(88 and 100 %, respectively). However, loss of HBeAg was achieved in a minority of patients (29 %) during the entecavir treatment.

In most patients without HBeAg loss at the end of the entecavir treatment, serum ALT and HBV DNA levels increased even during the interferon-α treatment. Hepatitis flare (defined as a rise in ALT equivalent to 10 times higher than the upper limit of normal and more than twice the baseline value) occurred in 3 patients after the withdrawal of entecavir. Although peak ALT levels in these patients were 693, 721, and 876 IU/L, respectively, none had jaundice or hepatic decompensation. At the end of the interferon-α treatment, the percentages of patients with normal ALT, HBV DNA $<10^4$ copies/mL, and loss of HBeAg were 75, 71, and 33 %, respectively.

Lastly, 24 weeks after the completion of the sequential therapy, a sustained biochemical, virological, and serological response was achieved in 5 (21 %) of the 24 patients. No patient had loss of serum HBsAg in response to the sequential therapy.

Changes in HBsAg and HBcrAg during and after sequential therapy

Changes in serum HBsAg and HBcrAg levels during and after the sequential therapy with entecavir and interferon-α are shown in Fig. 2. The serum HBsAg level did not change significantly during or after the therapy (Fig. 2a). The serum HBcrAg levels were significantly decreased at the start ($P < 0.0001$) and at the end of interferon-α treatment ($P < 0.0001$), but returned to baseline levels after completion of the sequential treatment (Fig. 2b). The serum HBsAg level did not differ significantly between patients with a sustained response and those with no response (Fig. 2c). In contrast, the serum HBcrAg level was significantly lower in patients with a sustained response than in those with no response at the end of the interferon-α therapy ($P = 0.013$) and 24 weeks post-treatment ($P = 0.031$) (Fig. 2d).

Characteristics of patients at the start of entecavir treatment

The baseline demographic, biochemical, virological, and histological characteristics of patients at the start of entecavir treatment, classified according to the response to sequential therapy, are listed in Table 1. The mean age of patients with a sustained response was more than 10 years less than that of the patients with no response, but this difference did not reach statistical significance ($P = 0.102$). There were no significant differences between the two groups with respect to sex ratio, proportion of patients with a history of interferon treatment, ALT level, HBV DNA level, ratios of HBV genotypes, ratios of precore or basal core promoter mutants, or histopathological findings in the liver.

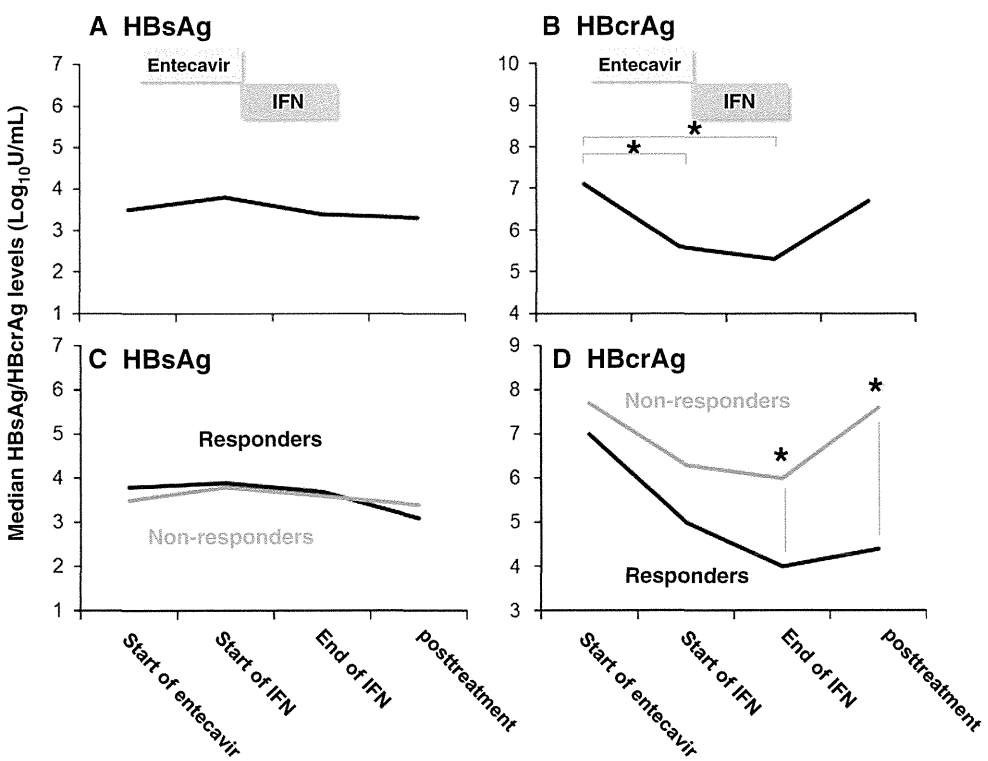

Fig. 2 Changes in serum levels of hepatitis B surface antigen (*HBsAg*) and hepatitis B core-related antigen (*HBcrAg*) during and after sequential therapy with entecavir and interferon-α. Serum HBsAg levels did not change during or after therapy (**a**). As compared with the baseline value, the serum HBcrAg level was significantly decreased at the start ($P < 0.0001$) and at the end of interferon-α treatment ($P < 0.0001$) (*asterisks*) (**b**). When sustained responders were compared with non-responders, there was no significant difference in the serum HBsAg level (**c**). In contrast, the serum HBcrAg level was significantly lower in sustained responders than in non-responders at the end of interferon-α therapy ($P = 0.013$) and 24 weeks post-treatment ($P = 0.031$) (*asterisks*) (**d**)

Table 1 Baseline characteristics of patients at the start of entecavir treatment

Characteristics	Sustained responders ($n = 5$)	Non-responders ($n = 19$)	P values
Age (years)	29 ± 6	41 ± 5	0.10
Male sex (%)	5 (100 %)	18 (95 %)	0.99
History of interferon treatment (%)	3 (60 %)	12 (63 %)	0.99
ALT (IU/L)	85 (65, 322)	210 (79, 531)	0.37
HBV DNA ($\log_{10}$ copies/mL)	7.7 ± 0.4	7.8 ± 0.8	0.31
Genotype (A/B/C/D)	0/0/5/0	1/0/18/0	0.99
Precore (wild/mixed/mutant)	0/4/1	9/9/1	0.12
Basal core promoter (wild/mixed/mutant)	1/0/4	5/8/6	0.070
Grade of inflammation (mild/moderate/severe)	2/3/0	9/7/2	0.60
Stage of fibrosis (mild/moderate/severe/cirrhosis)	2/2/0/1	10/5/3/0	0.19

Values are means ± SDs for normally distributed variables, and medians (with the interquartile range) for non-normally distributed variables

ALT alanine aminotransferase, *HBV* hepatitis B virus

Table 2 Characteristics of patients at the start of interferon-α treatment

Characteristics	Sustained responders ($n = 5$)	Non-responders ($n = 19$)	P values
ALT (IU/L)	24 (23, 35)	20 (15, 32)	0.27
ALT normal (%)	5 (100 %)	16 (84 %)	0.99
HBV DNA ($\log_{10}$ copies/mL)	2.1 ± 0.3	2.3 ± 0.4	0.18
HBV DNA negative (%)	3 (60 %)	6 (32 %)	0.33
HBeAg loss (%)	4 (80 %)	3 (16 %)	0.015

Values are means ± SDs for normally distributed variables, and medians (with the interquartile range) for non-normally distributed variables

HBeAg hepatitis B envelope antigen

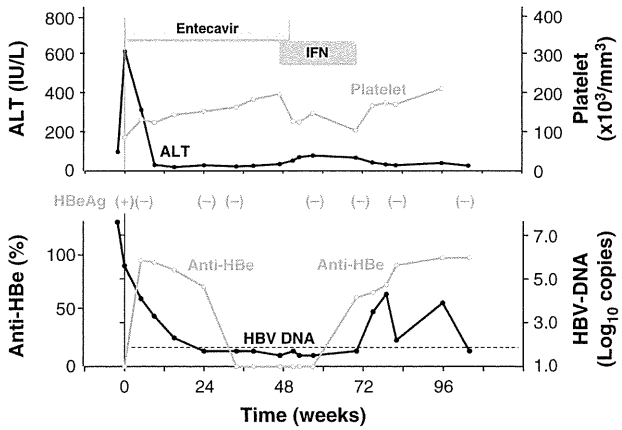

Fig. 3 Changes in platelet count, ALT, HBeAg, anti-HBe, and HBV DNA in a 24-year-old man with sustained response to sequential therapy with entecavir and interferon-α. In the *upper panel*, the changes in ALT levels (*filled circles*) and platelet counts (*open circles*) are shown. In the *lower panel*, the changes in HBV DNA (*filled circles*) and anti-HBe (*open circles*) titers are shown. During 1 year of entecavir treatment, the platelet count rose from 87,000 to 199,000/mm^3. After the patient was switched to interferon-α, his anti-HBe antibody titer increased. At the most recent hospital visit, the patient's ALT level was normal, HBeAg was negative, and HBV DNA was negative; the patient has remained drug-free since the completion of treatment

Characteristics of patients at the start of interferon-α treatment

The characteristics of the patients at the start of interferon-α treatment, classified according to the response to sequential therapy, are shown in Table 2. The responders and non-responders did not differ significantly with respect to ALT level or HBV DNA level at the start of interferon-α treatment. The proportion of patients in whom HBeAg was lost during entecavir treatment was significantly higher among those with a sustained response than among those with no response ($P = 0.015$). In another comparison, a sustained response was achieved in 4 (57 %) of the 7 patients with loss of HBeAg during entecavir treatment, as compared with 1 (5.9 %) of the 17 patients without loss of HBeAg during treatment; this difference was also statistically significant ($P = 0.015$).

Case presentation

A 24-year-old man with no response to previous treatment with interferon-α was referred to us (Fig. 3). His ALT level was 617 IU/L, HBV DNA level was 7.6 $\log_{10}$ copies/mL, and HBV genotype was C. A precore stop codon mutation at *nt* 1896 and basal core promoter mutations at *nt* 1762 and *nt* 1764 were detected. A liver biopsy showed moderate inflammation and cirrhosis. Although the patient was young, interferon-α was not indicated because of a low platelet count and concern about exacerbation of hepatitis. However, during 1 year of entecavir treatment, his ALT level became normal, and his platelet count rose from 87,000 to 199,000/mm^3. After switching to interferon-α, his HBV DNA rose transiently, but his anti-HBe antibody titer increased. At the most recent hospital visit (up to 35 weeks after the completion of treatment), his ALT level was normal and HBeAg and HBV DNA were negative; the

patient has remained drug-free since the completion of treatment.

Discussion

Several groups have evaluated protocols for sequential therapy with lamivudine and interferon-α, and their protocols were similar to that originally described by Serfaty et al. [10]. Manesis et al. [11], from Greece, where HBV genotype D is predominant, found that in HBeAg-*negative* patients, the rate of sustained biochemical and virological response was 22 %, which did not differ from that obtained in an age/sex-matched historical control group treated with interferon-α alone. In another report from Greece [12], sequential combination therapy significantly prevented the emergence of resistance to lamivudine, but the rate of sustained virological response was only 17 % among HBeAg-*negative* patients. A group from China, where genotype B or C is predominant, reported very similar results [13]. To date, only the study by Moucari et al. [17] has used adefovir dipivoxil instead of lamivudine. Sustained virological response was achieved in 50 % of their subjects, although only 20 HBeAg-*negative* patients were included.

In Japan and other countries in East Asia, genotype C is the most prevalent type of HBV [18, 19], and most patients with chronic hepatitis B acquire the virus perinatally or in early childhood [7]. The rates of response to interferon are thus lower than those reported in Europe and the United States. In our previous study [14], using a sequential therapy protocol similar to that described by Serfaty et al. [10], we found that the rate of sustained response was only 29 % among 24 HBeAg-*positive* patients. The patients with a sustained response were significantly younger and had a significantly lower HBV DNA level at the start of interferon than did those with no response. The rate of HBeAg loss during lamivudine treatment was slightly but not significantly higher among sustained responders than among non-responders. Minami and Okanoue [15] also found that patients who lost HBeAg during lamivudine treatment were more likely to have a sustained response to sequential therapy. Okuse et al. [16] reported that sequential therapy was effective for patients with acute exacerbations of chronic hepatitis B, particularly those in whom HBeAg had become negative during lamivudine treatment.

One objective of sequential therapy is to lower the viral load by the use of a nucleos(t)ide analogue, thereby restoring sensitivity to interferon treatment. In clinical studies, a low HBV DNA level is predictive of a favorable response to interferon-α [30, 31]. In basic studies, a high viral load is associated with T-cell hyporesponsiveness [32], and treatment with nucleos(t)ide analogues restores cellular immune response in chronic HBV infection [33]. Although lamivudine had been administered for about half a year before the start of interferon administration in previous studies (including ours) [10–16], we administered entecavir, a more potent antiviral agent, for about 1 year in the present study. Treatment with entecavir was given for a longer period because it has been reported in previous studies that patients in whom HBeAg and HBV DNA levels were lowered by lamivudine were more likely to have a sustained response and because few entecavir-resistant variants emerge within the first few years [34]. However, the use of entecavir for a longer duration did not raise the rate of off-treatment sustained response to sequential therapy in the present study, although the rate of on-treatment biochemical and virological responses was higher with entecavir than that obtained with lamivudine in our previous study [14].

Another objective of sequential therapy is to prevent the relapse of hepatitis after discontinuation of the nucleos(t)ide analogue through the use of interferon-α. Nucleos(t)ide analogues rapidly decrease serum HBV DNA levels by suppressing the reverse transcription of pregenomic HBV RNA, but viral relapse commonly occurs after the cessation of treatment. This high risk of viral relapse may be attributed to the persistence of HBV replicative intermediate covalently closed circular DNA (cccDNA) in the liver even during nucleos(t)ide treatment. The measurement of HBV antigens in serum is thus clinically important as a surrogate marker of intrahepatic cccDNA. In particular, a decline in serum levels of HBsAg is strongly associated with response to interferon-α [35]. The HBcrAg assay measures serum levels of all antigens transcribed from the precore/core gene, including hepatitis B core and e antigens, by using monoclonal antibodies that recognize common epitopes of the denatured antigens [23, 24]. Matsumoto et al. [36] recently proposed a model for predicting relapse of hepatitis after discontinuation of nucleos(t)ide analogue administration, in which cut-off values were set at 1.9–2.9 $\log_{10}$ IU/mL of HBsAg and 3.0–4.0 $\log_{10}$ U/mL of HBcrAg at the withdrawal of treatment. In our study, only one patient had a decrease in HBsAg to between 1.9 and 2.9 $\log_{10}$ IU/mL and another one had a decrease in HBcrAg to between 3.0 and 4.0 $\log_{10}$ U/mL at the withdrawal of entecavir (data not shown), probably because of an insufficient duration of entecavir treatment in our protocol. The finding that at least 21 % of our patients with insufficient HBsAg and HBcrAg decline during entecavir treatment achieved a sustained response to sequential therapy suggests that switching to interferon-α contributes to the safe termination of nucleos(t)ide analogue treatment in some patients.

The major advantages of interferon-α include a finite course of treatment, the opportunity to obtain an off-treatment durable response to therapy, and absence of drug resistance. The advantages of nucleos(t)ide analogues include good tolerance and potent antiviral activity associated with high rates of on-treatment response to therapy. Guidelines proposed by the Japanese Study Group of the Standardization of Treatment of Viral Hepatitis basically recommend interferon-α as the first-line treatment for patients with chronic hepatitis B who are younger than 35 years, to attain a 'drug-free state'; and entecavir for patients who are 35 years or older, to persistently suppress HBV DNA [37]. Consistent with the findings of previous studies [14–16], our results show that sequential therapy is best indicated for patients who have lost HBeAg during nucleoside analogue treatment, because such patients have a higher probability of a sustained response. As shown in Fig. 3, patients who are young but have exacerbation of hepatitis, cirrhosis, or both, were also good candidates for sequential therapy, because interferon-α is generally not recommended for such patients because of concern about hepatic decompensation, and the preceding use of a nucleos(t)ide analogue can reduce such risk.

Our study had several limitations. First, it was not a randomized controlled trial. The reported rate of HBeAg seroconversion obtained by 6-month interferon-α monotherapy among Japanese patients was about 20 % [38], which is similar to the rate obtained by the sequential therapy used in our study (21 %). As compared with our previous study of lamivudine [14], the rate of sustained response in our present study of entecavir did not differ significantly (21 % in the entecavir group vs. 29 % in the lamivudine group). Although the patients were not randomly assigned to treatment, the baseline characteristics of the subjects did not differ between those in our previous study of lamivudine and those in the present study of entecavir with respect to mean age, sex ratio, ALT level, HBV DNA level, ratios of HBV genotypes, ratios of precore or basal core promoter mutants, or histopathological findings (data not shown). Thus, we cannot conclude that sequential therapy with entecavir and interferon-α is more effective than interferon-α monotherapy or sequential therapy with lamivudine and interferon-α. Second, we gave patients non-pegylated interferon-α for 6 months, because pegylated interferon-α had not been approved for the treatment of chronic hepatitis B by the Japanese medical insurance system during the study period. Further studies are thus needed to evaluate the efficacy of sequential therapy with entecavir and pegylated interferon-α.

To our knowledge, this is the first study to report on the response to sequential therapy with entecavir and interferon-α in patients with chronic hepatitis B. In summary, an off-treatment sustained response to sequential therapy with entecavir and interferon-α was achieved in 21 % of HBeAg-*positive* patients with chronic hepatitis B in Japan, where genotype C is predominant. This rate of response was not higher than that in our previous study using lamivudine [14]. Patients who had loss of HBeAg during entecavir treatment were more likely to have a sustained response to sequential therapy.

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Conflict of interest Dr. Shuhei Nishiguchi has received research grants from Bristol-Myers K.K. and Otsuka Pharmaceutical Co., Ltd. Dr. Norifumi Kawada has received research grants from Bristol-Myers K.K. and Otsuka Pharmaceutical Co., Ltd.

Appendix

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Significant background rates of HBV and HCV infections in patients and risks of blood transfusion from donors with low anti-HBc titres or high anti-HBc titres with high anti-HBs titres in Japan: a prospective, individual NAT study of transfusion-transmitted HBV, HCV and HIV infections

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Vox Sanguinis

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Background The Japanese Red Cross (JRC) conducted a prospective study to evaluate the frequency of transfusion-transmitted HBV, HCV and HIV infections to assess the risk of transfusion of blood components routinely supplied to hospitals.

Study Design and Methods Post-transfusion specimens from patients at eight medical institutes were examined for evidence of infection with HBV (2139 cases), HCV (2091) and HIV (2040) using individual nucleic acid amplification testing (NAT). If these specimens were reactive, pre-transfusion specimens were also examined for the virus concerned by individual NAT. In the event that the pre-transfusion specimen was non-reactive, then all repository specimens from implicated donors were tested for the viruses by individual donation NAT. In addition, a further study was carried out to evaluate the risk of transfusion of components from donors with low anti-HBc titres or high anti-HBc with high anti-HBs titres.

Results Transfusion-transmitted HCV and HIV infections were not observed. One case of post-transfusion HBV infection was identified (rate, 0·0004675; 95% CI for the risk of transmission, 1 in 451–41 841). The background rates of HBV, HCV and HIV infections in patients prior to transfusion were 3·4% (72/2139), 7·2% (150/2091) and 0% (0/2040), respectively. Sixty-four anti-HBc- and/or anti-HBs-reactive blood components were transfused to 52 patients non-reactive for anti-HBc or anti-HBs before and after transfusion (rate, 0; 95% CI for the risk of transmission, <1 in 22).

Conclusion This study demonstrated that the current criteria employed by JRC have a low risk, but the background rates of HBV and HCV infections in Japanese patients are significant.

Key words: nucleic acid amplification testing, occult HBV infection, transfusion-transmitted viral infection.

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Introduction

In Japan, all blood components are collected from non-remunerated voluntary donors by the Japanese Red Cross (JRC). Subsequently, the components are screened by serological testing for syphilis, HBV, HCV, HIV, HTLV-1 and human parvovirus B19, and serologically non-reactive components (the criteria to determine the suitability of blood for supply, which are based on infectious markers, are shown in Table 1) are then subjected to nucleic acid amplification testing (NAT) for HBV DNA, HCV RNA, and HIV-1 RNA in a mini-pool (MP) using an automated multiplex assay system (AMPLINAT MPX; Roche Diagnostics) that can amplify these three viruses simultaneously [1]. When all the above-mentioned tests are non-reactive, the blood components are supplied to hospitals. An important point is that unlike some other countries [2], blood components from donors non-reactive for HBsAg and HBV DNA but reactive for anti-HBc titres of <1:32 or anti-HBc titres of ≥1:32 with anti-HBs ≥200 mIU/ml are considered suitable for transfusion in Japan on the basis of a study of the correlation between anti-HBc titres and HBV DNA levels in blood units without detectable HBsAg [3, 4].

Since the introduction of the automated NAT multiplex assay system by the JRC in October 1999, the risk of transmission of HBV, HIV and HCV via transfusion has reduced significantly in Japan [5, 6]. However, several cases of transfusion-transmitted viral infections, especially of HBV, continue to occur each year [7]. This is partly because the doubling time of HBV is longer than that of HCV or HIV [8, 9], and thus, the NAT window period is also longer. The other reason is that occult HBV-infected donors [3] with low anti-HBc titres and low levels of HBV DNA that are not detected by MP-NAT may not be identified [10].

In this study, we randomly selected five JRC blood centres (Hokkaido, Iwate, Osaka, Ehime, and Fukuoka) and eight hospitals within the jurisdiction of these centres and prospectively investigated the risk involved in routine blood transfusion to patients in these hospitals. In addition, we also examined the safety of blood transfusion from anti-HBc-positive donors with anti-HBc titres of <1:32 or anti-HBc titres of ≥1:32 with anti-HBs ≥200 mIU/ml.

Materials and methods

Serological tests on donated blood

All donated blood samples were serologically screened as shown in Table 1.

NAT

The NAT screening system used in Japan has been reported previously by Mine *et al.* [12]. In brief, NAT screening is performed using a multiplex system capable of simultaneous detection of HBV DNA, HCV RNA and HIV-1 RNA to reduce the cost and ensure that the test is completed within 72 h. Samples are tested in MPs of 50 with the ability to detect 185–550 IU/ml for HBV, 3050–5600 IU/ml for HCV

Table 1 Criteria for infectious and other markers

	Serological tests		
Pathogens	**Contents**	**Methods**	**Criteria**
Syphilis	Serodiagnosis	Treponema pallidum particle agglutination (TPPA)[a]	Non-reactive
HIV	Anti-HIV-1/2	Agglutination of gelatin particles coated with recombinant HIV-1/2 proteins[a]	Non-reactive
HCV	Anti-HCV	Passive hemagglutination (PHA[b]) or particle agglutination (PA[a])	Non-reactive
HBV	HBsAg	Reverse passive hemagglutination (RPHA[c])	Non-reactive
	Anti-HBs	PHA[c]	-[d]
	Anti-HBc	Haemagglutination inhibition (HI[c])	
HTLV-1	Anti-HTLV-1	PA[a]	Non-reactive
B19	Anti-B19	Receptor-mediated hemagglutination (RHA[e])	
Others	Serum ALT	Method of Wroblewski and LaDue (11)	≤60 IU/ml

[a]Fujirebio Inc., Tokyo, Japan.

[b]Dainabot Co. Ltd., Tokyo, Japan.

[c]Reagents prepared by JRC.

[d]Blood units with the following profile were excluded from being transfused: 1. Specimen reactive for HBsAg on RPHA, with the result subsequently confirmed by enzyme immune assay (EIA). 2. Specimen reactive for anti-HBc at a dilution of 1:32 or higher on HI and in which anti-HBs is either absent or at a level of not more than 200 mIU/ml.

[e]RHA using reagents prepared by JRC.

and 1650–3300 IU/ml for HIV in donations contained within the pool.

In this study, HBV DNA, HCV RNA and HIV-1 RNA from patients were individually tested using the modified methods of Iizuka *et al.* [4], Okamoto *et al.* [13] and Matsumoto *et al.* [14], respectively, at the JRC NAT centres in Hokkaido and Kyoto. The analytical sensitivity cut-off of ID-NAT was 3·7–11 IU/ml for HBV, 61–112 IU/ml for HCV and 33–66 IU/ml for HIV [1].

Criteria for blood transfusion

The serological test criteria for the release of blood donations in Japan are shown in Table 1. Donations must also be non-reactive for HBV DNA, HCV RNA and HIV RNA on 50-MP-NAT.

Study design

Informed consent was obtained from each patient before transfusion between November 2003 and December 2006 at eight hospitals [Asahikawa Medical College Hospital (Hokkaido); Iwate Medical University Hospital (Iwate); Osaka City University Hospital, Osaka City General Hospital, Osaka Red Cross Hospital (Osaka); National Hospital Organization Shikoku Cancer Center, Ehime Red Cross Hospital (Ehime); and Fukuoka University Hospital (Fukuoka)]. In total, 2139 patients who survived 3 months after transfusion (approximately 40% of patients died of their original disease or complications within 3 months) were enrolled in this study. Their pre-transfusion blood specimens had been collected and cryopreserved in these hospitals (Fig. 1).

Approximately 3 months after blood transfusion, post-transfusion specimens were collected from the patients and individually tested for HBV DNA, HCV RNA and HIV-1 RNA at the JRC NAT centres. In the case of neonates and elderly patients, when the specimen volume was insufficient to perform NAT for all the three viruses, the priority of examination was HBV DNA >HCV RNA >HIV-1 RNA.

If the post-transfusion specimen was non-reactive for all the three viruses, the study was terminated for the patient concerned. However, if the specimen was reactive, the patient's cryopreserved pre-transfusion specimen was tested for the virus concerned by NAT. If the pre-transfusion specimen was reactive, it was concluded that the patient was infected before transfusion. However, if the pre-transfusion specimen was non-reactive, all repository specimens from the implicated donors, which were drawn at the time of blood donation and cryopreserved at the JRC NAT centres, were also tested for the virus concerned by ID-NAT, as reported by Satake *et al.* [15]. If these specimens were non-reactive and the case was restricted to HBV, the remaining pre-transfusion specimen of the patient was serologically tested for anti-HBc, anti-HBs and/or HBsAg using an enzyme immunoassay

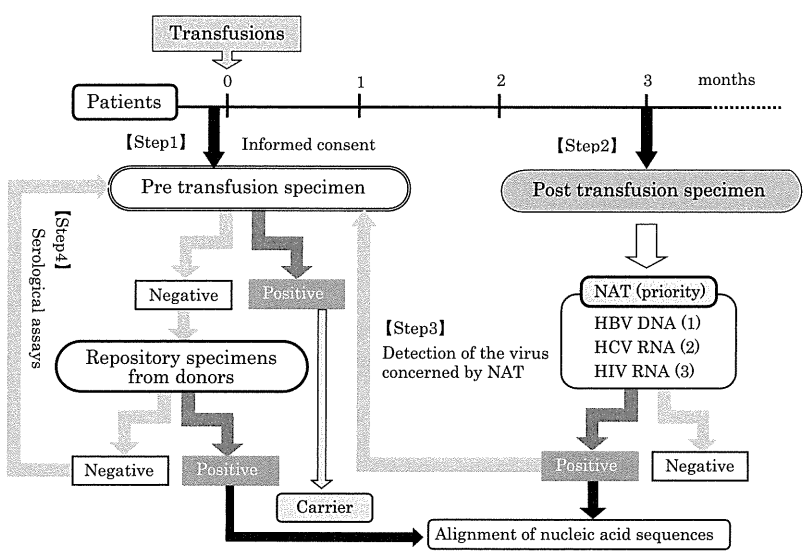

Fig. 1 Study design. Step 1: After obtaining informed consent from patients, pre-transfusion specimens were collected and cryopreserved. Step 2: Approximately 3 months after blood transfusion, post-transfusion specimens were collected from the patients at the eight hospitals and individually tested for HBV DNA, HCV RNA and HIV-1 RNA at the JRC nucleic acid amplification testing (NAT) centres. Step 3: If the post-transfusion specimen was reactive, the patient's pre-transfusion specimen was tested for the virus concerned by NAT. Step 4: If the pre-transfusion specimen was non-reactive (this was restricted to HBV alone), all repository specimens from the donors concerned were also tested for HBV DNA by ID-NAT. If these specimens were non-reactive, the remaining pre-transfusion specimen of the patient was then serologically tested for anti-HBc, anti-HBs and/or HBsAg.

(EIA) system in addition to the methods shown in Table 1.

Assessing the risk of blood transfusion from donors with low anti-HBc titres or high anti-HBc titres with high anti-HBs titres

We randomly selected 247 patients at the Osaka City University Hospital whose post-transfusion specimens were non-reactive for all the three viruses on NAT and 1009 blood components were transfused to these patients. Their pre- and post-transfusion specimens were tested for anti-HBs and anti-HBc. The repository specimens from the implicated donors were also examined to determine anti-HBs and anti-HBc titres and HBV DNA levels.

Results

The risk of transfusion-transmitted HBV, HCV and HIV infections

We examined 2139, 2091 and 2040 post-transfusion specimens for HBV DNA, HCV RNA and HIV-1 RNA, respectively, by NAT. The 2040 post-transfusion specimens were non-reactive for HIV-1 RNA (Table 2). Of the 2091 post-transfusion specimens, 150 specimens (7·2%) were reactive for HCV RNA (Table 2). However, the pre-transfusion specimens from the same 150 patients were also reactive for HCV RNA, indicating that the patients were already infected with HCV prior to the transfusion. Of the 2139 post-transfusion specimens, 73 (3·4%) specimens were reactive for HBV DNA (Table 2). Among these 73 patients, pre-transfusion specimens from 56 patients were reactive for HBV DNA, indicating that these patients were already infected with HBV prior to the transfusion. Pre-transfusion specimens from the remaining 17 patients were non-reactive for HBV DNA. Among these 17 patients, one patient who received 115 units of blood was judged to have transfusion-transmitted HBV infection on the basis of a donor-triggered look-back investigation on a donor, who was found to be reactive for HBV DNA at his next donation. The HBV DNA sequence of this donor was consistent with that of the patient. The repository specimens from the remaining 114 donors were non-reactive for HBV DNA.

Fourteen of the sixteen remaining patients were considered to have late-stage HBV infection because their pre-transfusion specimens were reactive for anti-HBc, and none of the repository specimens from the donors were reactive for HBV DNA. The other two patients were also considered to have late-stage infection because their HBsAg levels were relatively low (Table 3). According to additional information obtained from the hospital, one patient (No. 16 in Table 3) became infected with HBV several years ago and then periodically visited the hospital, and hospital records identified him as being HBsAg positive (AxSYM; Abbott Japan Co., Ltd, Tokyo, Japan). Considering their ages, diseases, therapies [16] administered to the patients, and follow-up observations by the hospitals, these 16 patients were strongly suggested to have occult hepatitis B infection (OBI).

The risk of transfusion of blood components from donors with low anti-HBc titres or high anti-HBc titres with high anti-HBs titres

None of the 1009 repository specimens were reactive for HBV DNA, but 86 of these specimens were reactive in the anti-HBc test at a titre of <1:32 (75 specimens) or ≥1:32

Table 2 Patient ages and HIV-1 RNA, HCV RNA and HBV DNA results

	Age		≤9	10–19	20–29	30–39	40–49	50–59	60–69	70–79	80–89	≥90	Total
HIV-1 RNA	Post-transfusion	Non-reactive	49	38	56	125	137	345	548	577	157	8	2040
		Reactive	0	0	0	0	0	0	0	0	0	0	0
	Total number		49	38	56	125	137	345	548	577	157	8	2040
HCV RNA	Post-transfusion	Non-reactive	55	38	59	128	134	330	517	518	154	8	1941
		Reactive	0	0	0	1	6	26	40	69	8	0	150
	Pre-transfusion	Non-reactive	0	0	0	0	0	0	0	0	0	0	0
		Reactive	0	0	0	1	6	26	40	69	8	0	150
	Total number		55	38	59	129	140	356	557	587	162	8	2091
HBV DNA	Post-transfusion	Non-reactive	79	43	61	129	135	334	546	574	156	9	2066
		Reactive	0	0	0	1	5	24	18	19	6	0	73
	Pre-transfusion	Non-reactive	0	0	0	0	0	5	4	5	3	0	17
		Reactive	0	0	0	1	5	19	14	14	3	0	56
	Total number		79	43	61	130	140	358	564	593	162	9	2139

Table 3 The details of 16 patients considered to have late-stage HBV infection

				Pre-transfusion				Post-transfusion			
No.	Age	Disease	Therapy	HBsAg	Anti-HBs (mIU/ml)	Anti-HBc	HBV DNA	HBsAg	Anti-HBs	Anti-HBc	HBV DNA
1	64	Heart disease	Operation	+	–	+	–	+	–	NT	+
2	54	Haematologic malignancy	HSCT	–	–	+	–	–	–	+	+
3	77	Gastric cancer	Chemotherapy	–	–	+	–	–	–	NT	+
4	60	AML	Chemotherapy	–	–	+	–	–	–	NT	+
5	56	Haematologic malignancy	HSCT	–	+	+	–	NT	–	+	+
6	76	Macroglobulinemia	HSCT	–	+ 2100	+	–	–	+	+	+
7	72	Oesophageal cancer	Chemotherapy	–	+ 134·2	+	–	–	+	+	+
8	57	Aplastic anaemia	HSCT	–	+ 5·2	+	–	–	+	+	+
9	89	Orthopaedic disorder	Operation	–	+ 34·9	+	–	–	+	+	+
10	70	Heart disease	Operation	–	+ 42·1	+	–	–	+	NT	+
11	77	Intracerebral haemorrhage	Operation	–	+ 1·4	+	–	–	+	NT	+
12	58	Gastric cancer	Chemotherapy	NT	+ 7·5	+	–	–	+	NT	+
13	58	Haematologic malignancy	HSCT	–	+	+	–	NT	NT	NT	+
14	82	Heart disease	Operation	NT	NT	+	–	–	+	+	+
15	80	Cancer	Chemotherapy	+2·52	–	−48·8	–	−1·69	–	+56·8	+
16	67	Gynaecological cancer	Chemotherapy	−1·87	–	–	–	+2·35	–	–	+

+, reactive or positive; –, non-reactive or negative; NT, not tested; HSCT, haematopoietic stem cell transplantation; AML, acute myelocytic leukaemia; HBsAg, anti-HBs and anti-HBc (Nos. 15 and 16) measured by EIA (AxSYM) in the hospital (normal range = HBsAg, S/N of <2·00; anti-HBc, % INH (inhibition) of <50·0) because the specimen volume was not sufficient to perform RPHA, PHA and HI.

Table 4 Analysis of blood components (n = 1009) transfused to 247 randomly selected patients negative for all three viruses on NAT

		Anti-HBc (HI) 2^n									
		0	1	2	3	4	5	6	7	8	Total
Anti-HBs (PHA) 2^n	0	896	14	13	6	3	Excluded from blood transfusion				932
	1	5	2		1	1					9
	2	8		2	3	1					14
	3	3	1	1	2	1					8
	4	3		3		3					9
	5			2	1		1	1		1	6
	6	3		1	1		1	1	1		8
	7	2		2	4	3				1	12
	8	1			1	2					4
	9	1				1			2	1	5
	10	1									1
	11							1			1
	Total	923	17	24	19	15	2	3	3	3	1009
			75				11				

NAT, nucleic acid amplification testing.

Values indicate the number of blood components with titres (2^n) of anti-HBc and anti-HBs transfused.

For example, '14' blood components with titres of anti-HBc and anti-HBs of 2^1 and 2^0, respectively, were transfused to patients.

with an anti-HBs titre of >1:32 that corresponds to 200 mIU/ml (11 specimens) (Table 4). All of the 86 donations met criteria for release for transfusion in Japan (Table 1). Of the 247 patients tested, neither pre- nor post-transfusion specimens from 165 patients were reactive for anti-HBs or anti-HBc, although 52 of these patients received blood components (total of 64) that were serologically reactive for anti-HBs and/or anti-HBc (Fig. 2). In

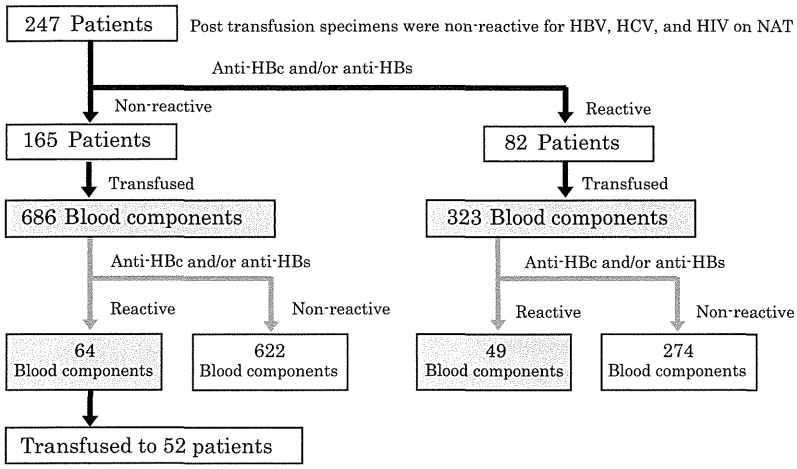

Fig. 2 The risk of transfusion of blood components with low anti-HBc and/or high anti-HBs titres. A total of 247 patients whose post-transfusion specimens were non-reactive for HBV, HCV and HIV on nucleic acid amplification testing were randomly selected, and their pre- and post-transfusion specimens were tested for anti-HBc and anti-HBs. One hundred and sixty-five patients showed non-reactivity for anti-HBc and anti-HBs in both their pre- and post-transfusion specimens, and they were transfused 686 blood components. The remaining 82 patients were reactive for anti-HBc or anti-HBs, and they were transfused 323 blood components. The repository specimens from the donors concerned were examined to determine the anti-HBc and anti-HBs titres. Of the 686 blood components, 64 were reactive for anti-HBc and/or anti-HBs and were transfused to 52 patients whose pre- and post-transfusion specimens were non-reactive for HBV DNA, anti-HBc and anti-HBs.

other words, 64 blood components that were serologically reactive for anti-HBs and/or anti-HBc were transfused to 52 patients, but no reactivity was observed for any of the HBV markers (HBsAg, anti-HBs, anti-HBc and HBV DNA) before and after transfusion.

Discussion

We commenced this study in November 2003 when the tests for post-transfusion hepatitis were not routinely performed in hospitals in Japan, including most of the hospitals that participated in this study. Medical insurance covered the cost of the tests for post-transfusion hepatitis only when a doctor suspected its possibility, and thus, a number of transmissions might have been missed. Similarly, anti-HBc and anti-HBs tests were also not performed before transfusions. Therefore, the JRC conducted this study to try to identify more accurately the transmission rate of infections in all patients receiving blood transfusions in the specified hospitals. The number of patients participating in this study was, however, insufficient to develop statistically significant rates.

Immediately after this study started, a post-transfusion specimen from a patient was found to be reactive for HBV DNA. However, neither the pre-transfusion specimen from the patient nor the repository specimen from the donor concerned was reactive for HBV DNA. Consequently, the remaining pre-transfusion specimen was tested and found to be reactive for anti-HBc. Therefore, it was concluded that the patient had been infected with HBV before transfusion–i.e., he had so-called OBI [17–20]. This demonstrates that OBI is an important issue among patients in Japan, especially in older patients [15] and patients receiving immunosuppressive therapies such as chemotherapy [21–23]. Since the completion of this study, medical insurance has been available to cover the cost of laboratory tests performed to evaluate viral markers of HBV, HCV and HIV (including anti-HBc) in all patients receiving blood transfusions. Furthermore, considering the significant background rates of HCV (7·2%) and HBV (3·4%) infections seen in Japanese patients, the Ministry of Health, Labor and Welfare has developed guidelines for the timing and testing required to support early detection of transfusion-transmitted HBV, HCV and HIV infections (Table 5). Pre-transfusion specimens can be tested for HBsAg, anti-HBs, anti-HBc, HCV core antigen, anti-HCV and anti-HIV. If these specimens are non-reactive for all the viral markers, post-transfusion specimens are tested for HBV DNA, HCV core antigen and anti-HIV. If any of the viral markers are reactive in pre-transfusion specimens, there is no requirement to undertake further testing for the viruses concerned in post-transfusion specimens. Of course, we can cryopreserve pre-transfusion specimens as performed in this study, and if the post-transfusion specimens are reactive for HBV DNA, HCV core antigen or anti-HIV, the cryopreserved pre-transfusion specimens can then be tested for the relevant viral markers.

The reasons for the high background rates of HBV and HCV infections, especially among older patients, are unclear; however, these rates might partly be the result of the reuse of needles and syringes for vaccination during

Table 5 The guideline of test markers for early detection of transfusion associated HBV, HCV and HIV infections in Japan

	Pre-transfusion	Post-transfusion	
Virus	**Test markers**	**When to test**	**Test markers**
HBV	HBsAg Anti-HBs Anti-HBc	Approximately 3 months later	HBV DNA
HCV	HCV core antigen Anti-HCV	1–3 months later	HCV core antigen
HIV	Anti-HIV	2–3 months later	Anti-HIV

childhood to save costs, a practice that lasted until the 1980s, or to the use of plasma anticoagulant products such as fibrinogen and factor VIII, which were not pathogen inactivated in the 1980s and 1990s. In fact, according to a report by Tanaka *et al.* [24], the prevalence of HBV and HCV in first-time blood donors was 0·63% (1·5% estimated for donors above 50 years) and 0·49% (2% estimated for donors above 50 years and 3% for donors above 60 years), respectively.

Transfusion-transmitted HCV or HIV infection was not observed in this study. The patient with confirmed transfusion-transmitted HBV infection was a 61-year-old man with acute myeloid leukaemia, and he underwent hematopoietic stem cell transplantation. On 29 November 2003, the patient received a platelet transfusion. The platelet component was derived from a donor on November 27. The donor's next donation was on 30 December 30 2003, and his blood sample was found to be reactive for HBV DNA using the 50 donation MP-NAT. A look-back study of the donor revealed that HBV DNA was detectable by ID-NAT in the repository specimen collected on 27 November 2003. Serum drawn from the patient on 26 January 2004 (on the same day when the result of look-back was obtained) was also reactive for HBV DNA, but the DNA level was too low (30 copies/ml) to sequence. His pre-transfusion specimen was non-reactive for HBsAg, anti-HBc, anti-HBs and HBV DNA. A total of 115 units of blood including this platelet component transfused to him were implicated, and the repository specimens from the donations were tested for HBV by ID-NAT. All specimens except the one identified previously were non-reactive for HBV DNA. Approximately 4 months later (1 June 2004), the patient's HBV DNA level was elevated (≥1000 copies/ml) along with leukaemia recurrence, and the specified HBV DNA sequences were consistent with those of the original donor. As the HBV DNA level in the patient was monitored, immediate administration of lamivudine (when the HBV DNA level was >1000 copies/ml), a nucleoside analogue reverse transcriptase inhibitor, prevented the development of acute hepatitis.

Despite the implementation of NAT screening, several cases of transfusion-transmitted HBV infection continue to occur each year in Japan [25]. One reason may be that only a few patients are immunized with a hepatitis B vaccine because only selective vaccination against HBV is carried out in Japan (medical staff, coworkers and babies born to HBV carrier mothers). In addition, donors in the early and late stages of HBV infection may have low HBV DNA levels that are detectable in ID-NAT but not by 50-NAT [26]. The patient discussed earlier is a typical case of transfusion of a blood component from a donor with an early acute HBV infection. The risk of HBV transmission identified in this study was 0·0004675 (95% CI for the risk of transmission, 1 in 451–41 841). However, data from donor-triggered look-back studies involving more than 10 000 cases between 2000 and 2004 [15] have been used to assess the residual risks of transfusion-transmission of these three viruses. On the basis of data reported by transfusion monitoring hospitals in Tokyo, the number of patients receiving blood transfusions was calculated to be 1·2 million per year in Japan [27]. The risks of transfusion-transmitted HBV, HCV and HIV infections were estimated at 13–17 cases per year (1 in 70 588–92 307), 1 case every 2–4 years (1 in 2 400 000–4 800 000), and 1 case in 4 years (1 in 4 800 000), respectively. In fact, 74, 41 and 0 cases of HBV, HCV and HIV infections, respectively, associated with transfusion were reported to the JRC in 2007. Investigation of these confirmed transfusion as the cause of 13 cases of HBV and 1 case of HCV [28].

Hollinger [29] has indicated that the reagents used in Japan to test blood donations and the criteria used by the JRC for the release of donations are different from those used in Western countries [30–33]. In Japan, if a specimen is reactive for anti-HBc at a titre of <1:32 based on a hemagglutination inhibition test or is ≥1:32 along with a passive hemagglutination inhibition assay revealing an anti-HBs titre of ≥200 mIU/ml, the blood components can be transfused to patients [3, 4]. We attempted to improve our understanding of the risk of routine transfusion of blood components to patients when these criteria are used. Pre-transfusion specimens, cryopreserved in Osaka City University hospitals, were tested for anti-HBs and anti-HBc. The specimens were non-reactive for HBsAg in all 247 patients tested but were serologically reactive for anti-HBs and/or anti-HBc in 82 patients. The remaining 165 patients whose pre-transfusion specimens were non-reactive for HBsAg, anti-HBc, anti-HBs and HBV DNA were transfused with 686 blood components. The repository specimens of the 686 donors concerned were tested for HBsAg, anti-HBs, anti-HBc and HBV DNA. Specimens of 64 of the donors were reactive for anti-HBs and/or anti-HBc, and their blood components (64) were transfused to 52 patients. None of

the HBV markers changed in those patients receiving these components indicating that the blood components with low anti-HBc and/or high anti-HBs titres and with non reactive results for HBV DNA by MP-NAT have a low risk (rate, 0; 95% confidence interval for the risk of transmission, <1 in 22) (Fig. 2).

The JRC implemented a chemiluminescent EIA system (Fujirebio Inc., Tokyo, Japan) in 2008 replacing the earlier agglutination method. We have continued the same strategy of using blood from donors with low anti-HBc titres (cut-off index <12) or high anti-HBc titres (cut-off index≥12) with high anti-HBs titres (≥200 mIU/ml) for transfusion because discarding these blood components (86/1009, 8·5% in Table 4) would have a huge influence on our ability to maintain a stable blood supply to hospitals. However, most of these donors are ≥50 years in most cases [34], and it is likely that we will be able to review this approach and adopt a policy of only issuing anti-HBc-negative blood components in the future. Meanwhile, we will continue to evaluate the residual risk of blood transfusion from donors with low anti-HBc titres or high anti-HBc titres with high anti-HBs titres.

Collaborating institutes

Asahikawa Medical College Hospital (Hokkaido).
Iwate Medical University Hospital (Iwate).
Osaka City University Hospital (Osaka).
Osaka Red Cross Hospital (Osaka).
Osaka City General Hospital (Osaka).
Ehime Red Cross Hospital (Ehime).
National Hospital Organization Shikoku Cancer Center (Ehime).
Fukuoka University Hospital (Fukuoka).

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Conflict of interest

The authors have no conflict of interest to declare regarding this manuscript.

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Hepatitis B Virus-Specific miRNAs and Argonaute2 Play a Role in the Viral Life Cycle

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Abstract

Disease-specific serum miRNA profiles may serve as biomarkers and might reveal potential new avenues for therapy. An HBV-specific serum miRNA profile associated with HBV surface antigen (HBsAg) particles has recently been reported, and AGO2 and miRNAs have been shown to be stably associated with HBsAg in serum. We identified HBV-associated serum miRNAs using the Toray 3D array system in 10 healthy controls and 10 patients with chronic hepatitis B virus (HBV) infection. 19 selected miRNAs were then measured by quantitative RT-PCR in 248 chronic HBV patients and 22 healthy controls. MiRNA expression in serum versus liver tissue was also compared using biopsy samples. To examine the role of AGO2 during the HBV life cycle, we analyzed intracellular co-localization of AGO2 and HBV core (HBcAg) and surface (HBsAg) antigens using immunocytochemistry and proximity ligation assays in stably transfected HepG2 cells. The effect of AGO2 ablation on viral replication was assessed using siRNA. Several miRNAs, including miR-122, miR-22, and miR-99a, were up-regulated at least 1.5 fold ($P<2E-08$) in serum of HBV-infected patients. AGO2 and HBcAg were found to physically interact and co-localize in the ER and other subcellular compartments. HBs was also found to co-localize with AGO2 and was detected in multiple subcellular compartments. Conversely, HBx localized non-specifically in the nucleus and cytoplasm, and no interaction between AGO2 and HBx was detected. SiRNA ablation of AGO2 suppressed production of HBV DNA and HBs antigen in the supernatant.

Conclusion: These results suggest that AGO2 and HBV-specific miRNAs might play a role in the HBV life cycle.

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Introduction

Hepatitis B virus (HBV) is a partially double-stranded DNA virus in the Hepadnaviridae family [1]. New therapies are urgently needed for the 350 million chronically infected individuals who face a significantly elevated lifetime risk of cirrhosis and hepatocellular carcinoma [2,3]. Recent insight into the role of non-coding RNAs in the liver has highlighted potential applications of microRNAs (miRNAs) in HBV diagnosis and treatment [4,5,6,7,8,9].

MiRNAs are a class of short non-coding RNAs involved in post-transcriptional gene regulation of multiple pathways [10]. In contrast to messenger RNAs, exosome-free extracellular miRNAs may be nuclease-resistant and remain in circulation for long periods of time by being stably bound to AGO2, a component of the RNA-induced silencing complex [11]. The origin and function of these extracellular miRNAs is unclear, but they may serve as biomarkers for liver injury and cancer [4]. Elucidating the function of hepatic miRNAs in HBV infection is important in the development of strategies to eradicate the virus and assess the risk of HCC. A number of miRNAs have been shown to be up- or down-regulated in HBV infection [4,12,13]. Noting that the defective hepatitis delta virus co-opts HBsAg subviral particles for export, Novellino et al. hypothesized that HBsAg subviral particles might also sequester miRNAs from the liver [5]. Using HBsAg immunoprecipitation, they identified a set of liver-specific and immune regulatory AGO2-bound miRNAs associated with HBsAg.

These reports suggest that AGO2 and a specific subset of miRNAs may participate in HBV replication, either as part of a host anti-HBV defense or as viral strategy to exploit or evade the RISC machinery. In this study, we examined serum miRNA expression in chronic HBV and healthy individuals and found a specific subset of miRNAs that are over-expressed in HBV-positive

patients and in which miR-122 was strongly up-regulated. To determine whether components of the miRNA system are associated with other HBV components, we performed subcellular localization experiments with viral proteins and AGO2.

Materials and Methods

Study Subjects

We performed a series of experiments to compare miRNA profiles of healthy and HBV-infected individuals in serum and liver tissue. All patients had chronic hepatitis B and agreed to provide blood samples for a viral hepatitis study. Patient profiles are shown in Table 1. Histopathological diagnosis was made according to the criteria of Desmet et al. [14]. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki, and all patients provided written informed consent. This study was approved a priori by the ethical committee of Hiroshima University.

miRNA Expression Levels in Serum

miRNA expression in serum samples was measured using the Toray Industries miRNA analysis system, in which serum miRNA samples were hybridized to 3D-Gene human miRNA ver12.1 chips containing 900 miRNAs (Toray Industries, Inc., Tokyo, Japan). MiRNA gene expression data were scaled by global normalization, and differential expression was analyzed using the limma package in the R statistical framework. Serum was collected from 20 patients with high HBV DNA and HBsAg levels and with either high (>42 IU/l) or low (≤42 IU/l) ALT levels. Serum from the 10 low ALT patients was analyzed as a mixture, whereas serum from each of the 10 high ALT patients was analyzed both separately and as a mixture. For comparison with healthy controls we collected separate mixtures of serum from 10 healthy females and 12 healthy males. Serum samples from each healthy female were also measured separately. All healthy controls were negative for HBsAg, HBcAb, and HCV Ab. For comparison with miRNA expression in hepatocytes, miRNA expression was measured in non-tumor biopsy tissue from an HBV-infected patient and compared to non-cancerous liver tissue samples from two patients without HBV or HCV infection.

Table 1. Clinical characteristics of chronic hepatitis B virus patients (n = 248).

Factor	Value
Age	44 (15–76)
Sex (male/female)	169/77
Alanine aminotransferase (IU/l)	56 (10–1867)
Aspartate aminotransferase (IU/l)	43.5 (15–982)
HBV DNA (IU/ml)	6.3 (1.8–9.1)
Liver fibrosis (1/2/3/4)	69/102/46/26
Necroinflammatory activity (0/1/2/3/4)	1/70/127/45/0
γ-glutamyl transpeptidase (IU/l)	43 (9–459)
Alpha-fetoprotein (μg/l)	6.15 (0–9400)
Promthrombin time (s)	93 (0–146)
Albumin (g/dl)	4.4 (0–5.2)
Platelets (x10^4/mm^3)	16.75 (1–36)
HBsAg (IU/l)	2765 (0.05–239000)
HBeAg (−/+)	115/127
HBeAb (−/+)	113/128

Continuous variables are shown as median and range, and categorical variables are shown as counts.
Fibrosis and necroinflammatory activity were scored according to the criteria of Desmet et al. [14].
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Quantitative Real-time Polymerase Chain Reaction miRNA Analysis

Using real-time polymerase chain reaction (RT-PCR) we measured the expression of 19 miRNAs in serum from 248 patients with chronic HBV infection and from 10 healthy females and 12 healthy males. Circulating microRNA was extracted from 300 μl of serum samples using the mirVana PARIS Kit (Ambion, Austin, TX) according to the manufacturer's instructions. RNA was eluted in 80 μl of nuclease free water and reverse transcribed using TaqMan MicroRNA Reverse Transcription Kit (Life Technologies Japan, Tokyo, Japan). *Caenorhabditis elegans* miR-238 (cel-miR-238) was spiked to each sample as a control for extraction and amplification steps. The reaction mixture contained 5 μl of RNA solution, 2 μl of 10× reverse transcription buffer, 0.2 μl of 100 mM dNTP mixture, 4 μl of 5× RT primer, 0.25 μl of RNase inhibitor and 7.22 μl of nuclease free water in a total volume of 20 μl. The reaction was performed at 16°C for 30 min followed by 42°C for 30 min. The reaction was terminated by heating the solution at 85°C for 5 min. MiRNAs were amplified using primers and probes provided by Applied Biosystems using TaqMan MicroRNA assays according to the manufacturer's instructions. The reaction mixture contained 12.5 μl of 2× Universal PCR Master Mix, 1.25 μl of 20× TaqMan Assay solution, 1 μl of reverse transcription product and 10.25 μl of nuclease free water in a total volume of 25 μl. Amplification conditions were 95°C for 10 min followed by 50 denaturing cycles for 15 sec at 95°C and annealing and extension for 60 sec at 60°C in an ABI7300 thermal cycler. For the cel-miR-238 assay, a dilution series using chemically synthesized miRNA was used to generate a standard curve that permitted absolute quantification of molecules.

Pathway Analysis

Target genes of differentially expressed miRNAs were predicted based on agreement among three miRNA prediction tools, miRanda, miRBase, and TargetScan. Gene Set Enrichment Analysis (http://www.broadinstitute.org/gsea) was used to identify significantly over-represented gene ontology (GO) terms among the predicted targets.

Plasmid Construction

The construction of wild-type HBV 1.4 genome length, pTRE-HB-wt, was described previously [15]. We used pTRE2 vector without pTet-off vector and doxycycline because a sufficient amount of HBV transcript was produced from internal HBV promoters, and transcription from the pTRE2 promoter is negligible under these conditions. The nucleotide sequence of the HBV genome that we cloned into plasmids pTRE-HB-wt was deposited into GenBank under accession number AB206817.

Cell Culture

HepG2 cells, derived from a human hepatoma cell line, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum at 37°C and under 5% CO_2. For the production of stably transfected cell lines, HepG2 cells were transfected with 20μg of the plasmid pTRE-HB-wt by calcium precipitation and the transfected cells were selected with

400μg/ml hygromycin-included DMEM. Sixty colonies were isolated, and clones that were positive for both HBs and HBe antigens were selected. Finally, one cell line named T23 was selected and used for further experiments. T23 cells continuously produced more than 6 log copies/ml of HBV DNA in supernatant over more than 12 months (data not shown).

Immunocytochemistry

Co-localization between AGO2 and several HBV proteins (HBc, HBs, and HBx) was analyzed using immunocytochemistry, followed by cellular localization assays using antibodies targeting various sub-cellular compartments. HepG2 or T23 cells were seeded in 2-well chamber plates and harvested 48 hours after seeding. The cells were washed with PBS and fixed with 4% (v/v) paraformaldehyde. After fixation, the cells were stained with several primary antibodies (Table S1). The bound antibodies were detected with an Alexa 488-conjugated antibody against rabbit IgG (1:2000) or Alexa 568-conjugated antibody against mouse IgG (1:2000), respectively (Molecular Probes, Eugene, OR). Nuclei were counterstained with 6-diamidino-2-phenylindole (DAPI) (Vector laboratories, Burlingame, CA). The stained cells were examined with a Fluoview FV10i microscope (Olympus, Tokyo, Japan).

In situ Proximity Ligation Assay

We used proximity ligation assays (PLA) to determine whether AGO2 and HBc physically interact. PLA is a recent method to detect protein-protein interactions using protein-DNA conjugates that can be detected using fluorescence microscopy [16]. PLA improves on traditional immunoassays by directly detecting even weak or transient protein interactions [16]. HepG2 and T23 cells were seeded in 2-well chamber plates and harvested 48 hours after seeding. The cells were washed with PBS and fixed with 4% (v/v) paraformaldehyde. After fixation, the cells were stained with primary antibodies. The primary antibodies used are listed in Table S1. After overnight incubation with primary antibody at 4°C, PLA was performed using Duolink II PLA probe anti-rabbit plus and anti-mouse minus and Duolink II Detection Reagents Orange (Olink, Uppsala, Sweden) following the manufacturer's protocol. Nuclei were counterstained with DAPI. Imaging was performed using a Fluoview FV10i microscope.

Analysis of Supernatant HBV Production by RNA Interference Against AGO2

To investigate the necessity of AGO2 for HBV production, we performed RNA interference assay using T23 cells that are HepG2 cells stably transfected with the plasmid pTRE-HB-wt. We used Silencer Select Pre-designed siRNA small interfering RNA targeting *AGO2* (#s25932, Ambion, Austin, TX) and Silencer Select Negative Control #1 siRNA for control (Ambion). T23 cells were transfected with one of the siRNA oligonucleotides (10 nM) using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. To examine the knockdown effect of siRNAs against *AGO2* by real-time quantitative RT-PCR, T23 cells transfected with siRNAs were harvested 72 hours after transfection. Total RNA was isolated using the QuickGene RNA cultured cell kit S (Fujifilm, Tokyo, Japan). One μg of each RNA sample was reverse transcribed with the SuperScript VILO cDNA Synthesis kit (Invitrogen). First-strand complementary DNA (cDNA) was amplified with specific primers for the coding sequence of *AGO2*. The primers were as follows: forward, 5′-CCAGCATACTACGCTCACCT-3′; reverse, 5′-CAGAGTGTCTTGGTGAACCTG-3′. We quantified *AGO2* mRNA with EXPRESS SYBR Green ER qPCR Supermix Universal (Invitrogen) according to the manufacturer's instructions. Amplification and detection were performed using the Mx3000P Multiplex quantitative PCR system (Stratagene, La Jolla, CA). Results were normalized to the transcript levels of the housekeeping reference gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Three to seven days after transfection, the culture media were collected to examine HBV production in supernatant. HBs antigen was measured quantitatively using the Abbott chemiluminescence immunoassay kit (Abbott Japan, Tokyo, Japan). HBV DNA levels were determined by Cobas TaqMan HBV standardized real-time PCR assay (Roche Molecular Systems, Pleasanton, CA). Results are expressed in log10 international units/ml. We also evaluated viability of cells using the Cell Counting kit-8 (Dojindo Laboratories, Kumamoto, Japan) at 3, 5 and 7 days after transfection, according to the manufacturer's instructions. All assays were performed in triplicate, and the results are expressed as mean ± SD.

Statistical Analysis

All analyses were performed using the R statistical package (http://www.r-project.org). Continuous variables are reported using the median and range. Moderated t statistics or Mann Whitney U tests were used to detect significant associations, as appropriate, and P-values were adjusted for multiple testing based on the false discovery rate.

Results

MiRNA Microarray Results

We performed miRNA microarray analysis to identify HBV-associated differences in serum miRNA profiles between 10 chronic HBV patients and 10 healthy controls (Fig. S1). 26 miRNAs with an absolute log fold change greater than 1.5 were found to be significantly ($P_{FDR} < 0.05$) up-regulated in serum of HBV patients, and 8 miRNAs were significantly down-regulated (Table 2). MiR-122, miR-22, and miR-99a levels were the most strongly up-regulated in serum of HBV-infected patients, and levels of miR-575, miR-125a-3p, and miR-4294 were the most down-regulated. We also examined miRNAs associated with presence of HBe antigen or HBe antibody, but no miRNAs were significant following correction for multiple testing (data not shown).

Analysis of Serum Sample Mixtures from HBV-infected Patients and Healthy Controls

In addition to individual serum samples, we also examined 4 pooled serum samples as follows: 10 healthy males, 10 healthy females, 10 HBV patients with low ALT levels, and 10 HBV patients with high ALT levels (Fig. S2). In agreement with results from individual analysis, miR-122 and miR-99 levels were significantly higher in serum from HBV serum samples compared to healthy control samples (Table 2). Corresponding results with a log change greater than 1.5 were found for several other miRNAs, including miR-22, miR-642b, miR-125b (up-regulated) and miR-575 and miR-4294 (down-regulated), but results were not significant following correction for multiple testing in the mixture samples due to the small number of samples compared.

RT-PCR Analysis

Serum levels of 19 miRNAs were analyzed using quantitative RT-PCR analysis of 250 chronic HBV patients and 20 healthy controls. Several miRNAs (miR-122, miR-22, miR-99a, miR-720, miR-125b, and miR-1275) were significantly up-regulated in

Table 2. Top 10 up- or down-regulated serum miRNAs associated with chronic HBV infection.

Sample	Direction	miRNA	logFC	AveExpr	t	P	P_{FDR}
Serum	Up	hsa-miR-122	5.97	9.09	12.84	3.27E−12	3.06E−09
		hsa-miR-99a	2.59	6.20	10.73	2.11E−10	2.19E−08
		hsa-miR-22	2.49	9.55	10.47	2.10E−10	2.19E−08
		hsa-miR-191	2.19	8.42	11.87	1.68E−11	3.93E−09
		hsa-miR-642b	2.03	10.07	9.93	5.92E−10	4.26E−08
		hsa-miR-125b	1.95	5.99	8.72	9.91E−09	4.21E−07
		hsa-miR-486-3p	1.79	9.09	8.01	3.19E−08	9.95E−07
		hsa-miR-378	1.78	5.97	9.94	9.00E−10	6.02E−08
		hsa-miR-320d	1.70	7.19	7.88	4.25E−08	1.21E−06
		hsa-miR-23b	1.69	8.99	7.62	7.64E−08	1.93E−06
	Down	hsa-miR-575	−2.10	8.35	−10.00	5.20E−10	4.05E−08
		hsa-miR-125a-3p	−1.99	7.22	−11.91	1.56E−11	3.93E−09
		hsa-miR-4294	−1.75	11.82	−11.37	4.07E−11	7.63E−09
		hsa-miR-92a-2*	−1.64	11.03	−7.70	6.36E−08	1.75E−06
		hsa-miR-1202	−1.59	8.60	−12.41	6.72E−12	3.14E−09
		hsa-miR-30c-1*	−1.31	6.29	−8.66	1.12E−08	4.35E−07
		hsa-miR-1275	−1.19	9.91	−7.50	1.00E−07	2.35E−06
		hsa-miR-3197	−1.05	11.46	−8.58	9.24E−09	4.21E−07
		hsa-miR-1908	−1.03	13.75	−9.05	3.49E−09	2.04E−07
Mixture	Up	hsa-miR-122	6.80	9.09	20.51	1.09E−06	0.001
		hsa-miR-99a	2.58	6.34	9.32	9.80E−05	0.037
		hsa-miR-22	2.07	8.60	3.16	0.020	0.528
		hsa-miR-125b	2.03	6.29	5.09	0.002	0.264
		hsa-miR-1915*	1.80	8.32	6.24	0.001	0.158
		hsa-miR-3648	1.69	14.16	5.06	0.002	0.264
		hsa-miR-642b	1.64	9.82	4.49	0.004	0.377
		hsa-miR-1288	1.39	6.43	3.56	0.012	0.528
		hsa-miR-325	1.30	4.91	2.87	0.047	0.586
		hsa-miR-486-3p	1.29	8.98	3.87	0.009	0.480
	Down	hsa-miR-575	−1.95	8.43	−6.38	0.001	0.158
		hsa-miR-4294	−1.79	11.95	−5.99	0.001	0.158
		hsa-miR-654-3p	−1.35	5.36	−2.99	0.042	0.569
		hsa-miR-1202	−1.24	8.52	−3.97	0.008	0.480
		hsa-miR-1237	−1.06	7.52	−3.10	0.022	0.531
		hsa-miR-744	−1.03	9.51	−2.91	0.028	0.545

Expression levels were compared using moderated t-statistics, and P-values were corrected for multiple testing using the false discovery rate.
logFC: log2 fold-change between patients with chronic HBV infection relative to healthy individuals.
AveExpr: The average log2 expression level for each miRNA over all samples.
t: moderated t-statistic for patients with chronic HBV infection compared to healthy individuals P for each miRNA.
P: uncorrected P-value for t-test.
P_{FDR}: P-value adjusted for multiple testing based on the false discovery rate.
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serum from HBV-infected patients (Table 3). Agreement of microarray and RT-PCR results was strongest for up-regulation of miR-122, miR-22, and miR-125b in serum of HBV patients. To determine whether there is a linear relationship between HBV markers and HBV-associated miRNAs, we analyzed the correlation between HBsAg and 6 up-regulated miRNAs. MiR-122, miR-99a, and miR-125b levels were found to be significantly correlated with HBsAg levels with $R^2>0.5$ (Fig. S3). These three miRNAs were also significantly correlated with HBV DNA titers, with R^2 of about 0.4 (Fig. S4). MiR-122 and miR-22 were significantly but diffusely associated with serum ALT levels ($R^2>0.2$; Fig. S5). To identify miRNAs associated with different phases of HBV infection, we also analyzed the 6 significantly up-regulated miRNAs with respect to the presence of HBe antigen and antibody. MiR-122, miR-99a, miR-720, and miR-125b were each highly significantly elevated in chronic HBV patients who were positive for the HBe antigen (P<4.0E−07; Fig. S6). Similarly, each miRNA was significantly elevated in chronic HBV patients who were negative for the HBe antibody (P<9.1E−05; Fig. S7).

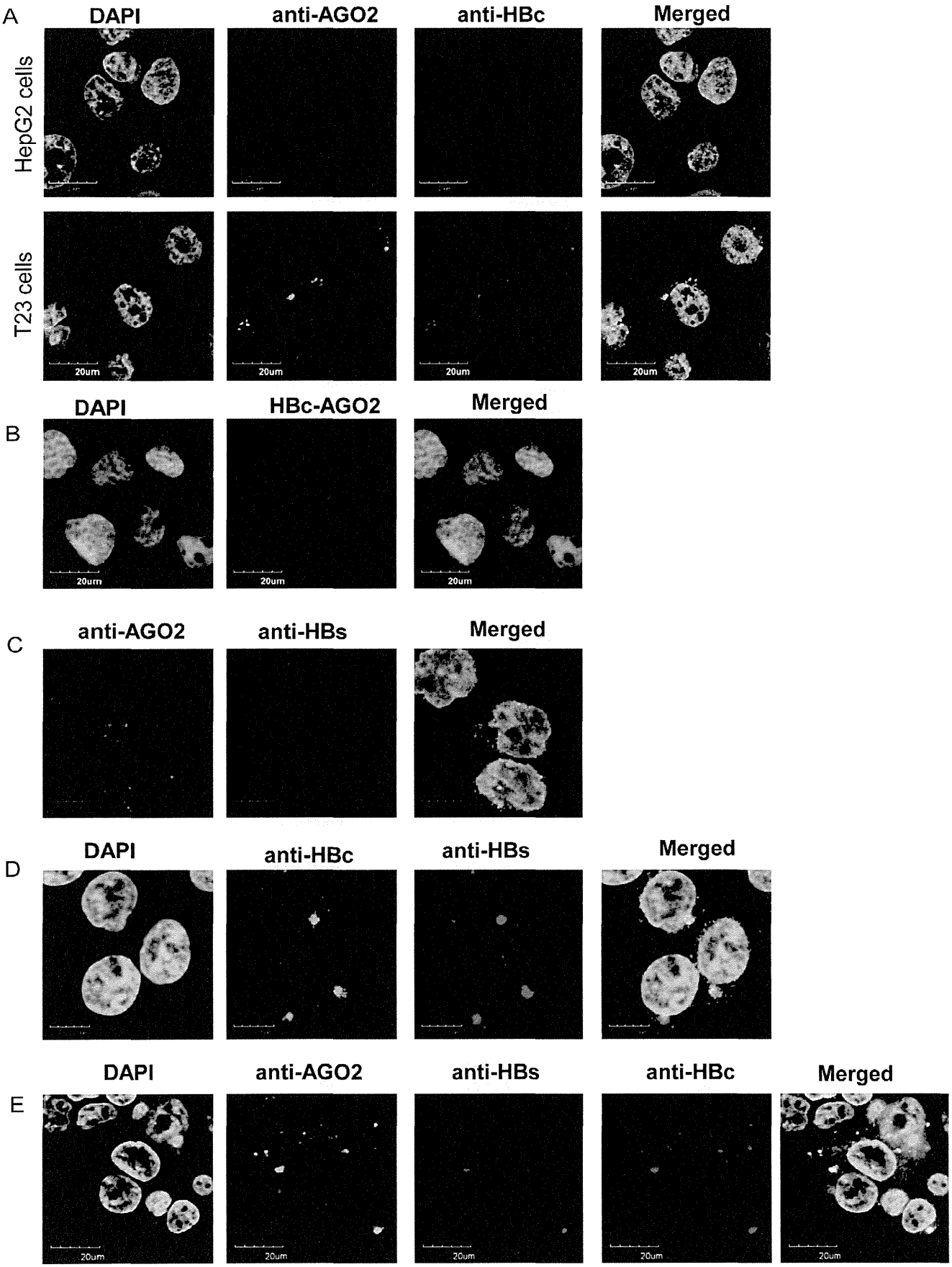
A
DAPI
anti-AGO2
anti-HBc
Merged
HepG2 cells
T23 cells
20um
B
DAPI
HBc-AGO2
Merged
C
anti-AGO2
anti-HBs
Merged
D
DAPI
anti-HBc
anti-HBs
Merged
E
DAPI
anti-AGO2
anti-HBs
anti-HBc
Merged

Figure 1. Co-localization of HBcAg and HBsAg with AGO2 in stably transfected T23 cells. A) Anti-AGO2 and anti-HBc staining overlapped in stably transfected T23 cells, but not in HepG2 control cells, suggesting an interaction between HBc and AGO2. B) HBc-AGO2 was detected in T23 but not HepG2 cells using proximity ligation assays (PLA), suggesting a protein-protein interaction between HBcAg and AGO2. C) Overlap of anti-AGO2 and anti-HBs staining suggests co-localization of HBs and AGO2. D) Anti- HBc, and anti-HBs staining overlapped in T23 cells, which may indicate that HBc and HBs co-localize. E) Overlap of anti-AGO2, anti-HBc, and anti-HBs staining in T23 cells suggests that all three proteins may co-localize.
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Pathway Analysis

Predicted gene targets of up-regulated miRNAs were most strongly associated with the GO term PROTEIN_TYROSINE_PHOSPHATASE_ACTIVITY (P = 5.24E−3), and down-regulated miRNAs were associated with the term POSITIVE_REGULATION_OF_JNK_ACTIVITY (P 9.47e−4). Predicted target genes associated with phosphatase activity and dephosphorylation included MTMR3, PTPN18, DUSP5, PTPN2, DUSP2, and PPP1CA.

MiRNA Expression in Liver Biopsy Samples

We compared miRNA expression in non-cancerous liver biopsy samples from a patient with chronic HBV to two uninfected patients (Table S2, Fig. S8). MiRNA levels were highly correlated between liver tissue and serum in all patients (P = <0.001; $R^2 = 0.57$), including the top HBV-associated miRNAs identified by microarray and RT-PCR analysis in this study.

Co-localization of HBcAg and HBsAg with AGO2

Using immunocytochemistry and PLA analysis, we found that HBV core protein and AGO2 co-localized within T23 cells (Fig. 1A–B), suggesting a potential protein-protein interaction between HBcAg and AGO2. AGO2 also co-localized with HBs in T23 cells (Fig. 1C), indicating a potential interaction between HBs and AGO2. Overlap between anti-HBc and anti-HBs staining (Fig. 1D) and between anti-AGO2, anti-HBc, and anti-HBs (Fig. 1E) suggests that these three proteins may co-localize. No overlap was observed between anti-AGO2 and anti-HBx staining in HepG2 cells transfected with HBx expression plasmid (p3FLAG-HBx) nor in control cells, suggesting that HBx does not interact with AGO2 (data not shown).

Subcellular Localization

We also examined HBcAg sub-cellular localization using immunocytochemistry and PLA analysis and found that HBcAg localized to several intracellular compartments, including the ER, autophagosomes, endosomes, and Golgi (Fig. 2). No evidence was found for interaction with mitochondria (data not shown). Using immunocytochemistry, HBsAg was also found to localize diffusely to several intracellular compartments, including the ER, endosomes, autophagosomes, Golgi, mitochondria, processing bodies, multi-vesicular bodies, and the nuclear envelope (Fig. 3). HBx localized non-specifically in the nucleus and cytoplasm, and no sub-cellular location could be ascertained (Fig. S9).

RNA Interference against AGO2

Antisense RNA directed against AGO2 strongly suppressed AGO2 expression (Fig. 4A) and resulted in lower HBV DNA (Fig. 4B) and HBsAg (Fig. 4C) levels in the supernatant. Cell viability was not significantly reduced (Fig. 4D).

Discussion

In this study, we report a set of miRNAs that were up-regulated in serum of HBV infected individuals compared to healthy

Table 3. Quantitative RT-PCR results of selected miRNAs associated in serum of chronic HBV patients.

Factor	Total (n = 270)	HBV (n = 248)	Healthy (n = 22)	P
hsa-miR-122/cel-miR-238	0.1513 (0.0068–2.5)	0.1635 (0.0068–2.5)	0.02074 (0.013–0.04)	1.19E−13
hsa-miR-22/cel-miR-238	0.3 (0.06–1.7)	0.3028 (0.06–1.7)	0.2252 (0.11–0.48)	6.35E−03
hsa-miR-99a/cel-miR-238	0.09121 (0.0046–2.4)	0.102 (0.0086–2.4)	0.0136 (0.0046–0.051)	4.61E−12
hsa-miR-720/cel-miR-238	0.1206 (0.024–3.7)	0.1345 (0.031–3.7)	0.04274 (0.024–0.12)	8.93E−11
hsa-miR-125b/cel-miR-238	0.09732 (0.0066–3.1)	0.1131 (0.0066–3.1)	0.02255 (0.0066–0.05)	1.92E−11
hsa-miR-1275/cel-miR-238	0.4842 (0.099–1.6)	0.5046 (0.099–1.6)	0.4044 (0.24–0.6)	0.010781066
hsa-miR-1826/cel-miR-238	0.5023 (0.14–4.6)	0.5583 (0.26–4.6)	0.33 (0.14–1.4)	7.23E−03
hsa-miR-1308/cel-miR-238	2.831 (1.1–6.9)	2.578 (1.1–6.9)	3.113 (2.3–4.7)	0.223164946
hsa-miR-923/cel-miR-238	3.8 (1.8–9.6)	4.141 (1.8–9.6)	3.01 (2–5)	0.104331611
hsa-miR-1280/cel-miR-238	1.089 (0.36–5)	1.332 (0.6–5)	0.5275 (0.36–0.8)	1.06E−05
hsa-miR-26a/cel-miR-238	1.221 (0.34–3.4)	1.221 (0.34–3.4)	1.231 (0.82–2.4)	0.532171224
hsa-let-7a/cel-miR-238	0.9608 (0.2–2.5)	0.9211 (0.2–2.5)	1.074 (0.71–1.9)	0.235258945
hsa-let-7f/cel-miR-238	1.134 (0.052–2.6)	1.126 (0.052–2.6)	1.143 (0.8–1.7)	0.639411853
hsa-let-7d/cel-miR-238	1.147 (0.35–1.9)	1.106 (0.35–1.8)	1.231 (0.73–1.9)	2.88E−01
hsa-miR-638/cel-miR-238	1.23 (0.3–7)	1.082 (0.3–7)	1.366 (0.68–4)	0.288244047
hsa-miR-1908/cel-miR-238	1.369 (0.45–3.2)	1.357 (0.45–1.9)	1.447 (0.7–3.2)	0.370765019
hsa-miR-34a/cel-miR-238	0.07502 (0.013–1.2)	0.108 (0.026–1.2)	0.02738 (0.013–0.044)	1.41E−05
hsa-miR-886-5p/cel-miR-238	1.627 (0.54–3.6)	1.773 (0.54–3.6)	1.55 (0.97–2.7)	0.478520977

Expression levels were compared using the Mann-Whitney U test.
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