of the figure. The phylogenetic tree was constructed with UPGMA (unweighted pair-group method using arithmetic average).

Discussion

The patient was negative for HBsAg in 1999, but anti-hepatitis B core antibody (anti-HBc) was not assayed at that time; therefore, we cannot completely deny the possibility that she had been an HBV carrier since childhood. However, her daughter was negative for HBV markers. From these data of the patient and her daughter, it is strongly suspected that the patient was not infected with HBV in 1999, but was infected after delivery (by blood transfusion in 1999 or sexually in 2007-2008), and developed chronic infection. We presumed that hers was an adult case of acute HBV infection that progressed to chronic infection. In 2008, her ALT level was within normal range, with high levels of HBV-DNA and HBeAg; therefore, she was diagnosed as an asymptomatic HBV carrier in the immune-tolerance phase at that time. She was not presumed to be an acutely infected patient; therefore, anti-HBc and IgM-type anti-HBc were not examined in 2008. Subsequently, it was proven that she was negative for HBsAg in 1999. We are of the opinion that history taking in 2008 was inadequate. It is important to keep in mind that there is a possibility that some HBV carriers are infected through horizontal transmission in adulthood and progress to chronic infection status even in Japan, where genotypes B and C are predominant.

It is possible that the infectious route in the present case was the blood transfusion of 1999. However, the screening of HBV markers in donated blood is performed stringently in Japan, so this possibility is unlikely. The most probable infectious route was sexual transmission between 2007 and 2008. It has been reported that only one-third of adults experience symptoms of acute hepatitis at the onset of HBV infection, while the majority have subclinical disease (3). She displayed no clinical features of acute hepatitis between 1999 and 2008, and elevation of ALT was not detected when she consulted our department. There are a few reports stating that chronicity of acute HBV infection relates to mild elevation of ALT or subclinical cases (3, 8). Mild or no elevation of ALT may relate to failure of immune clearance by virus-infected cells, and may result in the transition from acute to chronic infection.

The reason for the transition to chronic state in the present case is obscure. The severity of acute HBV infection and its clinical outcomes have been reported in relation to viral factors, host factors, and factors of the infectious source (9-11), however little is known about the factors relating to mild immune reactions or chronicity of HBV in acutely infected patients. Host factors and viral factors are suspected to affect the progression to chronic infection in acute HBV infected patients. The rate of chronicity in immunocompromised hosts has been known to be high, but the present patient was negative for HIV and HTLV-1, and she was not an immunocompromised host. Genetic factors of host such as human leukocyte antigen (HLA) may affect the chronicity, however they were not analyzed in the present case because informed consent for the examination of genetic DNA could not be obtained from her. Regarding viral factors, the HBV in the present case seems to be no peculiar strain from the point of view of the phylogenetic analysis. Precore mutation at nt position 1986 and core promoter mutations at nt 1862 and nt 1864 are known to be related to severe hepatitis and the HBV in the present case did not have these mutations. The absence of these mutations might be related to chronicity because HBV without these mutations may relate to mild immune response and low levels of elevation of ALT in acutely infected patient (11). Nucleotide sequences of HBV relating to chronicity of acute infection should be further investigated in the future. Regarding HBV genotypes, it has been reported that genotype B is related to severe hepatitis, and genotype A is related to chronicity. It has been reported that chronicity of acute infection by genotype C is very low (6). These studies were conducted based on analysis of the follow-up data of acute hepatitis with clinical features. However, to date there is no follow-up data for subclinical cases such as the present case. The present

case may indicate that the chronicity in subclinical cases of acute genotype C infection might not be very low. Moreover, a high rate of chronicity for genotype A was also obtained from the follow-up data of acute hepatitis with clinical features; therefore, chronicity in subclinical cases of genotype A infection might be higher than that of cases with clinical features. This problem should be clarified in the future.

We suspected sexual transmission of HBV in the present case. In many countries, including Japan, it is known that the majority of adult patients with acute hepatitis B were infected sexually (12). The ratio of genotype A infection among acute hepatitis patients is rapidly increasing in Japan, followed by an increase in HBV genotype A carriers who were suspected to have been horizontally infected as adults (13-15). In this context, it has become a matter of discussion whether universal HBV vaccination should be introduced in Japan, where HB vaccination subjects are restricted to high-risk groups, of which the main recipients are newborns from HBsAg-positive mothers (16). The present case may indicate that a considerable number of patients with chronic genotype C infection who were infected sexually or horizontally as adults might exist, and the presence of such cases should be included in the discussion of universal vaccination programs.

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

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Original Article

Longitudinal assessment of liver stiffness by transient elastography for chronic hepatitis B patients treated with nucleoside analog

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Aim: To evaluate the association between liver stiffness measured by transient elastography (FibroScan) and the efficacy of long-term nucleoside analog (NA) treatment for patients with chronic hepatitis B.

Methods: Study 1: Forty-four chronic HBV patients had liver stiffness measured by FibroScan and underwent liver biopsy. Study 2: Group A: 22 patients started NA treatment at entry and FibroScan was done annually for 3 years. Group B: 23 patients started NA treatment prior to pretreatment FibroScan measurement, and FibroScan was done for from 3 to 5 years after the start of NA treatment.

Results: Study 1: The FibroScan values were significantly correlated with fibrosis stage ($r = 0.672$, $P < 0.0001$). Optimal cutoff of FibroScan values were 6.1 kPa for ≥ F1, 6.3 kPa for ≥ F2, 8.9 kPa for ≥ F3 and 12.0 kPa for F4. Study 2: For Group A, the baseline median FibroScan value was 8.2 kPa. FibroScan values significantly decreased annually for 3 years after the start of NA treatment (6.4 kPa, 5.8 kPa and 5.3 kPa at years 1, 2 and 3, respectively). For Group B, the FibroScan values did not significantly improve over the 3 years after the start of NA treatment.

Conclusions: Liver stiffness, measured by transient elastography, of chronic hepatitis B patients treated with NA showed a rapid decline in the first 3 years followed by a more steady transition for from 3 to 5 years irrespective of long term virological effect.

Key words: breakthrough hepatitis, hepatitis B virus, liver fibrosis, nucleoside analog, transient elastography

INTRODUCTION

CHRONIC HEPATITIS B virus (HBV) infection is a main cause of viral hepatitis. It is estimated that more than 350 million people are infected with HBV worldwide.[1,2] Morbidity and mortality by chronic HBV infection are a major public health concern. Lamivudine (LMV), an oral cytosine nucleoside analog (NA), was introduced for the treatment for HBV infection in 1998.[3,4] LMV can provide suppression of viral replication and biochemical improvement, which reduces the risk of developing serious liver diseases such as cirrhosis and hepatocellular carcinoma (HCC).[5–7]

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Although LMV has been shown to be highly effective in inhibiting HBV replication, the incidence of LMV-resistant virus is high, occurring in approximately 24% of patients after one year of treatment and in as many as 70% of patients after 4 years of treatment.[8] The emergence of LMV-resistance owing to the emergence of genotypic resistance by tyrosine, methionine, aspartate, or aspartate (YMDD) mutation may lead to viral and biochemical breakthrough and sometimes hepatitis flare-ups and rapid decompensation.[9] Adefovir dipivoxil (ADV) has been introduced for the treatment of chronic HBV infection, and ADV as an add-on to LMV has been used to reduce breakthrough.

Liver biopsy was for many years the gold standard for staging fibrosis. However, liver biopsy is no longer considered a perfect methodology because of the invasive nature of the procedure, sampling error, and inter-observer variability,[10] making improved testing

strategies necessary for accurate assessment of the liver status of patients with liver diseases. Recently, transient elastography (FibroScan; Echosens, Paris, France) has been proposed as a reliable, rapid, noninvasive and reproducible method for measuring liver stiffness.[11] FibroScan is increasingly being used as a noninvasive method for assessing liver fibrosis, and there have been many reports to date on patients with various liver diseases, such as chronic hepatitis C, primary biliary disease, non-alcoholic steatohepatitis, and chronic hepatitis B.[12–14] However, there are few reports of an association between the values as measured by FibroScan (FibroScan values) and the effectiveness of treatments for chronic liver diseases. We previously showed that FibroScan values were significantly correlated with the histological stage of percutaneous liver biopsy of patients with chronic hepatitis B and C.[15] Longitudinal assessment by FibroScan among patients with chronic hepatitis C treated with pegylated interferon alpha-2b and ribavirin was also done, with FibroScan shown to be a useful tool for the diagnosis of liver fibrosis and follow-up assessment of antiviral treatment.[16]

The aims of the present study were as follows: (Study 1) to test the correlation between liver histology and liver stiffness measured by FibroScan before NA treatment and (Study 2) to evaluate the association between liver stiffness measured by FibroScan and the efficacy of NA treatment for long-term in chronic HBV patients.

METHODS

Patients

IN STUDY 1, to evaluate the relationship between histological findings and FibroScan values, the liver stiffness of 44 patients with chronic HBV infection was measured by FibroScan and the patients underwent liver biopsy around the same time. The baseline laboratory results of the study population are summarized in Table 1.

In Study 2, 45 patients with chronic HBV infection undergoing long-term NA treatment had annual measurements of liver elasticity by FibroScan. These patients were divided into two groups according to the time of initiation of NA treatment: Since 2005 and between 2001 and 2004 (Groups A and B, respectively). In Group A, 22 patients started NA treatment at entry and FibroScan was done annually for 3 years (17 patients included in Study 1). In Group B, 23 patients started NA treatment prior to our pretreatment FibroScan measurement program, so FibroScan was done from 3 to 5 years after the start of NA treatment. The baseline laboratory results of the study population are summarized in Table 2.

Table 1 Baseline of characteristics of patients with chronic hepatitis B virus (HBV) infection (Study 1)

Characteristics	Liver biopsy $n = 44$
Male (%)	29 (64.4)
Age (years)	47.0 ± 13.9
Alanine aminotransferase (IU/L)	50.4 ± 29.0
Platelet count (10^9/L)	163 ± 53
Prothrombin time (%)	90.6 ± 11.3
α-fetoprotein (ng/mL)	5.5 ± 10.9
Serum type IV collagen (ng/mL)	165 ± 81
HBeAg positive No. (%)	19 (42.2)
Serum HBV DNA level (Log copies/mL)†	5.3 ± 1.5
FibroScan values (kPa)	6.3 (3.3–25.7)
Liver histology	
Stage of fibrosis (F0/F1/F2/F3/F4)	6/18/12/4/4
Grade of activity (A0/A1/A2/A3)	0/19/23/2

†Logarithmic transformed copies/mL.
Data is shown by the mean ± standard deviation, median (range) or *n* (%).
HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; NA, nucleoside analog.

All patients satisfied the following criteria: (1) positive for hepatitis B surface antigen and (2) a history of an increased alanine aminotransferase (ALT) level for over 6 months. Exclusion criteria for the study were: (1) positive for antibody to human immunodeficiency virus or positive for anti-hepatitis C virus; (2) clinical or biochemical evidence of hepatic decompensation; (3) excessive active alcohol consumption (> 60 g/day converted into ethanol) or drug abuse; (4) suspected hepatocellular carcinoma at entry; or (5) treatment with immunosuppressive agents within 12 months prior to enrollment. Patients who fulfilled the above criteria were recruited for treatment at Kyushu University Hospital.

Informed consent was obtained from all patients before enrollment. The study was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki.

Clinical and laboratory assessment

Clinical parameters included ALT, platelet count, α-fetoprotein (AFP), type IV collagen, prothrombin time (PT), HBV genotype and HBV DNA. Body mass index (BMI) was calculated as weight in kilograms/height in square meters. Serum levels of ALT, platelet

Table 2 Baseline (proior to NA treatment) of characteristics of patients with chronic hepatitis B virus (HBV) infection (Study 2)

Characteristics	Group A $n = 22$	Group B $n = 23$	*P*-values
Male (%)	14 (63.6)	16 (69.6)	0.4567
Age (years)	49.8 ± 8.1	50.7 ± 10.3	0.7652
Body mass index (kg/m^2)	22.9 ± 2.5	22.4 ± 2.5	0.1786
Alanine aminotransferase (IU/L)	54.6 ± 30.4	56.1 ± 28.3	0.8613
Platelet count (10^9/L)	137 ± 54	156 ± 51	0.2128
Prothrombin time (%)	88.1 ± 11.2	80.2 ± 9.9	0.0239
α-fetoprotein (ng/mL)	5.0 ± 3.0	5.5 ± 4.0	0.6135
Serum type IV collagen (ng/mL)	172 ± 58	Not evaluated	–
HBeAg positive No. (%)	7 (31.8)	7 (30.4)	0.9202
Serum HBV DNA level (Log copies/mL)†	5.9 ± 1.5	6.2 ± 1.3	0.4448
FibroScan values (kPa)	8.2 (4.2–28.5)	Not evaluated	–
Liver histology			
Stage of fibrosis (F0/F1/F2/F3/F4)	2/5/7/1/2	0/3/5/7/5	0.1051
Grade of activity (A0/A1/A2/A3)	0/3/13/1	0/3/13/4	0.5166
Not determined (*n*)	5	3	

†Logarithmic transformed copies/mL.
Data is shown by the mean ± standard deviation, median (range), or *n* (%).
HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; NA, nucleoside analog.

count, AFP, type IV collagen and PT were measured by standard laboratory techniques at a commercial laboratory (MBC Laboratory, Tokyo, Japan).

HBV genotyping and HBV DNA measurement

Hepatitis B virus genotyping was determined serologically by the polymerase chain reaction (PCR)-invader method with genotype specific probes.[17] Quantification of serum HBV DNA was performed by quantitative PCR assay (Amplicor HBV Monitor, Roche Diagnostics, Mannheim, Germany), over a detection range from 2.6 (corresponding to 400 copies/mL) to 7.5 log copies/mL. Virological breakthrough was defined as the reappearance of serum HBV DNA to a level more than 10-fold (1 log) the minimum during treatment.[18]

Monitoring the emergence of LMV-resistant mutants

The emergence of LMV-resistant mutants is mainly based on point mutation from methionine to valine/isoleucine at rt204 (rt204V/L) in the YMDD motif. YMDD mutation was detected by rapid PCR amplification across the YMDD-encoding gene locus and analysis of the hybridization kinetics of an integrated probe to infer its sequence using the LightCycler (Roche Diagnostics).[19,20]

Nucleoside analog treatment

Of the 45 patients treated with NA, 38 (84.4%) received LMV (Zeffix; Glaxo Smith Kline, UK) in a single oral daily dose of 100 mg and the other seven (15.6%) received entecavir (ETV) (Baraclude; Bristol-Myers Squibb, USA) in a single oral daily dose of 0.5 mg. Breakthrough hepatitis (BTH) by drug-resistant YMDD mutants developed in 13 (28.9%) of these patients. BTH patients received 10 mg ADV (Hepsera; Glaxo Smith Kline) in addition to LMV daily.

Transient elastography (FibroScan)

FibroScan was done for the right lobe of the liver through the intercostal spaces with the patient lying in the dorsal decubitus position with the right arm in maximal position. The tip of the probe transducer was covered with coupling gel and placed on the skin between the ribs at the level of the right lobe of the liver. The operator, assisted by an ultrasonic time-motion image, located a liver portion at least 6 cm thick and free of large vascular structures. Once the measurement area had been located, the operator pressed the probe button to start acquisition. The elasticity was automatically calculated by the apparatus, with the data shown as kilopascal (kPa). All examinations were performed by accomplished operators of our department. Only liver stiffness measurements

Table 3 Optimal cutoff of FibroScan values for the determination of histological fibrosis stage in 44 biopsy-received patients with chronic hepatitis B virus (HBV) infection (Study 1)

	Histological fibrosis stage by liver biopsy			
	F ≥ 1	F ≥ 2	F ≥ 3	F = 4
Cutoff value* (kPa)	6.1	6.3	8.9	12.0
AUROC	0.67	0.86	0.87	0.89
Sensitivity (%)	65.9	95.2	87.5	75.0
Specificity (%)	71.4	74.0	75.0	88.6
Positive predictive value (%)	93.1	74.1	41.2	37.5
Negative predictive value (%)	26.3	95.2	96.8	97.5
Positive likelihood ratio	2.30	3.66	3.50	6.58

*The optimal cutoff value is the one that gives the higher total sensitivity and specificity.
AUROC, area under the receiver operating characteristic curve.

obtained with at least six successful acquisitions and a success rate of at least 60% were considered reliable. The validity of FibroScan values depends on an interquartile range of all successful measurements (IQR/M) of less than 30% of median values.[21] The mean IQR/M of the present study was 22.1% and no cases of IQR/M > 30% were found.

Liver histology and quantification of liver biopsy

Liver biopsy was performed by experienced hepatologists with a 16G disposable needle (Bard Monopty; C.R.Bard, Covington, USA) under ultrasound guidance. The median liver biopsy length was 18 mm (minimum length 15 mm). Liver biopsy specimens were fixed in formalin and paraffin was embedded. All biopsy specimens were analyzed by two experienced pathologists who were blinded to the clinical data. For each specimen, the stage of fibrosis and the grade of activity were established according to the METAVIR score.[22] Fibrosis was staged on a 0–4 scale as follows: F0 = no fibrosis, F1 = portal fibrosis without septa, F2 = portal fibrosis and few septa, F3 = numerous septa without cirrhosis, F4 = cirrhosis. The grading of activity, including the intensity of the necroinflammation, was scored as follows: A0 = no histological activity, A1 = mild activity, A2 = moderate activity, A3 = severe activity.

Breakthrough hepatitis

Biochemical breakthrough usually lags behind virological breakthrough, and serum ALT level may remain normal for weeks to years after the development of antiviral resistance. BTH was defined as a serum ALT level over five times the upper limit of the normal range.

Statistical analysis

Statistical analysis was done with biomedical computer programs (BMDP) statistical software for the IBM 3090 system computer (BMBD Statistical Software, Inc., Los Angeles, CA, USA). Continuous data were expressed as median or mean ± standard deviation (SD). The paired *t*-test, unpaired *t*-test, Mann-Whitney *U*-test or Kruskal–Wallis non-parametric analysis of variance (ANOVA) was used for the analysis. Area under the receiver operating characteristic curve (AUROC) analysis was done to evaluate the relationship between histological findings and FibroScan values. The cutoff values were selected from the receiver operating characteristic curve to maximize the total sensitivity and specificity. A *P*-value less than 0.05 was regarded as statistically significant.

RESULTS

Relationship between liver fibrosis and FibroScan values at baseline

ALL PATIENTS WERE infected with HBV genotype C. The median values (interquartile range) of the patients were 5.0 kPa (3.5–6.0), 5.7 kPa (5.0–6.0), 7.0 kPa (6.2–10.1), 9.4 kPa (9.2–13.3) and 18.0 kPa (12.9–21.9) for F0, F1, F2, F3 and F4, respectively. The FibroScan values were significantly correlated with fibrosis stage ($r = 0.672$, $P < 0.0001$) and were also significantly increased in accordance with the grade of activity of the patients ($r = 0.321$, $P < 0.0001$). According to the progression of liver fibrosis stage, the mean grade of activity was higher (F0: 1.2, F1: 1.4, F2 1.8, F3 2.0 and F4 2.0), but the analysis did not reach significance. Table 3 shows the optimal liver stiffness cutoff values obtained by sensitivity, specificity and positive

likelihood ratios. Four threshold FibroScan values were identified: 6.1 kPa for ≥F1 (sensitivity 65.9%, specificity 71.4%); 6.3 kPa for ≥F2 (sensitivity 95.2%, specificity 74.0%); 8.9 kPa for ≥F3 (sensitivity 87.5%, specificity 75.0%) and 12.0 kPa for F4 (sensitivity 75.0%, specificity 88.6%). The corresponding AUROC were 0.67 for ≥F1, 0.86 for ≥F2, 0.87 for ≥F3 and 0.89 for F = 4.

Longitudinal FibroScan values 1–3 years after the start of the nucleoside analog treatment (Group A, Tables 4,6)

All patients were infected with HBV genotype C. The clinical and FibroScan data of Group A are shown in Table 4. For 19 patients (86.4%) the serum HBV DNA level became undetectable and that of the other three patients was reduced to under 4.0 log copies/mL on PCR by 6 months after the start of NA treatment. ALT level (54.6 ± 30.4 to 22.1 ± 7.7 U/L, $P < 0.0001$), PT (88.1 ± 11.2 to 96.3 ± 9.1%, $P < 0.0001$), AFP (5.0 ± 3.0 to 3.0 ± 1.6 ng/mL, $P = 0.0004$) and serum type IV collagen (172 ± 58 to 142 ± 37 ng/mL, $P = 0.0023$) were significantly improved during the first 3 years of NA treatment. The baseline median FibroScan value was 8.2 kPa and the mean value was 10.5 ± 6.5 kPa in Group A. FibroScan values significantly decreased annually for 3 years after the start of NA treatment (median value 6.4 kPa [mean value 8.7 ± 5.6 kPa], 5.8 kPa [7.4 ± 4.6 kPa] and 5.3 kPa [6.8 ± 4.0 kPa] at years 1, 2 and 3, respectively).

For non-BTH patients ($n = 18$, 81.8%), the baseline median FibroScan value was 7.7 kPa and the mean value was 10.3 ± 7.0 kPa. FibroScan values showed a significant, annual decrease for 3 years after the start of NA treatment (median value 6.3 kPa [mean value 8.4 ± 5.9 kPa], 5.4 kPa [7.2 ± 5.0 kPa] and 5.0 kPa [6.5 ± 4.3 kPa] at years 1, 2 and 3, respectively). For patients with an episode of BTH and viral breakthrough due to YMDD mutation, the serum HBV DNA level became undetectable on PCR by 6 months after the additional medication (ADV added-on LMV). For BTH patients ($n = 4$, 18.2%), the baseline median FibroScan value was 10.2 kPa and the mean value was 11.2 ± 4.3 kPa. FibroScan values improved due to NA treatment for the first 2 years (median value 10.8 kPa [mean value 10.2 ± 3.9 kPa], 7.9 kPa [7.9 ± 2.4 kPa] and 8.2 kPa [8.0 ± 2.2 kPa] at years 1, 2 and 3, respectively). However, three of four patients had BTH occur in the 3 year, therefore, FibroScan values did not improve.

Longitudinal FibroScan values 3–5 years after the start of nucleoside analog treatment (Study 2: Group B, Tables 5,6)

The clinical and FibroScan data of Group B are shown in Table 5. The serum HBV DNA level of 20 patients (87.0%) became undetectable and that of the other three patients was under 4.0 log copies/mL by PCR by 6 months after the start of treatment. For patients with an episode of BTH or viral breakthrough due to YMDD mutation, the serum HBV DNA level became undetectable by PCR by 6 months after the addition of ADV to LMV. Neither liver fibrosis nor the biochemical markers (ALT: 25.2 ± 16.8 to 24.2 ± 13.8 IU/L, PT: 91.1 ± 10.8 to 92.7 ± 8.7%, AFP: 3.1 ± 2.1 to 3.3 ± 3.1 ng/mL and serum type IV collagen: 154 ± 51 to 148 ± 48 ng/mL) of patients with or without BTH statistically improved over the 3 years after the start of NA treatment. At 3, 4 and 5 years after the start of NA treatment, the median (mean) FibroScan values were 6.1 (8.1 ± 5.2) kPa, 6.7 (8.2 ± 5.2) kPa and 5.9 (8.1 ± 5.1) kPa, respectively. For BTH patients ($n = 9$, 39.1%) at 3, 4 and 5 years after the start of NA treatment, the median (mean) FibroScan values were 10.4 (11.0 ± 6.4) kPa, 10.2 (11.1 ± 6.8) kPa and 9.6 (11.1 ± 6.6) kPa, respectively. Similarly, for non-BTH patients ($n = 14$, 60.9%), the median (mean) FibroScan values were 5.2 (6.2 ± 3.3) kPa, 5.2 (6.3 ± 2.8) kPa and 5.3 (6.3 ± 2.7) kPa, respectively. In both the BTH and non-BTH groups, FibroScan values did not significantly improve over the 3 years after the start of NA treatment.

Relationship between hepatocellular carcinoma and breakthrough hepatitis

Three of the patients (13.6%) in Group A and three (13.0%) in Group B developed HCC in the follow-up period: Four (66.7%) had FibroScan values consistently over 10 kPa. Patients no. 4 in Group A and no. 20 in Group B developed HCC after the start of NA treatment, in spite of low FibroScan values. Both of these patients had YMDD mutation detected by PCR and an elevated HBV DNA level before the occurrence of HCC.

In analysis of the relation between HCC and BTH, the incidence of HCC for BTH patients (4 of 13, 30.8%) was significantly higher than that of non-BTH patients (2 of 32, 6.3%) ($P = 0.0332$).

DISCUSSION

THIS STUDY DEMONSTRATED an association between liver stiffness measured by FibroScan and the efficacy of NA treatment of patients with chronic

Table 4 Clinical data and longitudinal FibroScan values in Group A (Study 2)

Patient No.	Age (years)	Sex	BMI (kg/m²)	Histology		FibroScan values (kPa)				ALT (IU/L)				HBeAg	HBV DNA (LogIU/mL)				BTH	HCC
				F-stage	A-grade	FS-0	FS-1	FS-2	FS-3	ALT-0	ALT-1	ALT-2	ALT-3		DNA-0	DNA-1	DNA-2	DNA-3		
1	48	M	22.5	2	2	17.3	14.0	12.9	12.6	53	30	35	29	–	5.5	–	–	4.2	+ (3)	+ (3)
2	56	F	21.1	not tested	not tested	10.4	9.6	7.9	8.6	27	18	16	13	+	6.8	–	–	5.4	+ (3)	–
3	53	M	23.0	2	2	9.9	12.0	7.9	7.7	67	84	20	19	+	7.5	5.9	–	–	+ (1)	–
4	42	M	23.4	not tested	not tested	7.1	5.1	5.0	5.2	44	23	20	21	+	7.7	3.2	–	5.4	+ (3)	+ (3)
5	42	F	21.4	0	2	4.2	4.0	3.8	2.5	25	14	15	16	–	5.5	–	–	–	–	–
6	65	M	26.3	1	1	5.2	4.3	4.0	3.4	35	17	20	34	–	3.4	–	–	3.0	–	–
7	34	M	24.2	1	2	5.4	4.8	3.8	3.9	74	36	45	36	+	7.5	–	–	–	–	–
8	56	F	23.6	0	1	5.5	4.8	4.7	4.0	87	17	15	16	–	7.0	–	–	–	–	–
9	57	M	22.5	not tested	not tested	5.6	4.8	4.0	4.0	43	37	35	32	–	7.3	–	–	–	–	–
10	51	M	28.6	1	2	5.8	6.2	5.6	5.6	55	28	27	21	–	5.4	–	–	–	–	–
11	49	F	22.9	2	3	7.0	5.3	4.6	2.9	59	18	12	12	+	6.2	–	–	–	–	–
12	32	F	29.1	1	2	7.2	5.4	5.2	4.8	65	28	27	27	–	4.8	–	–	–	–	–
13	37	M	22.3	1	1	7.5	6.4	6.1	5.2	99	17	12	18	+	7.7	3.4	–	–	–	–
14	52	M	23.8	3	2	7.9	5.2	3.9	4.8	96	17	19	11	–	4.1	–	–	–	–	–
15	55	M	22.5	not tested	not tested	8.5	6.3	6.0	5.8	47	26	25	35	–	7.3	–	–	–	–	–
16	55	M	20.8	2	2	8.6	7.4	6.2	5.3	42	20	25	27	–	5.0	–	–	–	–	–
17	51	F	21.1	2	2	9.4	7.4	5.9	5.4	36	29	23	24	–	6.1	–	–	–	–	–
18	48	M	23.9	2	2	12.8	7.2	5.2	4.8	45	22	29	22	–	5.5	–	–	–	–	–
19	53	M	21.6	4	2	13.3	11.5	9.6	8.6	13	14	12	12	–	3.7	–	–	–	–	–
20	56	F	22.7	2	2	17.3	14.2	10.9	10.4	21	16	18	20	–	4.5	–	–	–	–	–
21	55	F	21.2	4	2	25.7	22.1	17.6	15.1	141	29	21	24	–	6.0	–	2.7	3.0	–	+ (3)
22	49	M	27.5	not tested	not tested	28.5	24.0	21.2	18.0	27	19	20	17	–	3.4	–	–	–	–	–
					median	8.2	6.4	5.8	5.3											

FS-0, FibroScan values at baseline; FS-1, 2 and 3, FibroScan values at 1, 2 and 3 years after the start of nucleoside analog treatment, respectively.
M, male; F, female; BMI, body mass index; F-stage, fibrosis stage; A-grade, activity grade; ALT, alanine aminotranseferase; BTH, breakthrough hepatitis; HCC, hepatocellular carcinoma.
ALT-0, ALT level at baseline; ALT-1, 2 and 3, ALT levels at 1, 2 and 3 years after the start of nucleoside analog treatment, respectively.
DNA-0, HBV DNA level at baseline; DNA-1, 2 and 3, HBV DNA levels at 1, 2 and 3 years after the start of nucleoside analog treatment, respectively.
Figure in parenthesis was the onset of BTH or HCC after the start of nucleoside analog treatment (unit: year).

Table 5 Clinical data and longitudinal FibroScan values in Group B (Study 2)

Patient No.	Age (years)	Sex	BMI (kg/m²)	Histology		FibroScan values (kPa)			ALT (IU/L)			HBeAg	HBV DNA (LogIU/mL)						BTH	HCC
				F-stage	A-grade	FS-3	FS-4	FS-5	ALT-0	ALT-3	ALT-5		DNA-0	DNA-1	DNA-2	DNA-3	DNA-4	DNA-5		
1	64	M	22.7	4	3	20.5	23.3	21.8	34	14	16	–	7.1	–	4.9	–	–	–	+ (2)	+ (2)
2	50	M	24.4	4	2	20.4	17.7	18.8	83	29	25	+	6.5	–	3.9	3.6	3.6	2.8	+ (2)	–
3	56	M	23.7	3	2	14.3	16.7	16.6	24	17	15	–	6.5	–	–	5.3	–	2.7	+ (3)	+ (5)
4	61	F	24.2	3	3	10.6	8.5	9.6	56	38	41	+	7.8	–	6.8	3.1	–	–	+ (2)	–
5	46	F	22.0	4	3	10.4	10.2	9.6	63	27	24	–	7.1	–	–	–	4.0	–	+ (4)	–
6	43	M	22.2	4	3	9.5	10.2	9.8	56	38	41	+	4.1	6.0	–	–	–	–	+ (1)	–
7	49	M	21.5	3	2	6.5	6.7	6.4	32	24	22	–	6.0	–	–	–	4.0	–	+ (4)	–
8	47	M	21.5	1	1	3.7	3.5	4.0	31	29	29	–	6.4	–	–	–	–	3.9	+ (5)	–
9	57	M	20.7	3	2	3.2	3.5	3.0	40	14	12	+	8.6	–	7.1	4.0	–	–	+ (2)	–
10	71	F	22.6	1	1	3.9	4.3	5.1	25	13	11	–	5.5	–	–	–	–	–	–	–
11	35	M	22.6	2	2	4.4	5.0	5.0	97	15	14	+	7.6	–	–	–	–	–	–	–
12	55	M	22.4	1	1	4.6	4.5	3.8	42	17	15	–	3.3	–	–	–	–	–	–	–
13	60	F	19.1	not tested	not tested	4.8	3.8	4.8	77	13	11	–	6.0	–	–	–	–	–	–	–
14	47	M	24.2	2	2	4.8	5.2	4.3	61	41	47	–	6.3	–	–	–	–	–	–	–
15	58	F	21.2	2	2	4.8	5.2	5.2	38	10	14	–	7.0	–	–	–	3.7	3.9	–	–
16	60	M	24.0	3	2	5.1	5.8	5.4	119	26	24	–	4.6	–	3.0	2.7	3.1	3.0	–	–
17	41	F	18.9	2	2	5.3	4.8	5.9	39	7	13	+	7.6	–	–	–	–	–	–	–
18	44	M	20.9	not tested	not tested	5.5	6.8	5.8	23	11	17	–	5.6	–	–	–	–	–	–	–
19	33	M	21.3	2	2	6.1	5.0	4.8	73	41	41	+	7.2	2.7	3.3	–	–	–	–	–
20	51	M	23.6	3	2	6.1	6.8	6.1	54	29	27	–	6.1	–	3.6	3.0	3.8	3.2	–	+ (2)
21	43	F	18.2	not tested	not tested	6.6	6.8	7.5	73	32	27	–	6.5	–	–	–	–	–	–	–
22	62	M	23.6	4	2	8.3	10.5	10.1	33	28	26	–	3.8	–	–	–	–	–	–	–
23	32	M	30.3	3	2	17.1	14.0	13.9	118	87	68	–	6.1	–	–	–	–	3.3	–	–
					median	6.1	6.7	5.9												

FS-3, 4 and 5, FibroScan values at 3, 4 and 5 years after the start of nucleoside analog treatment, respectively.
M, male; F, female; BMI, body mass index; F-stage, fibrosis stage; A-grade, activity grade; ALT, alanine aminotransferase; BTH, breakthrough hepatitis; HCC, hepatocellular carcinoma.
ALT-0, ALT level at baseline; ALT-3 and 5, ALT levels at 3 and 5 years after the start of nucleoside analog treatment, respectively.
DNA-0, HBV DNA level at baseline; DNA-1, 2, 3, 4 and 5, HBV DNA levels at 1, 2, 3, 4 and 5 years after the start of nucleoside analog, respectively.
Figure in parenthesis was the onset of BTH or HCC after the start of nucleoside analog treatment (unit: year).

Table 6 Longitudinal assessment of FibroScan values and biochemical parameters after the start of nucleoside analog treatment (Study 2)

	FibroScan (kPa)	*P*-value	ALT (IU/L)	*P*-value	PT (%)	*P*-value	AFP (ng/mL)	*P*-value	IV-C (ng/mL)	*P*-value
Group A										
All patients ($n = 22$)										
Baseline	8.2 (4.2–28.5)		54.6 ± 30.4		88.1 ± 11.2		5.0 ± 3.0		172 ± 58	
FS-1	6.4 (4.0–24.0)	<0.0001*	25.4 ± 14.8	0.0002*						
FS-2	5.8 (3.8–21.2)	<0.0001**	22.3 ± 8.3	0.3211**						
FS-3	5.3 (2.5–18.0)	0.0064***	22.1 ± 7.7	0.8494***	96.3 ± 9.1	<0.0001	3.0 ± 1.6	0.0004	142 ± 37	0.0023
Group B										
All patients ($n = 23$)										
Baseline	not tested		56.1 ± 28.3		80.2 ± 9.9		5.5 ± 4.0		not tested	
FS-3	6.1 (3.2–20.5)		25.2 ± 16.8	0.0001*	91.1 ± 10.8	0.0001*	3.1 ± 2.1	0.0004*	154 ± 51	
FS-4	6.7 (3.5–23.3)	0.7439#								
FS-5	5.9 (3.0–21.8)	0.6785##	24.2 ± 13.8	0.3530#	92.7 ± 8.7	0.2459#	3.3 ± 3.1	0.5401#	148 ± 48	0.1884#

*, compared to Baseline; **, ***, compared to FS-1; ***, compared to FS-2; #, compared to FS-3; ##, compared to FS-4.
Data are shown by the mean ± standard deviation or median (range).
FS-1, 2, 3, 4 and 5, FibroScan values at 1, 2, 3, 4 and 5 years after the start of nucleoside analog treatment, respectively.
ALT, alanine aminotransferase; PT, prothrombin time; AFP, α-fetoprotein; IV-C, type IV collagen.

hepatitis B. Recent reports showed that 12 months ETV treatment for chronic hepatitis B is associated with an improvement of FibroScan values.[23,24] The results suggested that a decrease in FibroScan values during the first year of NA treatment might be attributed to not only improvement of liver fibrosis but also to necroinflammation. We were able to investigate the association between the efficacy of NA treatment and FibroScan values for a much longer period than the previous reports. Suzuki *et al.*[25] found that a 3-year LMV therapy could induce histological improvements whether YMDD mutants accompanied by virological breakthrough and BTH appeared. Our findings also showed that liver stiffness markedly improved during the first 3 years of NA treatment, but that the degree of improvement was less after 3 years of NA treatment, irrespective of the long term virological effect.

First, we investigated the association between liver fibrosis by liver biopsy and FibroScan values. Liver biopsy is not always acceptable to patients and biochemical liver examinations are sometimes unreliable for determining the extent of liver fibrosis because patients who have advanced liver fibrosis may have normal ALT levels. We showed that the FibroScan values were significantly correlated with fibrosis stage ($r = 0.672$, $P < 0.0001$). However, the cutoff for chronic hepatitis B (F3 8.9 kPa, F4 12.0 kPa) was lower than that of chronic hepatitis C (F3 10.3 kPa, F4 14.9 kPa)[16] especially for predicting severe fibrosis and cirrhosis. Macronodular cirrhosis, characterized by large nodules delimited by thin septa, is commonly found in patients with HBV infection. A different type and/or extent of liver inflammatory infiltrate within liver blocks, characterized by interface and/or lobular hepatitis infected with HBV and by the portal tract lymphoid aggregate near or surrounding bile ducts infected with HCV, might account for the difference of cutoff values between chronic hepatitis B and C patients.

Usually, complete long-term suppression of HBV DNA is an essential goal of treatment for chronic hepatitis B. Liver fibrosis is potentially reversible after viral replication has subsided,[26] therefore, the longitudinal assessment of liver fibrosis treated with NA is very important. We previously showed a dramatic reduction of FibroScan values for both virological and biochemical response by chronic hepatitis C patients treated with pegylated interferon alpha-2b and ribavirin treatment.[16] In this study of chronic hepatitis B patients who underwent NA treatment, the FibroScan values showed a rapid decline without BTH in the first 3 years. In the treatment of chronic hepatitis C, sustained virological clearance is

an ultimate aim. However, even successful response to NA by chronic hepatitis B patients does not bring about clearance. In fact, our data showed that in both the BTH and non-BTH groups, FibroScan values did not significantly improve over the 3 years after the start of NA treatment.

Previous reports have shown a well-marked association between elevated HBV DNA and progression to cirrhosis and HCC.[27] Even when ALT levels were normal, HBeAg negative patients who had HBV DNA over 4.0 log copies/mL had an increased risk of developing liver cirrhosis or HCC. Although HBV genotypes also influence the pathological features of patients, the virologic and molecular mechanisms involved remain largely not understood. Epidemiologic studies have shown that each genotype has a distinct geographic and ethnic distribution.[28,29] HBV genotypes A and D occur frequently in Africa, Europe and India, while genotypes B and C are prevalent in Asia. In general, genotype C has been shown to be associated with more progressive hepatitis than genotype B.[30] Recently, the intracellular expression of HBV DNA and hepatitis B core protein were shown to be higher for genotypes B and C than for genotypes A and D in an experimental study.[31] The intracellular accumulation of HBV DNA and antigens may play a role in liver cell damage, and moreover, the higher replication capacity of genotype C may explain the association with more severe histological liver damage than is seen for other genotypes and continued higher FibroScan values than in the case of long term HCV infection, irrespective of well-controlled antiviral treatment.

The introduction of LMV has resulted in improved suppression of HBV replication, improving histological necroinflammation and fibrosis. However, LMV-resistant HBV (the emergence of YMDD mutation) has appeared with prolonged LMV treatment, which can lead to viral or biochemical breakthrough. Some patients with hepatic cirrhosis suffer severe deterioration after BTH. Among patients who received LMV and maintained an ALT level of under 40 IU/L, the rate of YMDD motif mutant and BTH occurrences were 11%, 3% (1 year), 42%, 13% (3 years) and 61%, 19% (5 years).[32] The rate of YMDD motif mutant and BTH were low after three or more years of LMV treatment. In our data, once a BTH was experienced, FibroScan values were less likely to show improvement, which may indicate the emergence of HCC. Development of HBV resistance to LMV is typically indicated by an increase in HBV DNA followed by an increase in serum ALT level. With the advent of ETV, the frequency of viral breakthrough has been dramatically reduced;[33] however, the viral kinetics must be carefully determined.

In an analysis of the relationship between FibroScan values and the development of HCC, higher FibroScan values were shown to be associated with the development of HCC in a large prospective study of patients with chronic liver disease.[34] FibroScan values of over 10 kPa were associated with a significantly increased risk of subsequent HCC development and mortality for both chronic hepatitis B and C, irrespective of virological response owing to antiviral treatment.[35,36] Foucher *et al.*[34] suggested that the cutoff value established with a negative predictive value of greater than 90% was 53.7 kPa for HCC in patients with chronic liver diseases. Masuzaki *et al.*[35] reported that patients with chronic hepatitis C with higher FibroScan values had a significantly higher risk of HCC, with a hazard risk of 16.7 with 10.1–15 kPa, 20.9 with 15.1–20 kPa, 25.6 with 20.1–25 kPa and 45.5 with over 25 kPa, as compared to under 10 kPa. In the present study, 6 (13.3%) of 45 patients developed HCC. For those who underwent NA treatment and achieved virological response; however, the FibroScan values of most (66.7%) were consistently over 10 kPa during the follow-up period. Therefore, patients with a high FibroScan value need careful attention to prevent the development of HCC, even after achieving virological response by NA treatment.

Some limitations of FibroScan must be taken into account, such as whether or not the results are reliable. FibroScan values may be influenced by ALT flares, with a risk of overestimating liver stiffness because ALT flares reflect liver cell inflammation, edema and swelling.[36] In fact, FibroScan values of our patients transiently increased with the onset of BTH, thus FibroScan was done only after ALT level had returned to normal owing to the addition of ADV. Although the machine provides no feedback when FibroScan measurement is unsuccessful, Castéra *et al.*[37] recommended that successful measurements be validated by use of the following criteria: success rate of under 60%, IQR/M of more than 30% of median values, obesity, particularly BMI greater than 30 kg/m^2 and limited operator experience (fewer than 500 examinations) are the main determinants of unreliable FibroScan measurement. Another limitation is the small size of the study population; therefore, the clinical correlations with BTH or HCC development cannot be made appropriately.

In conclusion, transient elastography is a useful tool for the evaluation of liver stiffness and follow-up assessment of NA treatment of patients with chronic HBV infection. Liver stiffness, measured by transient elastog-

raphy, of chronic hepatitis B patients treated with NA showed a rapid decline in the first 3 years followed by a more steady transition from years three to five irrespective of the long term virological effect.

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No viremia of pandemic (H1N1) 2009 was demonstrated in blood donors who had donated blood during the probable incubation period

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BACKGROUND: In the spring of 2009, the novel swine-origin influenza A (pandemic [H1N1] 2009) virus emerged and spread globally. Although no established cases of transfusion-transmitted influenza have been reported, the widespread outbreak of pandemic (H1N1) 2009 caused serious concern regarding the safety of blood products. The Japanese Red Cross Blood Centers have intercepted blood products with accompanying postdonation information indicating possible pandemic (H1N1) 2009 infection. To study the risk of transmission of pandemic (H1N1) 2009 by blood transfusion, we searched for the viral genome in such products using nucleic acid amplification technology.

STUDY DESIGN AND METHODS: Between June and December 2009, blood components were collected from 579 blood donors who were diagnosed as or strongly suspected of having pandemic (H1N1) 2009 within 7 days after donation. Viral RNA was extracted from plasma and red blood cell (RBC) products, and RNA samples were subjected to real-time reverse transcription–polymerase chain reaction of the hemagglutinin and matrix genes of the pandemic (H1N1) 2009 virus.

RESULTS: A total of 565 plasma and 413 RBC products from the 579 blood donors were available. No viral RNA of the pandemic (H1N1) 2009 was detected in any of the blood samples from the 579 blood donors.

CONCLUSION: No viremia of pandemic (H1N1) 2009 was demonstrated in any of the 579 blood donors who had most likely donated blood during the incubation period. It is considered that the risk of transmitting pandemic (H1N1) 2009 by blood transfusion is extremely low.

The novel swine-origin influenza A (pandemic [H1N1] 2009) virus was a triple-reassortant swine influenza virus that contains genes from human, swine, and avian influenza A viruses.[1-3] The pandemic (H1N1) 2009 virus emerged in early 2009 in Mexico and the United States[4,5] and rapidly spread worldwide including Japan[6] via human-to-human transmission because most people have no immunity to this new virus. Although no established cases of transfusion-transmitted influenza have been recognized and reported, the apparently high virulence reported in Mexico[7] raised a serious concern regarding the safety of blood products.

A few studies in the 1960s and 1970s have shown the viremia of seasonal influenza. Most data were obtained from blood samples that were collected after the onset of symptoms.[8-11] Only one instance of the detection of the virus in blood during the incubation period has been reported, but no virus has been detected from blood from the same individual at the onset of symptoms.[11] In recent reports, no viremia of seasonal influenza has been demonstrated.[12,13] Most studies have, thus, failed to demonstrate viremia in blood samples, but this is not unexpected considering that influenza is essentially a respiratory tract

ABBREVIATIONS: HA = hemagglutinin; JRCBSs = Japanese Red Cross Blood Centers; M gene = matrix gene; NIID = Japanese National Institute of Infectious Diseases; PDI = postdonation information.

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infection. There are almost no data on the viremia of pandemic (H1N1) 2009.

We previously reported that there was no viremia detected using nucleic acid amplification technology (NAT) in the blood samples obtained from 96 blood donors who showed symptoms of influenza within 7 days after the donation.[14] In this study, we report the result of our extended study in which 579 blood products from blood donors who were in the same presymptomatic period, as described above, were examined, together with the detailed profiles of such blood donors and the sensitivity of NAT used.

MATERIALS AND METHODS

Collection of blood

In May 2009, when the emergence of pandemic (H1N1) 2009 was confirmed in Japan, the Japanese Red Cross Blood Centers (JRCBCs) implemented the following measures to ensure the safety of blood products, in line with the notification from the Japanese Ministry of Health, Labour and Welfare: screening of blood donors for fever, refusing donation from blood donors who had returned to Japan from abroad within 4 weeks, and refusing donation from donors who had close contact with a patient suspected of having pandemic (H1N1) 2009 within 7 days.

From May 2009 to March 2010, the JRCBCs asked blood donors to provide postdonation information (PDI) on diagnosis of pandemic (H1N1) 2009 in addition to the usual information required. To acquire the PDI, the JRCBCs distributed handbills and explained to all blood donors in all blood collection sites: if they had any symptom of influenza within 7 days after blood donation, and it was diagnosed as pandemic (H1N1) 2009 in medical institutions, they were requested to contact the JRCBCs. If the PDI indicated a possible pandemic (H1N1) 2009 infection after the donation was given to the JRCBCs, the supply of blood products from such blood donors was stopped or withdrawn. These blood products collected from June to December 2009 were used for this study. After the blood products were collected from the inventory or retrieved from medical institutions, they were stored at –20°C in aliquots until use. The time lags between the donation and collection of the blood products for this study were 2 to 30 (mean, 10.3) days for plasma samples and 2 to 18 (mean, 7.8) days for red blood cell (RBC) samples. From the time of donation to collection, these blood products were stored at the stipulated temperatures (plasma, –20°C; RBCs, 4°C). Informed consent to tests for infection was obtained from all blood donors at the blood collection sites.

All blood donors in this study were diagnosed as having pandemic (H1N1) 2009 at a medical institution within 7 days after donation. These blood donors were classified into laboratory-confirmed cases and suspected cases on the basis of the confirmation standard for the diagnosis of pandemic (H1N1) 2009 infection. Laboratory-confirmed cases refer to those diagnosed as having pandemic (H1N1) 2009 by the reverse transcription–polymerase chain reaction (RT-PCR) using respiratory specimens in public health institutes. Suspected cases refer to those diagnosed as having pandemic (H1N1) 2009 infection by the rapid diagnostic kits for influenza A infection or on the basis of the symptoms of influenza-like illness such as fever and acute respiratory symptoms, without performing RT-PCR.

According to the Infectious Agents Surveillance Report published by the Japanese National Institute of Infectious Diseases (NIID), the pandemic (H1N1) 2009 virus dominated 99% of the influenza viruses isolated or detected from the cases of influenza-like illness during the study period from June to December 2009.[15] Therefore, suspected cases in this study were expected to be cases of either pandemic (H1N1) 2009 or noninfluenza illness, with negligible possibility of seasonal influenza.

Evaluation of NAT detection sensitivity

NAT detection sensitivity was evaluated by spiking experiments using virus particles of the pandemic (H1N1) 2009 virus (A/California/04/2009 [H1N1]) contained in the viral culture supernatant donated by NIID. The viral genome copy number of the culture supernatant was determined by quantitative RT-PCR, using synthesized RNA molecules of the matrix (M) gene as standards. The synthesized RNA was obtained from the cloned M gene inserted into plasmid DNA (TOPO TA cloning kit, Invitrogen, Carlsbad, CA) by transcription using T7 RNA polymerase (Roche Diagnostics, Indianapolis, IN). The transcribed RNA was purified using a commercially available kit (RNeasy Plus Mini Kit, Qiagen, Gaithersburg, MD), and its quantity and quality were checked using a capillary electrophoresis system (Agilent 2100 Bioanalyzer and RNA 6000 Nano Kit, Agilent, Santa Clara, CA). A dilution series of the synthesized RNA sample was used to construct a standard curve to estimate the viral genome copy number of the culture supernatant.

The quantified culture supernatant of the pandemic (H1N1) 2009 virus was spiked into plasma and RBC samples from healthy volunteers, at inoculation doses from 20 to 2×10^5 genome equivalents (geq)/mL, and the NAT for the M and hemagglutinin (HA) genes was performed 20 times for each dose. The relationship between NAT positivity and pandemic (H1N1) 2009 virus concentration was analyzed by probit analysis. An input viral genome copy number with a 95% probability of a positive result was used as the detection limit.

NAT for detection of pandemic (H1N1) 2009 virus

Viral RNA was extracted from plasma and packed RBC samples using a kit for automated purification of viral

DNA and RNA (QIAamp Virus Biorobot MDx kit, Qiagen) and a viral nucleic acid purification kit (High Pure Viral Nucleic Acid Large Volume Kit, Roche Diagnostics), respectively. RNA samples were immediately subjected to the real-time RT-PCR of the M and HA genes of influenza A with a sequence detection system (PRISM 7900, Applied Biosystems, Foster City, CA) using an RT-PCR kit (Quanti-Tect Probe, Qiagen). The real-time RT-PCR of the HA gene was designed for the specific detection of the pandemic (H1N1) 2009 virus, whereas the real-time RT-PCR of the M gene was designed for the universal detection of type A influenza viruses. The sequences of the primers and probes used were synthesized in accordance with the protocols developed by NIID.[16] The forward and reverse primers were 5′-CCMAGGTCGAAACGTAYGTTCTCTCTA TC-3′ and 5′-TGACAGRATYGGTCTTGTCTTTAGCCAYTC CA-3′, respectively, for M gene and 5′-AGAAAAGA ATGTAACAGTAACACACTCTGT-3′ and 5′-TGTTTCCACAA TGTARGACCAT-3′, respectively, for HA gene. The TaqMan probes for M and HA genes were 5′-ATYTCGGCT TTGAGGGGGCCTG-3′ and 5′-CAATRTTRCATTTACC-3′, respectively. Each probe was labeled with a reporter dye (FAM) at the 5′ end, a nonfluorescent quencher and a minor groove binder at the 3′ end. Either 200 μL of plasma or 100 μL of RBC samples was used for each test. The real-time RT-PCR conditions comprised a 30-minute RT step at 60°C, a 10-minute initial PCR activation step at 95°C, and 45 amplification cycles at 95°C for 15 seconds and at 60°C for 45 seconds. To assess the analytical accuracy of NAT, a dilution series of pandemic (H1N1) 2009 virus particles in the viral culture supernatant as a positive control, and plasma and RBC samples obtained from healthy volunteers as negative controls were included in each assay.

Lookback investigation for blood recipients

As one of the operations in hemovigilance, JRCBCs have been collecting information on transfusion-transmitted infections and adverse transfusion reactions such as fever, urticaria, pain, nausea, hypotension, anaphylactic reaction or shock, dyspnea, and neuropsychiatric symptoms. If the blood products had already been released when the PDI indicating possible pandemic (H1N1) 2009 infection was acquired, we inform the medical institution of the blood product concerned. Patients who had received transfusion with the blood products involved with the pandemic (H1N1) 2009 infection were observed for influenza-like symptoms such as fever, respiratory symptoms, or systemic inflammatory reactions for a period of 7 days after transfusion.

RESULTS

Characteristics of blood donors

Between June and December 2009, the blood components were collected from 579 blood donors (314 male and 265 female). A total of 366 blood donors (190 male and 176 female) had laboratory-confirmed pandemic (H1N1) 2009 infection, and 213 blood donors (124 male and 89 female) had suspected pandemic (H1N1) 2009 infection.

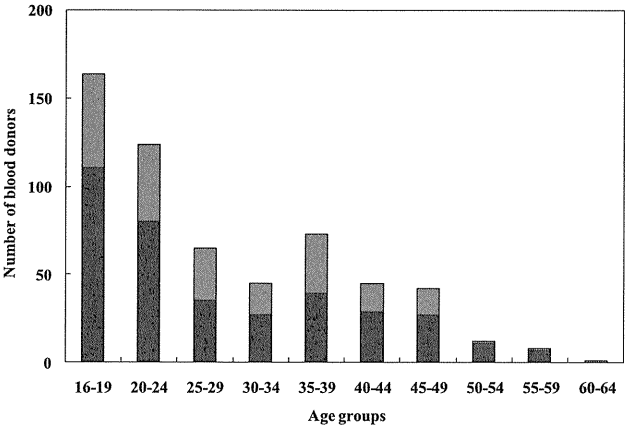

Fig. 1. Age distribution of the blood donors who had symptoms of pandemic (H1N1) 2009 after donation. Laboratory-confirmed cases (black bar) were diagnosed as pandemic (H1N1) 2009 by the RT-PCR method using respiratory specimens. Suspected cases (gray bar) were diagnosed by rapid diagnostic kits or on the basis of the symptoms of influenza-like illness, without performing RT-PCR. Sixty-one percent of the blood donors who showed symptoms of influenza after donation belonged to the young age group and were less than 30 years of age.

The ages of the 579 blood donors are shown in Fig. 1. Sixty-one percent of them were less than 30 years of age. The ratio of the donor number in the 16- to 29-year age group to the 579 blood donors were 2.2 times as high as the ratio of all blood donors in the 16 to 29 age group to the total number of donors who donated in 2009 in Japan. In each age group, there were no significant differences in the ratios between sexes (data not shown).

The time lag between the donation and the onset of influenza symptoms is shown in Fig. 2. Ten (1.7%) blood donors developed symptoms of influenza on the day of the donation, 74 (12.8%) within 1 day, 105 (18.1%) within 2 days, and 132 (22.8%) within 3 days after donation. That is, 321 (55.4%) blood donors showed symptoms of influenza within 3 days after donation.

The Infectious Agents Surveillance Report published by NIID indicated that the fall wave of pandemic (H1N1) 2009 appears to have peaked in late November 2009; this corresponded to the finding that 468 (80.8%) of the 579 blood donors donated between November and December 2009 (data not shown).

Evaluation of NAT detection sensitivity

For the plasma samples, the NAT showed a 100% detection probability for both M and HA genes at more than

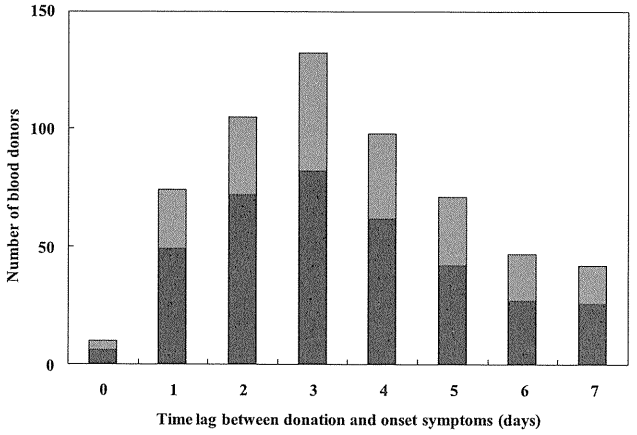

Fig. 2. Time lag between donation and onset of influenza symptoms. Laboratory-confirmed cases (black bar) were diagnosed as pandemic (H1N1) 2009 by the RT-PCR method. Suspected cases (gray bar) were diagnosed by rapid diagnostic kits or on the basis of the symptoms of influenza like illness, without performing RT-PCR. More than half of blood donors (55.4%) showed symptoms of influenza within 3 days after donation. It was speculated that many blood donors gave blood during the incubation period of the pandemic (H1N1) 2009 virus.

2000 geq/mL. The detection probabilities were 85 and 75% at 200 geq/mL and 50 and 15% at 20 geq/mL for the M and HA genes, respectively (Fig. 3A). For the RBC samples, the NAT showed a 100% detection probability for both M and HA genes at more than 20,000 geq/mL. The detection probabilities were 80 and 65% at 2000 geq/mL and 15 and 5% at 200 geq/mL for the M and HA genes, respectively (Fig. 3B).

The 95% detection limit of the NAT in the plasma samples was calculated to be 283 geq/mL (95% confidence interval [CI], 116-3287), corresponding to 57 geq per reaction (95% CI, 23-658) for the M gene, and 528 geq/mL (95% CI, 256-2368), corresponding to 106 geq per reaction (95% CI, 51-474) for the HA gene (Fig. 3A). For the RBC samples, the 95% detection limit of the NAT was calculated to be 3444 geq/mL (95% CI, 1784-12,697), corresponding to 344 geq per reaction (95% CI, 178-1270) for the M gene and 5292 geq/mL (95% CI, 2829-19,911), corresponding to 529 geq per reaction (95% CI, 283-1991) for the HA gene (Fig. 3B).

Detection of pandemic (H1N1) 2009 virus RNA

The NAT was performed using 565 plasma and 413 RBC samples obtained from 579 blood donors who showed symptoms of influenza within 7 days after donation. The samples consisted of 362 plasma and 271 RBC samples from the 366 blood donors who had laboratory-confirmed pandemic (H1N1) 2009 infection and 203 plasma and 142 RBC products from the 213 blood donors who had suspected pandemic (H1N1) 2009 infection. The NAT was performed in duplicate for the M and HA genes of the pandemic (H1N1) 2009 virus in each sample. None of the viral genome of the M or HA gene was detected in any of the plasma samples and RBC samples (Table 1).

Lookback investigation of blood recipients

In the lookback investigation of the donated blood products from the 579 blood donors, it was revealed that 36 platelet (PLT) products and 34 RBC products had already been used for transfusion when the PDI was acquired. Of the 36 blood donors who donated these PLT products, two showed symptoms of influenza on the next day of donation, and 10 and 13 showed symptoms 2 and 3 days after donation, respectively. Of the 34 blood donors who donated these RBC products, two and three showed symptoms of influenza 2 and 3 days after donation, respectively (Fig. 4). Of the blood donors who donated these PLT products and RBC products, 25 and 20 blood donors belonged to laboratory-confirmed cases, respectively.

In the 70 blood recipients who received blood products likely donated during the incubation period of the pandemic (H1N1) 2009 infection, influenza-like symptoms such as fever and acute respiratory symptoms and any transfusion adverse reactions were not observed for a period of 7 days after transfusion.

DISCUSSION

In this study, we examined blood samples from 579 blood donors who were diagnosed as or strongly suspected of having pandemic (H1N1) 2009 infection within 7 days after donation. Sixty-one percent of the blood donors involved in this study belonged to the young age group of less than 30 years of age. The ratio of the young age group to the 579 blood donors was higher than that of this age group to the total number of blood donors who donated in 2009 in Japan. It has been reported that the majority of patients with pandemic (H1N1) 2009 were children and young people.[17,18] According to the reports by the Centers for Disease Control and Prevention in the United States, more than 64% of the pandemic (H1N1) 2009 virus-infected individuals were 5 to 24 years old; only 1% were 65 years of age or older.[17] In this point, pandemic (H1N1) 2009 markedly differs from seasonal influenza. The ratio of the age groups of the blood donor cohort involved in this study reflected the ratio of the age groups of the pandemic (H1N1) 2009 virus-infected individuals reported in Japan and abroad.[17,19]

In this study, 10 blood donors showed symptoms of influenza on the day of the donation, 74 within 1 day, and 105 within 2 days after the donation. The incubation

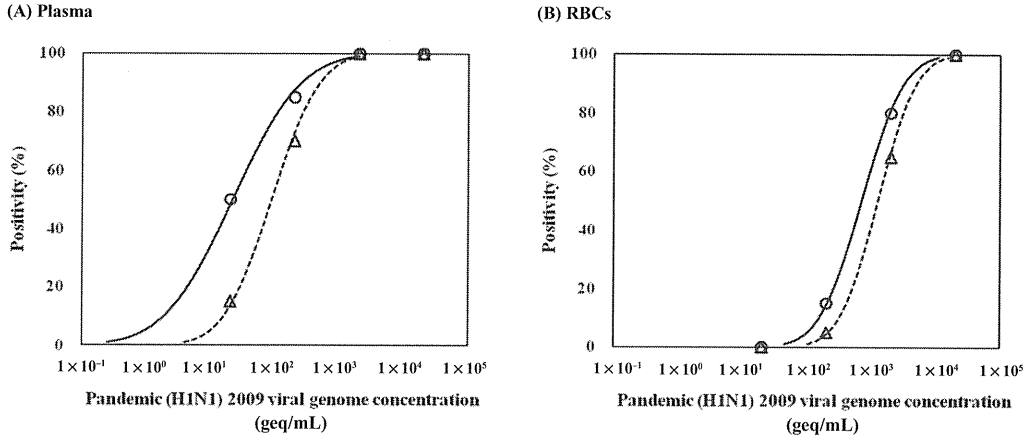

Fig. 3. Sensitivities of the NAT for plasma samples (A) and RBC samples (B). The quantified culture supernatant of the pandemic (H1N1) 2009 virus was spiked into the plasma and RBC samples from healthy volunteers, at doses from 20 to 2×10^5 geq per mL, and the NAT for the M (—) and HA (- - -) genes was performed 20 times for each concentration. The relationship between NAT positivity and pandemic (H1N1) 2009 virus concentration was analyzed by probit analysis. The 95% detection limit of the NAT for the plasma samples was calculated to be 283 geq/mL for the M gene and 528 geq/mL for the HA gene (A). For the RBC samples, the 95% detection limit of the NAT was calculated to be 3444 geq/mL for the M gene and 5292 geq /mL for the HA gene (B).

TABLE 1. Results of detection of pandemic (H1N1) 2009 virus RNA in plasma or RBCs drawn from blood donors who were diagnosed as pandemic (H1N1) 2009 after donation

Cases	Total number of blood donors	Number of samples tested		NAT-positive samples			
				Plasma		RBCs	
		Plasma	RBC	M gene	HA gene	M gene	HA gene
Laboratory-confirmed case*	366	362	271	0	0	0	0
Suspected case†	213	203	142	0	0	0	0
Total	579	565	413	0	0	0	0

* Pandemic (H1N1) 2009 was diagnosed by the RT-PCR method using respiratory specimens.
† Pandemic (H1N1) 2009 was diagnosed by the rapid diagnostic kits for influenza A or on the basis of the symptoms of influenza-like illness, without performing RT-PCR.

period of the pandemic (H1N1) 2009 virus is reported to be 1 to 7 days, with a mean of 2 days.[5,20] Therefore, it is speculated that many of the 579 blood donors gave blood during the incubation period of the pandemic (H1N1) 2009 virus. NAT was performed using specimens of this donated blood. No RNA of the pandemic (H1N1) 2009 virus was detected in any of the blood samples; no viremia of pandemic (H1N1) 2009 before clinical onset was demonstrated. In this study, however, some donors included in the 213 suspected cases were diagnosed by physicians on the basis of the symptoms of influenza-like illness without performing RT-PCR or rapid diagnostic tests. The possibility that donors with noninfluenza illness were not completely excluded from the suspected cases would weaken the power of this study.

Although influenza epidemics occur every winter season, no established cases of transfusion-transmitted influenza have been recognized and reported. A few studies published from the 1960s to the 1970s showed the presence of viremia of seasonal influenza;[8-11] however, among studies published in recent years, no viremia of seasonal influenza has been demonstrated yet.[12,13] In both pandemic (H1N1) 2009 and seasonal influenza virus infections, the peak viral load in the respiratory specimens was observed immediately after the onset of symptoms.[21,22] Although the mean viral load in the respiratory specimens of pandemic (H1N1) 2009 was 1.84×10^8 copies/mL, the virus was detected in none of the blood specimens obtained at the same time.[20] In addition to these data, we detected no viremia of pandemic (H1N1) 2009 in the present study. It thus seems that the occurrence of pandemic (H1N1) 2009 viremia before the onset of illness is extremely low. If there were cases in which influenza infections occurred by blood transfusion, a viremia condition would need to be present during the incubation period in which blood donation was performed. It is thus considered that the risk of the transmission of the pandemic (H1N1) 2009 virus by blood transfusion is extremely low.

The 95% detection limit of NAT for the plasma samples was calculated to be 283 geq/mL for the M gene and 528 geq/mL for the HA gene, whereas that for the RBC

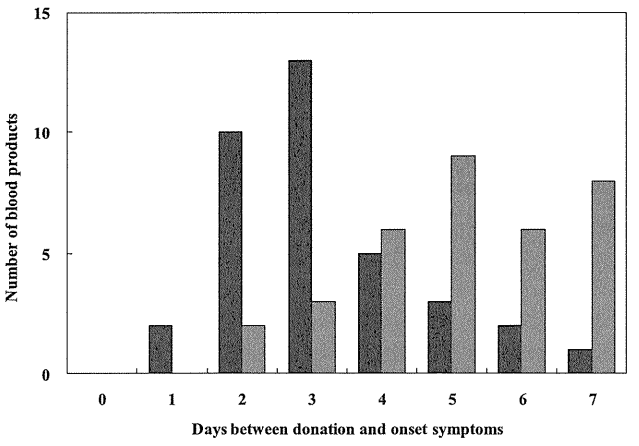

Fig. 4. Time lag between donation of blood implicated in lookback study and onset of symptoms. Thirty-six PLT products (black bar) and 34 RBC products (gray bar) likely donated during the incubation period of the pandemic (H1N1) 2009 had already been used for transfusion to 70 recipients when the PDI was acquired. In the lookback investigation, no influenza-like symptoms or any other observable symptoms were observed in the recipients after transfusion.

samples was calculated to be 3444 geq/mL for the M gene and 5292 geq/mL for the HA gene. The NAT sensitivity for the HA gene was lower than that for the M gene, for both plasma and RBC samples. This difference could be due to the secondary structure of the viral RNA or reverse-transcribed cDNA that might have decreased the sensitivity of the RT-PCR of the HA gene. The lower sensitivity in the packed RBC samples might be caused by the inhibitors of RT-PCR such as hemoglobin that contaminated the RNA solutions obtained from the RBC samples or the insufficient efficiency of the viral RNA extraction. Improved methods will be required to purify viral RNA from RBC samples. If low-level viremia below the NAT detection sensitivity should exist, it would not be detected using our assay.

In the first step in influenza infection and viral replication, influenza viruses bind through the HA transmembrane glycoprotein onto sialic acid residues on the surface of epithelial cells, typical in respiratory organs. After HA is cleaved by a protease, the cells import the virus by endocytosis.[23-25] HA cleavage is required to activate virus infectivity, and the activating proteases are mainly distributed in the respiratory organs and intestine in humans,[24,26] so that it should be difficult for influenza viruses to acquire infectivity in blood. In theory therefore the risk of the direct transmission of influenza via blood is considered to be extremely low. In fact, we showed, in this study, that the transfusion of 36 PLT and 34 RBC products from the blood donors who likely donated during the incubation period of the pandemic (H1N1) 2009 virus caused apparently no transmission of the virus.

The risk of virus infection by blood transfusion has decreased owing to the introduction of a screening test for donated blood, but there is still residual risk caused by pathogens that are excluded from targets of the current screening test or newly spread in humans. The influenza virus is one of the potentially unrecognized pathogens in the blood supply. This study showed that the viremia of pandemic (H1N1) 2009 during the incubation period is highly unlikely to occur and that it does not pose a noticeable risk to the safety of the blood supply. The main infection routes of the influenza virus are droplet infection and contagious infection. During a pandemic, many people are easily infected by the influenza virus. Compared with the risk of infection by the influenza virus via respiratory droplets, the risk of transmission by transfusion is almost negligible. In this point, pandemic (H1N1) 2009 markedly differs from any other currently known viral threats to the blood supply.

Regarding influenza viruses with high pathogenicity, we do not know the risk of their transmission by transfusion. The HA protein of highly pathogenic avian influenza virus can be cleaved by proteases that are produced in many different tissues. As a results, these viruses can replicate in many organs of the bird, not just the respiratory organs.[27] In severe cases of highly pathogenic avian influenza A (H5N1) virus-infected humans, viremia has been reported.[28-30] New studies will be required when a new type of influenza emerges in the future.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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