(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

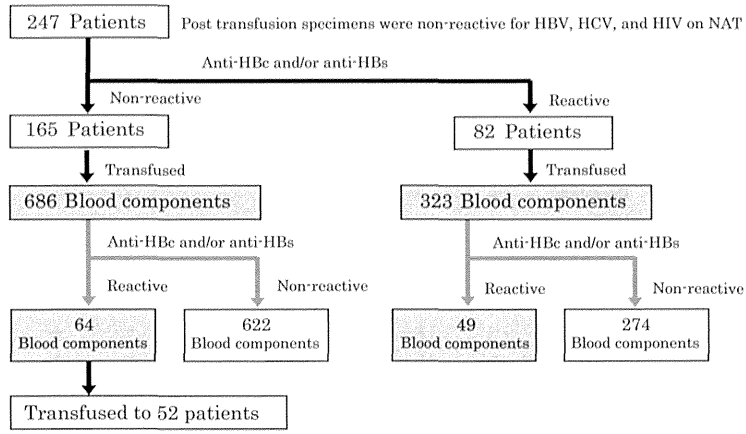

Fig. 2 The risk of transfusion of blood components with low anti-HBc and/or high anti-HBs titres. A total of 247 patients whose post-transfusion specimens were non-reactive for HBV, HCV and HIV on nucleic acid amplification testing were randomly selected, and their pre- and post-transfusion specimens 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).
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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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Differences in serum microRNA profiles in hepatitis B and C virus infection

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Microarray

Summary *Objectives:* Patients infected with chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) are at greater risk of cirrhosis and hepatocellular carcinoma. The objective of this study was to identify virus-specific serum microRNA profiles associated with liver function and disease progression. Microarray analysis of serum microRNAs was performed using the Toray 3D array system in 22 healthy subjects, 42 HBV patients, and 30 HCV patients. Selected microRNAs were then validated by qRT-PCR in 186 HBV patients, 107 HCV patients, and 22 healthy subjects.

Results: Microarray analysis showed up-regulation of a number of microRNAs in serum of both HBV and HCV patients. In qRT-PCR analysis, miR-122, miR-99a, miR-125b, miR-720, miR-22, and miR-1275 were up-regulated both in HBV patients relative to healthy subjects, and all except

List of abbreviations: HBV, Hepatitis B virus; HCV, Hepatitis C virus; HCC, hepatocellular carcinoma; qRT-PCR, quantitative real-time polymerase chain reaction; HBsAg, HBV surface antigen; HBeAg, HBe antigen; HBeAb, HBe antibody; HBcAg, HBV core antigen; γGTP, γ-glutamyl transpeptidase.

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miR-1275 were up-regulated in HBeAg-positive patients relative to HBeAg-negative patients. Specific microRNAs were independently associated with different aspects of HBV infection. MiR-122 was independently associated with HBV DNA level, whereas miR-125b was independently associated with levels of HBV DNA, HBsAg, and HBeAg. MiR-22 and miR-1275 were independently associated with serum γ-glutamyl transpeptidase levels.

Conclusions: Serum microRNA levels reflect differences in the etiology and stage of viral hepatitis.

Introduction

Chronic infection with hepatitis B virus (HBV), a partially double-stranded DNA virus, and hepatitis C virus (HCV), a single stranded RNA virus, increases the risk of cirrhosis and hepatocellular carcinoma (HCC). Despite improvements in antiviral therapy, many patients fail to respond to current therapies.[1–3] Therefore, non-invasive methods are needed for early detection of changes in liver function. One such approach is to measure changes in levels of small RNAs present in the serum of infected patients. In addition to messenger RNA, transfer RNA, and ribosomal RNA, there are many other classes of RNAs, many of which act to fine-tune gene expression and may play a role in disease pathogenesis. MicroRNAs are among the most important classes of non-coding RNA and consist of short linear RNA sequences that range in size from 19 to 24 nucleotides. MicroRNAs may influence gene expression by binding to a partially complementary region in the 3′ untranslated region of a targeted messenger RNA, thereby inhibiting translation or promoting degradation of the transcript. Because a single microRNA may regulate multiple genes, and a single gene may be regulated by multiple microRNAs, microRNAs may form complex regulatory networks.[4] Viral pathogenesis and inflammation may disrupt these intricate networks, resulting in changes in microRNA levels inside and outside of the cell. Given the liver's dual blood supply and central role in circulation, pathogenic changes in gene expression in the liver are likely to be reflected in changes in microRNA profiles in the serum.

Understanding the origin and function of serum microRNAs is important in the development of strategies to eradicate HCV and HBV and to monitor the degree of liver damage. Analysis of differential microRNA expression in liver tissues has revealed HCV- and HBV-specific microRNAs as well as microRNAs associated with the stage of liver disease.[5–9] MicroRNA levels in the liver have been found to be correlated with serum levels for a number of microRNAs,[10,11] suggesting that serum microRNAs might act as a surrogate measure of microRNA activity in the liver. While RNA typically has a short-half life and is quickly degraded by RNases, microRNAs tend to exist stably in serum when bound to argonaute proteins such as AGO2 as part of the RNA-induced silencing complex, the molecular scaffold that facilitates interaction of a microRNA with its target sequence.[12] Circulating microRNAs may exist in this form as vesicle-free ribonucleoprotein complexes, or they may be transported within HBV surface antigen (HBsAg) particles or contained within exosomes/microvesicles.[12–14] However, serum microRNAs are typically concentrated in exosomes.[15]

Exosomes are 30–150 nm endosome-derived microvesicles that are released from multiple cell types and are detectable in blood, urine, saliva, and other body fluids. Exosomes are involved in removal of cellular waste products as well as cell–cell communication and immune activation but may also be exploited by pathogens and contribute to tumor proliferation. Exosomes contain characteristic RNA transcripts, including microRNAs, transfer RNAs and other types of non-coding RNAs[16] and have been shown to affect gene expression in recipient cells. MiR-99a, miR128, miR-124, miR-22, and miR-99b account for 49% of identified exosome-associated microRNAs.[16] While exosomal RNA profiles vary by cell type, they do not completely mirror the RNA profile of the parent cell due to selective sorting and may change in response to cellular conditions.[16] Hepatocyte-derived exosomes are enriched for gene products involved in lipoprotein metabolism and xenobiotic processing and therefore have potential as a diagnostic tool by reflecting hepatic changes linked to disease.[17] Interferon-stimulated release of exosomes containing antiviral products and internalization by HBV-infected hepatocytes may also play a role in antiviral defense by bypassing viral interference in interferon signal transduction.[18] It is likely that analysis of serum microRNA profiles will provide insight into disease progression and antiviral activity in the liver, particularly in the case of HBV infection.

In order to investigate the relationship between serum microRNA profiles and viral hepatitis, we performed microarray and quantitative real-time polymerase chain reaction (qRT-PCR) analysis to identify host microRNAs that differ between healthy subjects and patients with chronic HBV or HCV infection as well as between HBeAg-positive and negative patients.

Methods

Study subjects

All patients had either chronic hepatitis B or C infection and were negative for HIV and HCC. No patients were co-infected with both HBV and HCV. All healthy subjects were negative for HBsAg and HCV antibody. Patient profiles are shown in Table 1. Histopathological diagnosis was determined as in Desmet et al.[19] The study was approved *a priori* by the ethical committee of Hiroshima University and conforms to the ethical guidelines of the 1975 Declaration of Helsinki. All patients provided written informed consent.

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Microarray analysis of serum microRNA expression levels

Host microRNA expression in serum samples was measured using the Toray Industries microRNA analysis system, in which serum microRNA samples were hybridized to 3D-Gene human microRNA ver17.1 chips containing 1200 microRNAs (Toray Industries, Inc., Tokyo, Japan). Serum from 42 patients with chronic HBV infection and 30 patients with chronic HCV infection were compared with serum from 12 healthy males and 10 healthy females using a separate microarray for each sample.

Quantitative RT-PCR microRNA analysis

A subset of microRNAs was selected for validation using qRT-PCR based on preliminary microarray results and a search of the literature. Expression of 7 microRNAs was measured in serum from 186 HBV patients, 107 HCV patients, and 22 healthy subjects. Circulating microRNA was extracted from 300 μl of serum samples using the mirVana PARIS Kit (Ambion Inc., 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 Ltd, Tokyo, Japan). Each sample was spiked with *Caenorhabditis elegans* miR-238 (cel-miR-238) as a control for extraction and amplification. The reaction mixture contained 5 μl of RNA solution, 2 μl of 10x reverse transcription buffer, 0.2 μl of 100 mM dNTP mixture, 4 μl of 5x 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. MicroRNAs were amplified using primers and probes provided by Applied Biosystems Inc. using TaqMan MicroRNA assays according to the manufacturer's instructions. The reaction mixture contained 12.5 μl of 2x Universal PCR Master Mix, 1.25 μl of 20x 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 s at 95 °C and annealing and extension for 60 s at 60 °C in an ABI7300 thermal cycler. For the cel-miR-238 assay, a dilution series using chemically synthesized microRNA was used to generate a standard curve that permitted absolute quantification of molecules. A separate internal normalization factor was not used.

Statistical analysis

MicroRNA microarray expression data was normalized using cyclic loess and analyzed using moderated *t*-tests using the limma package in the R statistical framework (http://www.r-project.org). *P*-values were adjusted for multiple testing using the false discovery rate (P_{FDR}). qRT-PCR expression levels were compared between healthy subjects and HBV or HCV using the non-parametric Mann–Whitney *U* test. Association between qRT-PCR microRNA levels and clinical parameters such as HBsAg, HBV DNA, HBeAg, HBeAb, AST, and ALT were evaluated using multiple linear regression. Factors that were significant at 0.05 in univariate analysis were included as candidates in the multivariate model, and forward-backward stepwise selection based on Akaike information criterion (AIC) was used to identify independently associated factors.

Pathway analysis

Target genes of differentially expressed microRNAs were predicted using the miRWalk database (http://www.umm.

Table 1 Clinical characteristics of healthy controls and patients with chronic viral HBV or HCV infection. Continuous variables are shown as median and range, and categorical variables are shown as counts.

Factor	Healthy(N = 22)	Hepatitis B virus (N = 186)	Hepatitis C virus (N = 107)
Age	33 (27–45)	48 (22–79)	64 (24–85)
Sex (male/female)	12/10	122/64	47/60
Alanine aminotransferase (IU/l)	18.5 (15–22)	73.5 (10–1867)	30.5 (18–145)
Aspartate aminotransferase (IU/l)	13.5 (6–44)	47.5 (15–982)	33.5 (11–141)
γ-glutamyl transpeptidase (IU/l)	20 (11–52)	41.5 (9–459)	22 (8–161)
rs8099917 genotype (TT/GT/GG/unknown)	5/0/0/17	89/76/3/18	–
Liver fibrosis (1/2/3/4/unknown)	–	65/76/28/3/14	39/35/11/4/18
Necroinflammatory activity (1/2/3/unknown)	–	58/80/34/14	32/48/9/18
Alpha-fetoprotein (ug/l)	–	6.1 (<5.0–2510.0)	5.0 (<5.0–104.8)
Promthrombin time (s)	–	95 (35–123)	98 (71–116)
Albumin (g/dl)	–	4.4 (2.8–4.9)	4.3 (3.5–5.0)
Platelets ($x10^4/mm^3$)	–	17.4 (5.0–35.7)	17.6 (5.3–29.8)
rs8099917 genotype (TT/GT/GG/unknown)	5/0/0/17	89/76/3/18	–
HBV DNA (IU/ml)	–	6.7 (<2.1–≥9.1)	–
HBsAg (IU/l)	–	3650 (1.2–239000)	–
HBeAg (−/+)	–	82/104	–
HBeAb (−/+)	–	88/98	–
HBV genotype (A/B/C/unknown)	–	3/14/129/40	–
HCV RNA (Log IU/ml)	–	–	6.5 (1.7–7.3)
HCV genotype (1a/1b/2a/2b/3a)	–	–	5/42/18/9/1/32

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Table 2 Top up- or down-regulated serum microRNAs associated with chronic HBV or HCV infection. MicroRNAs that have been detected in exosomes are noted.

Contrast	Direction	miRNA	logFC	AveExpr	t	P	P_{FDR}	Exosome
HBV-Healthy	Up	hsa-miR-122	2.80	8.30	7.63	2.42E-11	3.23E-09	exosome
	Up	hsa-miR-3648	1.39	13.63	8.26	1.20E-12	2.14E-10	
	Up	hsa-miR-642b	1.07	9.64	9.16	1.63E-14	9.76E-12	
	Up	hsa-miR-22	1.04	8.16	5.12	1.70E-06	3.01E-05	exosome
	Up	hsa-miR-1246	1.02	10.75	5.29	8.59E-07	1.78E-05	
	Up	hsa-miR-486-3p	0.89	8.32	7.43	6.06E-11	5.66E-09	
	Up	hsa-miR-191	0.80	7.65	6.04	3.46E-08	1.30E-06	exosome
	Up	hsa-miR-1915*	0.63	7.64	4.85	5.22E-06	7.76E-05	
	Up	hsa-miR-3665	0.62	14.38	5.69	1.58E-07	4.54E-06	
	Up	hsa-miR-658	0.61	7.72	8.80	9.24E-14	3.70E-11	exosome
	Up	hsa-miR-550a	0.59	7.24	10.56	2.00E-17	2.40E-14	
	Up	hsa-miR-320b	0.57	7.22	7.13	2.43E-10	2.08E-08	
	Up	hsa-miR-320a	0.54	7.29	6.63	2.47E-09	1.41E-07	exosome
	Up	hsa-miR-320c	0.54	7.05	6.67	2.00E-09	1.24E-07	
	Up	hsa-miR-3663-3p	0.51	10.69	5.63	2.08E-07	5.67E-06	
	Up	hsa-miR-99a	0.51	6.56	5.30	8.38E-07	1.78E-05	exosome
	Down	hsa-miR-223	−0.89	7.69	−5.15	1.56E-06	2.79E-05	exosome
	Down	hsa-miR-4294	−0.86	10.91	−5.50	3.59E-07	8.98E-06	
	Down	hsa-miR-575	−0.75	7.63	−6.05	3.31E-08	1.28E-06	exosome
	Down	hsa-miR-1268	−0.57	11.77	−6.83	1.00E-09	6.66E-08	
	Down	hsa-miR-1202	−0.54	8.10	−5.40	5.51E-07	1.25E-05	
	Down	hsa-miR-1275	−0.52	8.92	−5.06	2.20E-06	3.71E-05	
HCV-Healthy	Up	hsa-miR-122	1.81	8.30	4.74	8.05E-06	7.37E-05	exosome
	Up	hsa-miR-3648	1.52	13.63	8.63	2.04E-13	2.23E-11	
	Up	hsa-miR-642b	1.42	9.64	11.67	1.12E-19	6.69E-17	
	Up	hsa-miR-24	1.11	8.80	6.58	3.06E-09	5.92E-08	exosome
	Up	hsa-miR-3925-5p	1.10	7.28	7.98	4.61E-12	2.49E-10	
	Up	hsa-miR-296-3p	1.10	7.76	7.30	1.10E-10	3.56E-09	
	Up	hsa-miR-3162-5p	1.08	8.42	8.30	9.94E-13	7.95E-11	
	Up	hsa-miR-3622b-5p	1.08	7.82	6.13	2.33E-08	3.77E-07	
	Up	hsa-miR-3665	1.06	14.38	9.27	9.51E-15	1.90E-12	
	Up	hsa-miR-3917	1.01	7.99	7.59	2.92E-11	1.11E-09	
	Up	hsa-miR-762	1.01	14.16	10.63	1.48E-17	5.93E-15	
	Up	hsa-miR-4258	0.96	8.57	7.00	4.39E-10	1.15E-08	
	Up	hsa-miR-4257	0.92	7.83	9.45	4.05E-15	9.73E-13	
	Up	hsa-miR-663	0.86	10.87	5.38	5.82E-07	7.27E-06	exosome
	Up	hsa-miR-4299	0.86	7.19	7.65	2.13E-11	9.33E-10	
	Up	hsa-miR-486-3p	0.83	8.32	6.65	2.20E-09	4.48E-08	
	Up	hsa-miR-149*	0.78	10.33	7.73	1.49E-11	6.88E-10	exosome
	Up	hsa-miR-4259	0.74	7.74	5.06	2.22E-06	2.32E-05	
	Up	hsa-miR-1469	0.74	10.93	5.28	8.83E-07	1.05E-05	
	Up	hsa-miR-3934	0.74	7.43	7.62	2.48E-11	1.03E-09	
	Up	hsa-miR-658	0.73	7.72	10.14	1.52E-16	4.57E-14	exosome
	Up	hsa-miR-3663-3p	0.73	10.69	7.65	2.18E-11	9.33E-10	
	Up	hsa-miR-671-5p	0.67	8.15	8.31	9.52E-13	7.95E-11	exosome
	Up	hsa-miR-187*	0.67	8.45	8.20	1.61E-12	1.02E-10	
	Up	hsa-miR-3131	0.66	7.71	8.40	6.21E-13	6.21E-11	
	Up	hsa-miR-3154	0.64	8.13	6.32	1.00E-08	1.77E-07	
	Up	hsa-miR-320a	0.59	7.29	6.94	5.85E-10	1.40E-08	exosome
	Up	hsa-miR-4300	0.55	6.89	6.43	6.06E-09	1.12E-07	
	Up	hsa-miR-3126-5p	0.53	6.85	7.43	6.11E-11	2.16E-09	
	Up	hsa-miR-3153	0.51	6.99	5.16	1.46E-06	1.56E-05	
	Up	hsa-miR-550a	0.51	7.24	8.70	1.50E-13	1.80E-11	
	Up	hsa-miR-3616-3p	0.50	6.87	8.18	1.78E-12	1.07E-10	
	Up	hsa-miR-371-5p	0.50	7.70	5.91	6.09E-08	9.14E-07	
	Up	hsa-miR-3147	0.50	7.60	6.20	1.68E-08	2.88E-07	

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Table 2 (*continued*)

Contrast	Direction	miRNA	logFC	AveExpr	t	P	P_{FDR}	Exosome
	Down	hsa-miR-451	−2.00	10.87	−5.76	1.16E-07	1.68E-06	exosome
	Down	hsa-miR-223	−1.42	7.69	−7.91	6.28E-12	3.14E-10	exosome
	Down	hsa-miR-92a-2*	−1.30	10.11	−7.20	1.76E-10	5.03E-09	
	Down	hsa-miR-4294	−1.22	10.91	−7.42	6.33E-11	2.17E-09	
	Down	hsa-miR-575	−1.17	7.63	−9.06	2.67E-14	4.57E-12	exosome
	Down	hsa-miR-16	−1.13	7.77	−4.99	2.96E-06	2.96E-05	exosome
	Down	hsa-miR-1275	−0.75	8.92	−7.08	3.05E-10	8.52E-09	
	Down	hsa-miR-1915	−0.75	11.10	−12.24	7.86E-21	9.44E-18	
	Down	hsa-miR-1202	−0.69	8.10	−6.61	2.67E-09	5.34E-08	
	Down	hsa-miR-887	−0.68	8.13	−8.23	1.38E-12	9.30E-11	exosome
	Down	hsa-miR-1203	−0.64	8.50	−7.05	3.48E-10	9.49E-09	
	Down	hsa-miR-125a-3p	−0.62	6.90	−7.53	3.72E-11	1.35E-09	exosome
	Down	hsa-miR-17	−0.59	6.76	−5.00	2.79E-06	2.81E-05	exosome
	Down	hsa-miR-3141	−0.59	8.72	−7.02	4.11E-10	1.10E-08	
	Down	hsa-miR-20a	−0.59	6.60	−5.65	1.91E-07	2.57E-06	exosome
	Down	hsa-miR-1268	−0.58	11.77	−6.60	2.81E-09	5.52E-08	
	Down	hsa-miR-423-5p	−0.51	7.97	−7.75	1.38E-11	6.64E-10	
HCV-HBV	Up	hsa-miR-296-3p	0.80	7.76	6.07	3.06E-08	1.67E-06	
	Up	hsa-miR-3925-5p	0.74	7.28	6.09	2.79E-08	1.59E-06	
	Up	hsa-miR-4257	0.70	7.83	8.28	1.09E-12	4.34E-10	
	Up	hsa-miR-3162-5p	0.66	8.42	5.79	1.01E-07	4.67E-06	
	Up	hsa-miR-1469	0.65	10.93	5.28	8.82E-07	2.52E-05	
	Up	hsa-miR-149*	0.64	10.33	7.23	1.54E-10	2.65E-08	exosome
	Up	hsa-miR-3917	0.57	7.99	4.91	4.01E-06	8.74E-05	
	Up	hsa-miR-4299	0.53	7.19	5.36	6.43E-07	1.98E-05	
	Up	hsa-miR-762	0.52	14.16	6.27	1.25E-08	9.35E-07	

logFC: log2 fold-change; AveExpr: The average log2 expression level; t: moderated t-statistic; P: uncorrected *P*-value for *t*-test; P_{FDR}: *P*-value adjusted for multiple testing based on the false discovery rate.

uni-heidelberg.de/apps/zmf/mirwalk/ accessed on 14 September 2014)[20] based on maximum agreement among the following tools: DIANA-mT, miRanda, miRDB, miRWalk, RNAhybrid, PICTAR5, PITA, RNA22, and TargetScan. Gene set enrichment in canonical pathways was analyzed using Ingenuity Pathway Analysis software (Ingenuity Systems, CA, USA).

Results

MicroRNA microarray results

MicroRNA microarray analysis was performed to identify differentially expressed microRNAs in serum of patients with chronic HBV or HCV compared to healthy individuals and between patients with chronic HBV compared to patients with chronic HCV. A larger number of microRNAs were significantly up- or down-regulated in serum of HCV patients compared to HBV patients (Table 2, Suppl. Table 1). MiR-122 was strongly up-regulated in both patients with HBV (logFC = 2.77) and HCV (logFC = 1.81), but the fold change was modest for other microRNAs. Several microRNAs were associated with HBV infection, including miR-22, miR-99a, miR-1246, miR-320a and miR-320b (Table 2; Fig. 1A). Serum microRNA profiles of HBeAg-positive and negative patients were compared with healthy subjects (Table 3, Fig. 1B, Suppl. Table 2). Results were similar for both HBeAg-positive and negative patients, but several microRNAs, including miR-122, miR-194, miR-125b, miR-99a, and miR-100, were up-regulated in HBeAg-positive patients compared to HBeAg-negative patients. MicroRNAs were annotated based on whether or not they have been reported to be detected within exosomes (www.exocarta.org accessed on 12 September 2014)[21,22] and/or within circulating HBsAg particles.[14] Nearly all of the significantly up-regulated microRNAs have been reported to be detected in exosomes, and miR-122, miR-30a, miR-30b, and miR-30c have been detected in HBsAg particles. However, further research is necessary to confirm in which compartments these microRNAs are present in these patients.

Quantitative RT-PCR analysis

qRT-PCR was used to validate expression of selected microRNAs (Table 4). MiR-122, miR-99a, miR-125b, miR-720, miR-22, and miR-1275 were significantly up-regulated in serum of HBV patients (n = 185) compared to healthy subjects (n = 22). MiR-122 and miR-720, but not miR-1246, were significantly up-regulated in serum of HCV patients (n = 107) relative to healthy subjects (n = 10). Microarray and qRT-PCR expression levels from the same individual were correlated ($P < 0.05$; data not shown). MiR-99a, miR-125b, miR-122, miR-720, and miR-22, but not miR-1275, were significantly elevated in HBeAg-positive versus HBeAg-negative individuals (Table 4; Fig. 2). In Fig.2, the points representing the highest

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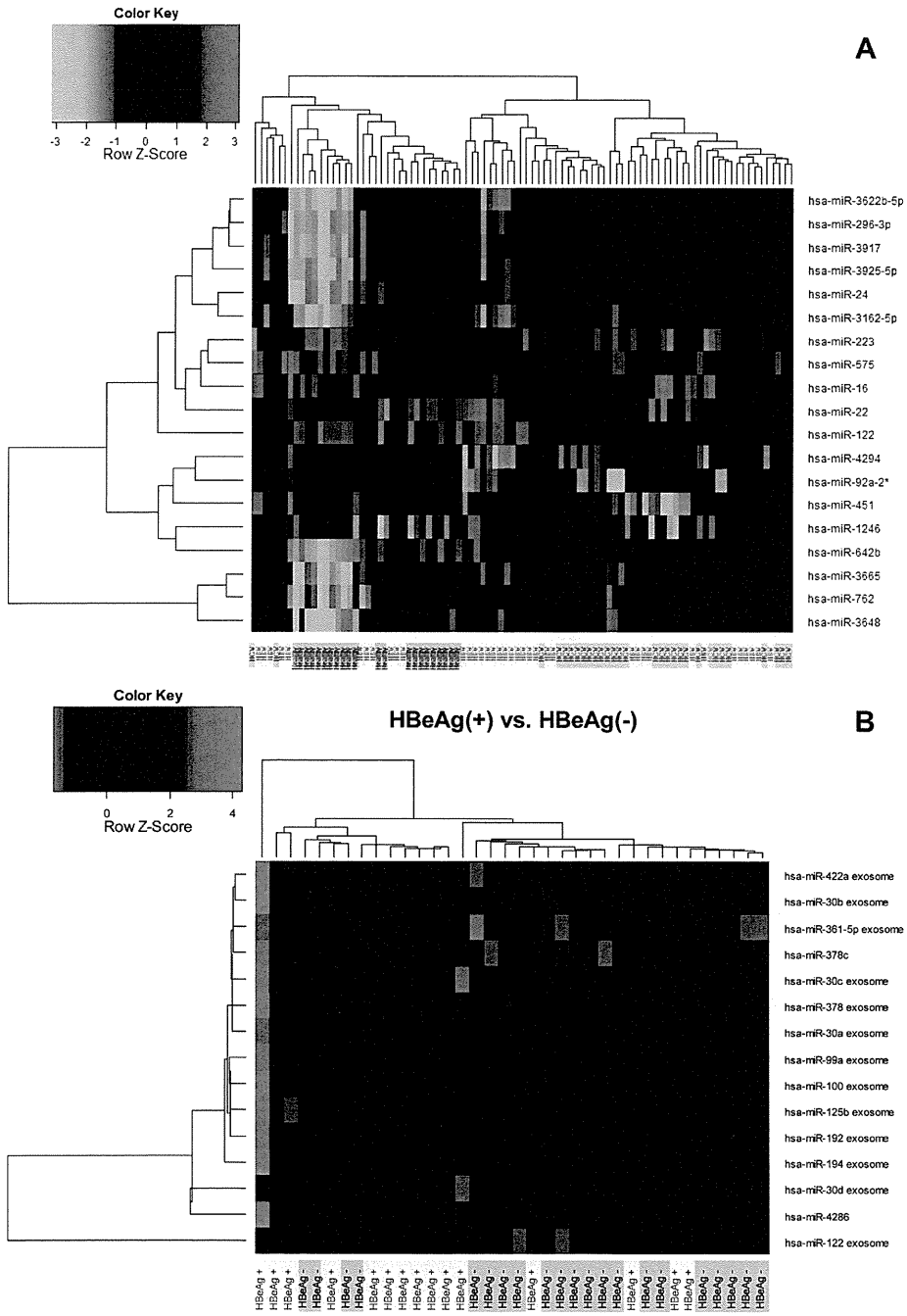

Figure 1 Heatmap of results of serum microRNA microarray analysis. Up-regulated microRNAs are shown in red, and down-regulated microRNAs are shown in green. Hierarchical clustering was performed in R using Euclidean distance and McQuitty clustering. A) Healthy (blue) versus HCV (purple) and HBV (yellow). B) HBe antigen-positive patients (yellow) versus HBe antigen-negative patients (purple). MicroRNAs that have been reported to be associated with exosomes are annotated based on a search of the Exocarta database.[21]

expression level for each of miR-122, miR-99a, and miR-125b corresponds to the same patient, who also had the highest HBsAg level (239000 IU/ml), but no other patients shared a similar rank pattern. The non-parametric Kruskal–Wallis test, based on the median instead of the mean, is robust to outliers and was used to prevent patients with high microRNA expression levels from having undo influence over the results. No internal normalization factors were selected *a priori*. However, because miR-1275 did not differ between HBeAg-positive and HbeAg-negative, it

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Table 3 Top up- or down-regulated serum microRNAs associated with HBeAg-positive or negative chronic HBV infection. MicroRNAs that have been detected in exosomes or HBsAg particles are noted.

Contrast	Direction	miRNA	logFC	AveExpr	*P*	P_{FDR}	Exosome	HBsAg
HBeAg(+) vs Healthy	Up	hsa-miR-122	3.9	8.1	3.48E-14	4.18E-11	exosome	HBsAg
	Up	hsa-miR-22	1.3	8.1	3.52E-07	3.52E-05	exosome	
	Up	hsa-miR-3648	1.2	13.0	3.47E-06	2.08E-04		
	Up	hsa-miR-1246	1.0	10.5	7.43E-06	3.43E-04		
	Up	hsa-miR-642b	1.0	9.1	3.94E-08	5.91E-06		
	Up	hsa-miR-486-3p	0.9	8.0	3.79E-06	2.15E-04		
	Up	hsa-miR-191	0.8	7.5	7.67E-07	5.76E-05	exosome	
	Up	hsa-miR-4286	0.8	7.3	3.74E-04	6.31E-03		
	Up	hsa-miR-194	0.8	6.5	1.66E-05	5.88E-04	exosome	
	Up	hsa-miR-99a	0.7	6.6	3.99E-06	2.15E-04	exosome	
	Up	hsa-miR-125b	0.7	6.7	9.17E-06	3.84E-04	exosome	
	Up	hsa-miR-30d	0.7	7.4	5.54E-06	2.66E-04	exosome	
	Up	hsa-miR-3665	0.6	14.0	5.11E-04	8.07E-03		
	Up	hsa-miR-320b	0.6	7.1	6.74E-09	1.35E-06		
	Up	hsa-miR-100	0.6	6.5	1.70E-05	5.88E-04	exosome	
	Up	hsa-miR-1915*	0.6	7.5	9.81E-04	1.39E-02		
	Up	hsa-miR-320a	0.6	7.1	8.21E-09	1.41E-06		
	Up	hsa-miR-320d	0.6	6.8	4.01E-07	3.70E-05		
	Up	hsa-miR-550a	0.6	7.1	3.38E-11	2.03E-08		
	Up	hsa-miR-320c	0.5	6.9	2.17E-07	2.61E-05		
	Up	hsa-miR-658	0.5	7.4	3.73E-09	1.00E-06	exosome	
	Down	hsa-miR-4294	−1.0	11.3	1.08E-04	2.50E-03		
	Down	hsa-miR-575	−0.7	8.0	4.65E-04	7.54E-03	exosome	
	Down	hsa-miR-92a-2*	−0.7	10.6	1.29E-03	1.69E-02		
	Down	hsa-miR-3197	−0.6	10.8	1.28E-04	2.84E-03		
	Down	hsa-miR-1268	−0.5	12.0	2.96E-05	8.89E-04		
	Down	hsa-miR-1275	−0.5	9.2	4.72E-04	7.54E-03		
HBeAg(−) vs Healthy	Up	hsa-miR-122	2.1	7.6	1.68E-06	4.39E-05	exosome	HBsAg
	Up	hsa-miR-3648	1.5	13.3	2.78E-09	2.09E-07		
	Up	hsa-miR-642b	1.2	9.3	2.15E-11	6.45E-09		
	Up	hsa-miR-1246	1.0	10.6	3.12E-05	4.31E-04		
	Up	hsa-miR-486-3p	0.9	8.1	7.30E-11	1.75E-08		
	Up	hsa-miR-22	0.8	8.0	1.07E-03	7.36E-03	exosome	
	Up	hsa-miR-191	0.8	7.5	5.11E-06	1.04E-04	exosome	
	Up	hsa-miR-3622b-5p	0.7	7.6	1.49E-03	9.54E-03		
	Up	hsa-miR-658	0.7	7.6	4.34E-10	5.21E-08	exosome	
	Up	hsa-miR-4258	0.6	8.3	3.39E-05	4.58E-04		
	Up	hsa-miR-1915*	0.6	7.5	3.79E-06	8.93E-05		
	Up	hsa-miR-24	0.6	8.5	6.50E-04	4.97E-03	exosome	HBsAg
	Up	hsa-miR-3665	0.6	14.1	3.08E-05	4.30E-04		
	Up	hsa-miR-550a	0.6	7.1	7.37E-14	8.84E-11		
	Up	hsa-miR-663b	0.6	9.3	4.31E-05	5.56E-04		
	Up	hsa-miR-3663-3p	0.6	10.5	2.75E-09	2.09E-07		
	Up	hsa-miR-320b	0.5	7.1	5.71E-07	1.90E-05		
	Up	hsa-miR-762	0.5	13.9	1.21E-05	2.02E-04		
	Up	hsa-miR-320c	0.5	7.0	1.50E-06	4.10E-05		
	Up	hsa-miR-3917	0.5	7.7	7.78E-04	5.66E-03		
	Up	hsa-miR-135a*	0.5	8.4	2.13E-04	2.09E-03	exosome	HBsAg
	Up	hsa-miR-663	0.5	10.7	1.66E-03	1.04E-02	exosome	
	Up	hsa-miR-3934	0.5	7.3	3.00E-07	1.09E-05		
	Up	hsa-miR-320a	0.5	7.1	1.58E-06	4.21E-05		
	Down	hsa-miR-451	−1.5	11.3	9.61E-06	1.72E-04	exosome	
	Down	hsa-miR-223	−1.0	8.0	7.28E-05	8.56E-04	exosome	HBsAg
	Down	hsa-miR-16	−0.8	8.0	1.39E-03	9.03E-03	exosome	
	Down	hsa-miR-4294	−0.8	11.3	7.84E-07	2.30E-05		

(*continued on next page*)

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Table 3 (*continued*)

Contrast	Direction	miRNA	logFC	AveExpr	*P*	P_{FDR}	Exosome	HBsAg
	Down	hsa-miR-575	−0.8	7.9	1.40E-06	3.89E-05	exosome	
	Down	hsa-miR-92a-2*	−0.8	10.5	9.47E-06	1.72E-04		
	Down	hsa-miR-1202	−0.6	8.3	2.12E-08	1.16E-06		
	Down	hsa-miR-1268	−0.6	11.9	1.99E-09	1.71E-07		
	Down	hsa-miR-1275	−0.5	9.1	4.35E-06	9.41E-05		
	Down	hsa-miR-17	−0.5	6.8	1.38E-05	2.24E-04	exosome	HBsAg
	Down	hsa-miR-20a	−0.5	6.7	2.58E-05	3.83E-04	exosome	
HBeAg(+) vs HBeAg(−)	Up	hsa-miR-122	2.8	8.3	1.57E-07	1.50E-04	exosome	HBsAg
	Up	hsa-miR-194	0.7	6.5	2.49E-07	1.50E-04	exosome	
	Up	hsa-miR-4286	0.6	7.3	3.97E-04	3.17E-02		
	Up	hsa-miR-30d	0.6	7.4	8.35E-06	2.01E-03	exosome	
	Up	hsa-miR-125b	0.5	6.7	1.07E-05	2.14E-03	exosome	
	Up	hsa-miR-99a	0.5	6.6	2.00E-04	1.85E-02	exosome	
	Up	hsa-miR-100	0.5	6.5	1.75E-04	1.75E-02	exosome	
	Up	hsa-miR-192	0.4	6.8	4.52E-05	6.23E-03	exosome	
	Up	hsa-miR-378	0.4	6.6	2.20E-06	6.61E-04	exosome	
	Up	hsa-miR-30a	0.3	6.5	8.66E-05	9.45E-03	exosome	HBsAg
	Up	hsa-miR-422a	0.3	6.5	1.50E-06	6.00E-04	exosome	
	Up	hsa-miR-30c	0.3	6.6	7.59E-05	9.11E-03	exosome	HBsAg
	Up	hsa-miR-378c	0.3	6.4	2.61E-04	2.23E-02		
	Up	hsa-miR-30b	0.2	6.5	4.67E-05	6.23E-03	exosome	HBsAg
	Up	hsa-miR-361-5p	0.2	6.4	3.11E-05	5.33E-03	exosome	

was used to renormalize miR-99a, miR-125b, miR-122, miR-720, and miR-22 qRT-PCR expression data. *P*-values using renormalized data decreased by approximately one order of magnitude but remained highly significant and did not affect any conclusions (data not shown).

Association between microRNA level and clinical factors in patients with chronic HBV

Multiple regression was used to identify associations among microRNA levels and clinical factors in HBV patients using

Table 4 Quantitative RT-PCR results of selected microRNAs in serum of chronic HBV or HCV patients and healthy controls and between HBeAg-positive and negative patients. Expression levels are shown as median and range and compared using the Mann–Whitney *U* test.

microRNA	Healthy (n = 22)	HBV (n = 185)	logFC	*P*	P_{FDR}
hsa-miR-122/cel-miR-238	0.021 (0.013–0.04)	0.204 (0.011–2.495)	3.31	1.54E-13	1.08E-12
hsa-miR-99a/cel-miR-238	0.014 (0.005–0.051)	0.132 (0.008–2.436)	3.24	3.64E-12	8.50E-12
hsa-miR-125b/cel-miR-238	0.023 (0.007–0.05)	0.146 (0.007–3.084)	2.70	3.36E-12	8.50E-12
hsa-miR-720/cel-miR-238	0.043 (0.024–0.123)	0.146 (0.035–3.732)	1.76	4.66E-11	8.15E-11
hsa-miR-22/cel-miR-238	0.226 (0.107–0.485)	0.335 (0.096–1.305)	0.57	4.69E-04	6.57E-04
hsa-miR-1275/cel-miR-238	0.405 (0.237–0.604)	0.517 (0.099–1.626)	0.35	4.90E-03	5.71E-03
microRNA	Healthy (n = 10)	HCV (n = 107)	logFC	*P*	P_{FDR}
hsa-miR-720/cel-miR-238	0.388 (0.232–0.749)	0.653 (0.198–1.731)	0.75	2.51E-03	7.53E-03
hsa-miR-122/cel-miR-238	0.671 (0.307–0.95)	1.096 (0.1–8.542)	0.71	1.78E-02	2.68E-02
hsa-miR-1246/cel-miR-238	2.893 (1.821–6.813)	4.360 (0.429–36.311)	0.59	7.28E-02	7.28E-02
microRNA	HBeAg-negative (n = 82)	HBeAg-positive (n = 103)	logFC	*P*	P_{FDR}
hsa-miR-99a/cel-miR-238	0.070 (0.009–0.585)	0.250 (0.008–2.436)	1.84	4.55E-11	1.59E-10
hsa-miR-125b/cel-miR-238	0.100 (0.007–0.507)	0.253 (0.012–3.084)	1.34	7.70E-10	1.80E-09
hsa-miR-122/cel-miR-238	0.143 (0.011–0.678)	0.337 (0.017–2.495)	1.24	8.60E-12	6.02E-11
hsa-miR-720/cel-miR-238	0.119 (0.035–0.517)	0.185 (0.040–3.732)	0.64	4.24E-06	7.42E-06
hsa-miR-22/cel-miR-238	0.302 (0.096–1.305)	0.391 (0.103–1.049)	0.37	2.36E-04	3.30E-04
hsa-miR-1275/cel-miR-238	0.494 (0.099–1.626)	0.541 (0.186–1.376)	0.13	1.07E-01	1.25E-01

logFC: log2 fold-change; P: uncorrected *P*-value for Mann-Whitney *U* test; P_{FDR}: *P*-value adjusted for multiple testing based on the false discovery rate.

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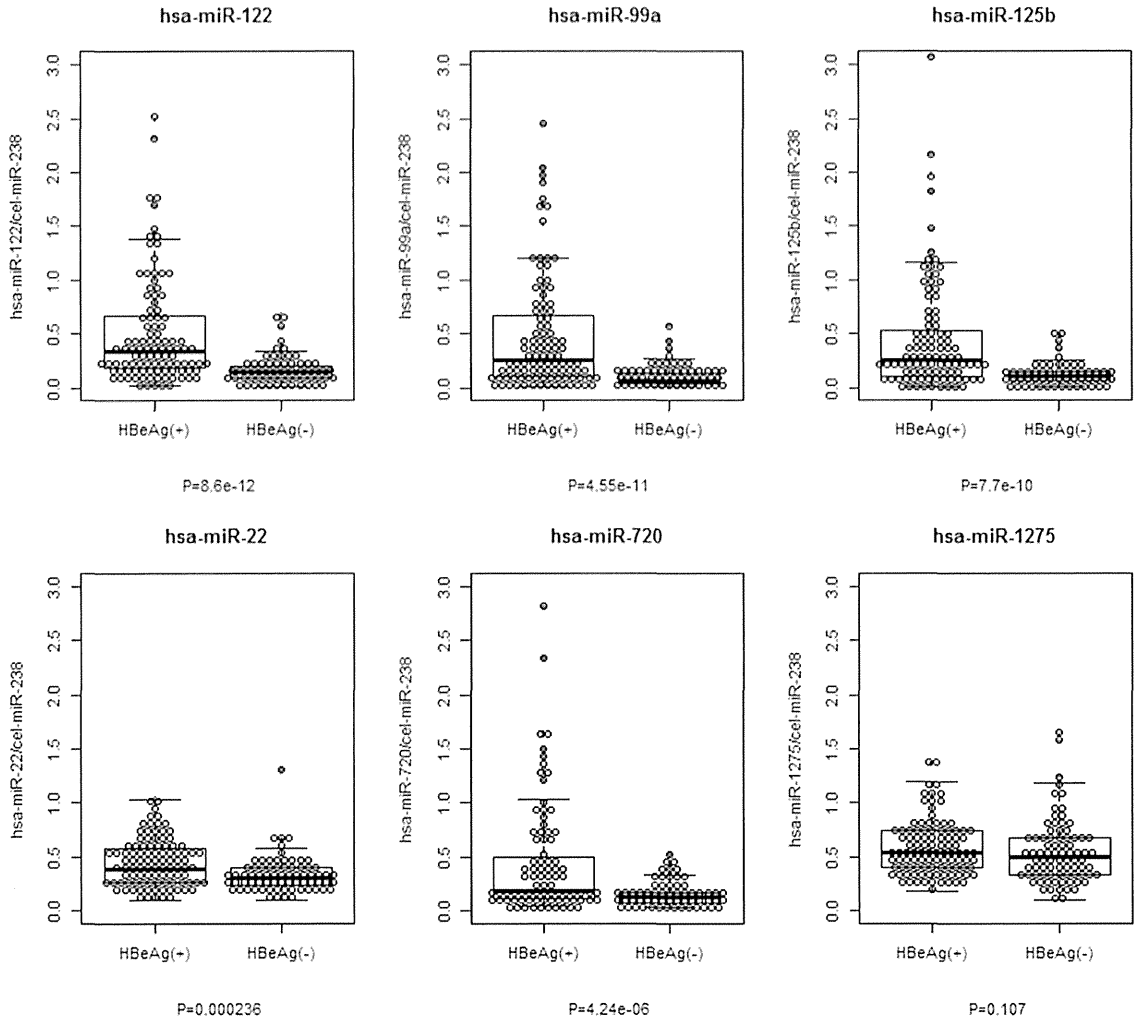

Figure 2 Serum microRNA expression in HBe antigen positive and negative individuals. qRT-PCR microRNA expression levels normalized by cel-miR-238 are shown. *P*-values represent the difference in median values using the non-parametric Kruskal–Wallis rank sum test.

qRT-PCR data (Table 5). MiR-122 was independently associated only with HBV DNA level, whereas miR-125b was independently associated with HBV DNA, HBsAg, HBeAg, and HBeAb levels. MiR-99a was also independently associated with HBeAb levels, and miR-720 was independently associated with HBsAg. While these microRNAs were associated with viral components, miR-22 and miR-1275 were independently associated with γGTP levels. rs8099917 SNP genotype TT in the IFNL3 locus was independently associated with necroinflammatory activity. MiR-125b was the strongest independent factor associated with HBeAg levels, and miR-125b and miR-99a and HBV DNA were each independently associated with HBeAg level. Pairwise expression levels of serum microRNAs were highly correlated, e.g., miR-22 and miR-99a (R^2 = 0.97), miR-99a and miR-125b (R^2 = 0.96), and miR-122 and miR-125b (R^2 = 0.96).

Pathway analysis

To determine which pathways HBV or HCV-associated microRNAs affected, gene targets were predicted using the miRWalk database, and predicted gene targets were compared against pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Predicted targets were found to be significantly overrepresented in the "Pathways in Cancer" gene set. Several of the genes in this set (*AKT1*, *AKT3*, *PTEN*, *BCL2*, *CDKN1B*, *CCND1*, and *TP53*) were also targeted by multiple microRNAs as part of a complex regulatory network. To further examine differences between HBV and HCV infection, predicted gene targets were analyzed using Ingenuity Pathway Analysis software. Significant associations were found between predicted targets and "Cancer," "Cell Cycle," and "Cell Death and Survival" networks in HCV patients and between

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Table 5 Univariate and multivariate linear/logistic regression analysis of associations between clinical data and quantitative RT-PCR serum microRNA levels (relative to cel-miR-238) in patients with chronic HBV infection. Independent factors (bold) were determined using forward-backward stepwise selection based on the Akaike information criterion (AIC) using factors with a univariate *P*-value less than 0.05.

Variable	Factor	N	Coef.	P_{uni}	Coef.	P_{multi}	
HBV DNA (IU/ml)	**hsa-miR-122**	185	2.6	6.1E-17	3.8	7.43E-05	***
	hsa-miR-22	185	3.1	4.3E-06			
	hsa-miR-99a	185	2.3	3.7E-15			
	hsa-miR-720	185	1.5	4.0E-08	−0.5	1.08E-01	
	hsa-miR-125b	185	2.3	2.1E-13	−1.8	2.57E-02	*
	hsa-miR-1275	184	0.4	4.1E-01			
	HBsAg (IU/l)	185	0.0	6.7E-11			
	HBeAg (IU/l)	185	0.0	2.5E-13			
	HBeAb (+/−)	185	−2.2	1.8E-18	−1.5	9.76E-10	***
	rs8099917 TT	167	0.8	5.0E-03			
	AST	185	0.0	4.2E-04	0.0	6.60E-02	.
	ALT	185	0.0	7.4E-04			
	γ-GTP(IU/l)	179	0.0	2.3E-01			
	Liver fibrosis	171	0.2	3.8E-01			
	Activity	171	0.9	4.0E-06	0.6	2.00E-05	***
	Genotype C	145	−0.3	5.4E-01			
HBsAg (IU/l)	hsa-miR-122	185	62950.0	7.6E-60			
	hsa-miR-22	185	59425.0	1.1E-08			
	hsa-miR-99a	185	60936.0	6.9E-66			
	hsa-miR-720	185	41920.0	5.1E-31	14228.0	4.47E-08	***
	hsa-miR-125b	185	62707.0	9.0E-62	51193.0	7.20E-39	***
	hsa-miR-1275	184	2856.0	7.2E-01			
	HBeAg (IU/l)	185	34.0	3.6E-18			
	HBeAb (+/−)	185	−25347.0	1.7E-09			
	rs8099917 TT	167	12077.0	1.2E-02			
	HBV DNA (IU/ml)	185	7119.0	6.7E-11			
	AST	185	−10.3	6.6E-01			
	ALT	185	1.2	9.1E-01			
	γ-GTP	179	−12.6	7.3E-01			
	Liver fibrosis	171	−5283.0	8.4E-02			
	Activity	171	3301.0	3.1E-01			
	Genotype C	145	−16648.0	4.3E-02			
HBeAg (IU/l)	hsa-miR-122	185	751.0	2.8E-20			
	hsa-miR-22	185	872.0	1.3E-06			
	hsa-miR-99a	185	700.0	1.7E-19			
	hsa-miR-720	185	464.0	2.1E-11			
	hsa-miR-125b	185	741.0	3.4E-20	544.0	4.90E-13	***
	hsa-miR-1275	184	101.0	4.6E-01			
	HBsAg (IU/l)	185	0.0	3.6E-18			
	HBeAb (+/−)	185	−609.0	3.8E-19	−395.0	3.14E-10	***
	rs8099917 TT	167	121.0	1.4E-01			
	HBV DNA (IU/ml)	185	135.0	2.5E-13			
	AST	185	0.9	3.3E-02	0.6	3.50E-02	*
	ALT	185	0.4	2.2E-02			
	γ-GTP	179	0.4	5.3E-01			
	Liver fibrosis	171	−22.3	6.7E-01			
	Activity	171	94.1	9.2E-02			
	Genotype C	145	−1.5	9.9E-01			
HBeAb (+/−)	hsa-miR-122	184	−52.1	1.0E-12			
	hsa-miR-22	184	−65.8	2.4E-05			
	hsa-miR-99a	184	−49.8	7.4E-13	−55.3	3.90E-03	**
	hsa-miR-720	184	−32.2	1.3E-07			
	hsa-miR-125b	184	−46.4	2.6E-10	51.3	9.53E-03	**

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Table 5 (*continued*)

Variable	Factor	N	Coef.	P_{uni}	Coef.	P_{multi}	
	hsa-miR-1275	183	−19.4	9.6E-02			
	HBsAg (IU/l)	184	0.0	1.3E-10			
	HBeAg (IU/l)	184	−0.1	7.4E-18	0.0	8.67E-07	***
	rs8099917 TT	166	−10.6	1.2E-01			
	HBV DNA (IU/ml)	184	−13.9	5.4E-20	−8.7	2.84E-08	***
	AST	184	−0.1	8.4E-02			
	ALT	184	0.0	2.9E-02			
	γ-GTP	178	0.0	6.9E-01			
	Liver fibrosis	170	−1.2	7.8E-01			
	Activity	170	−3.9	4.1E-01			
	Genotype C	144	−11.4	3.3E-01			
ALT (IU/l)	hsa-miR-122	185	17.0	6.3E-01			
	hsa-miR-22	185	337.0	1.0E-06	48.2	1.29E-01	
	hsa-miR-99a	185	−18.8	5.7E-01			
	hsa-miR-720	185	15.5	5.8E-01			
	hsa-miR-125b	185	−1.6	9.6E-01			
	hsa-miR-1275	184	9.0	8.6E-01			
	HBsAg (IU/l)	185	0.0	9.1E-01			
	HBeAg (IU/l)	185	0.1	2.2E-02			
	rs8099917 TT	167	18.2	5.5E-01			
	HBV DNA (IU/ml)	185	25.1	7.4E-04			
	AST	185	1.9	2.6E-66	1.8	2.20E-47	***
	γ-GTP	179	2.0	2.1E-20	0.4	6.05E-04	***
	Liver fibrosis	171	35.8	7.6E-02			
	Activity	171	74.8	4.3E-04	−19.0	4.58E-02	*
	Genotype C	145	30.0	5.6E-01			
AST (IU/l)	hsa-miR-122	185	0.2	9.9E-01			
	hsa-miR-22	185	148.0	8.2E-06			
	hsa-miR-99a	185	−15.1	3.4E-01			
	hsa-miR-720	185	4.1	7.6E-01			
	hsa-miR-125b	185	−7.3	6.6E-01			
	hsa-miR-1275	184	10.3	6.8E-01			
	HBsAg (IU/l)	185	0.0	6.6E-01			
	HBeAg (IU/l)	185	0.0	3.3E-02			
	rs8099917 TT	167	18.3	2.0E-01			
	HBV DNA (IU/ml)	185	12.6	4.2E-04			
	ALT	185	0.4	2.6E-66	0.4	1.05E-59	***
	γ-GTP	179	0.9	8.1E-18			
	Liver fibrosis	171	27.2	4.8E-03			
	Activity	171	48.6	1.5E-06	17.4	1.98E-04	***
	Genotype C	145	4.0	8.7E-01			
γ-GTP (IU/l)	hsa-miR-122	179	−5.3	6.4E-01			
	hsa-miR-22	179	46.4	4.2E-02	−48.0	1.95E-02	*
	hsa-miR-99a	179	−10.1	3.4E-01			
	hsa-miR-720	179	3.9	6.7E-01			
	hsa-miR-125b	179	−9.7	3.8E-01			
	hsa-miR-1275	178	33.9	4.3E-02	43.2	2.70E-03	**
	HBsAg (IU/l)	179	0.0	7.3E-01			
	HBeAg (IU/l)	179	0.0	5.3E-01			
	rs8099917 TT	161	10.9	2.7E-01			
	HBV DNA (IU/ml)	179	3.0	2.3E-01			
	AST	179	0.4	8.1E-18			
	ALT	179	0.2	2.1E-20	0.2	5.35E-19	***
	Liver fibrosis	166	24.1	1.7E-04	15.9	1.59E-03	**
	Activity	166	23.5	7.4E-04			
	Genotype C	140	15.7	3.3E-01			

(*continued on next page*)

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Table 5 (*continued*)

Variable	Factor	N	Coef.	P_{uni}	Coef.	P_{multi}	
Liver fibrosis	hsa-miR-122	171	−0.3	6.4E-02			
	hsa-miR-22	171	0.0	9.3E-01			
	hsa-miR-99a	171	−0.3	5.3E-02			
	hsa-miR-720	171	−0.1	4.6E-01			
	hsa-miR-125b	171	−0.2	7.7E-02			
	hsa-miR-1275	170	0.2	2.6E-01			
	HBsAg (IU/l)	171	0.0	8.4E-02			
	HBeAg (IU/l)	171	0.0	6.7E-01			
	rs8099917 TT	160	0.4	1.8E-04			
	HBV DNA (IU/ml)	171	0.0	3.8E-01			
	AST	171	0.0	4.8E-03			
	ALT	171	0.0	7.6E-02			
	γ-GTP	166	0.0	1.7E-04	0.0	3.79E-02	*
	Activity	171	0.6	4.8E-15	0.5	1.35E-09	***
	Genotype C	139	0.4	3.0E-02	0.4	2.63E-02	*
Activity	hsa-miR-122	171	0.2	1.6E-01			
	hsa-miR-22	171	0.4	1.3E-01			
	hsa-miR-99a	171	0.2	1.7E-01			
	hsa-miR-720	171	0.2	1.4E-01			
	hsa-miR-125b	171	0.2	1.1E-01			
	hsa-miR-1275	170	0.1	7.4E-01			
	HBsAg (IU/l)	171	0.0	3.1E-01			
	HBeAg (IU/l)	171	0.0	9.2E-02			
	rs8099917 TT	160	0.9	1.9E-17	0.6	3.80E-13	***
	HBV DNA	171	0.1	4.0E-06	0.1	1.51E-03	**
	AST	171	0.0	1.5E-06	0.0	5.66E-04	***
	ALT	171	0.0	4.3E-04			
	γ-GTP	166	0.0	7.4E-04			
	Liver fibrosis	171	0.5	4.8E-15	0.4	7.00E-11	***
	Genotype C	139	0.0	8.1E-01			

predicted targets and "Cancer," "Hematological Disease," and "Gastrointestinal Disease" networks in HBV patients. To determine if the HBV-associated serum microRNAs shared common transcriptional regulators, upstream transcription factors for each up-regulated microRNA were retrieved from ChIPBase (http://deepbase.sysu.edu.cn/chipbase/ accessed on 14 September 2014).[23] NRSF, JunD, c-Jun transcription have been reported to regulate expression of miR-125b, miR-22, and miR-99a. ZNF11 regulates both miR-125b and miR-99a, and NANOG, E2F4, and HNF4A have been reported to regulate miR-122 and miR-22.

Discussion

This study reports a set of microRNAs that were up- or down-regulated in serum of patients with chronic HBV or HCV compared to healthy subjects. MiR-122 was significantly up-regulated in serum of patients with HBV or HCV, whereas elevated miR-22, miR-99, and miR-125b levels were more characteristic of chronic HBV infection. A number of microRNAs were up-regulated in HBeAg-positive patients compared to HBeAg-negative patients. The HBeAg-associated microRNAs are regulated by a small set of shared transcription factors, including c-Jun, ZNF11, and HNF4A.[23] Expression levels of most HBeAg-associated microRNAs were highly correlated, but individual microRNAs were independently associated with different aspects of HBV infection. MiR-122 was independently associated with HBV DNA, whereas miR-125b was associated with multiple aspects of viral replication, including HBV DNA, HBsAg, and HBeAg, and miR-22 and miR-1275 were independently associated with serum levels of γGTP, a liver enzyme normally associated with alcoholic liver disease or biliary obstruction but which may be elevated in the event of severe viral hepatitis.[24] These results suggest that serum microRNA profiles might serve a diagnostic role in monitoring different aspects of viral infection, although their specific roles in pathogenesis of viral hepatitis remain to be worked out.

The presence of specific serum microRNA profiles associated with chronic HCV or HBV infection suggests involvement of these microRNAs in host-mediated antiviral defense or pathogenesis. Hepatic microRNAs enter the serum via apoptosis or necrosis, or they may be actively secreted within exosomes or viral particles.[14] MiR-122 is abundantly expressed in hepatocytes, and its presence in the serum has been shown to correlate with ALT levels and liver damage.[25,26] MiR-122 strongly suppresses HBV replication both through direct binding to HBV RNA as well as indirectly through cyclin G1-modulated p53 activity.[27–31] MiR-125a-5p, miR-199a-3p and miR-210 also

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inhibit viral replication by directly binding to and suppressing HBV RNA.[30,32,33] MiR-99a is abundantly expressed in the liver and in exosomes and acts as a tumor suppressor by targeting IGF-1R and inducing cell cycle arrest.[16,34] In addition, miR-99 suppresses activity of NF-κB, a transcription factor associated with inflammation and tumorigenesis.[35] In HCC, miR-99a may be severely down-regulated in liver tissue, which is associated with poor prognosis and shorter survival time.[34] As with miR-99a, miR-22 is also abundantly expressed in hepatocytes and exosomes and acts as a tumor suppressor.[16] MiR-22 induces cellular senescence by directly targeting CDKN1A, CDK6, SIRT1, and Sp1 HCC[36,37] and is down-regulated in HBV-related HCC.[37]

Two serum microRNAs investigated in this study (miR-1246 and miR-1275) are part of a set of 13 mitomiRs that have been reported to be significantly enriched in the mitochondrial RNA fraction.[38] Mitochondria play a central role in oxidative stress and apoptosis and are targeted by the HBV X (HBx) protein and the HCV p7 protein.[39] Most mitomiRs, including miR-1246 and miR-1275, are predicted to target COX1, ND5, or other components of the respiratory chain.[38] In this study miR-1275 was significantly up-regulated in patients with HBV and was independently associated with γGTP level, whereas miR-1246 was marginally up-regulated in patients with HCV. MiR-720 has been reported to target the oncogene *TWIST1* involved in tumor metastasis in breast cancer,[40] but its status as a microRNA has been challenged due to a possible mis-annotation of what may be a tRNA fragment instead.[41]

An unexpected result of this study is that serum levels of a number of microRNAs were elevated in HBeAg-positive patients compared to HBeAg-negative patients, even though expression levels of both HBeAg-positive and negative patients were both higher than in healthy subjects. The role of the HBe antigen in HBV infection remains unclear, as it is not required for infection but may serve an immunomodulatory role and contribute to chronic infection through vertical transmission by crossing the placenta. However, the HBV precore region that codes for the HBe antigen is highly conserved among hepadnaviruses, which also infect avian hosts lacking a placenta, suggesting that the protein has a more fundamental function. The precore protein contains a signal peptide, causing it to be secreted.[42] However, up to 30% of the protein is retained in the cytoplasm.[43] While secreted HBeAg may have an immunosuppressive role, intracellular HBeAg instead promotes inflammation.[44] However, HBeAg has been shown to inhibit Toll-like receptor signaling and suppress NF-kB and interferon-beta promoter activity.[45] HBeAg also inhibits IL-6 production by blocking activation of RIPK2-mediated activation of NF-kB.[46] Therefore HBeAg may have a complex roles in both intracellular and extracellular immune modulation.

Seroconversion of HBeAg-positive patients to HBe antibody (HBeAb)-positive patients is usually accompanied by a stop codon mutation within the precore open reading frame.[47] This region has been identified as a mutation hotspot for APOBEC3G, an interferon-stimulated deaminase that inhibits HBV replication by hyper-editing of single-stranded HBV DNA[22] as well as by directly blocking reverse transcription.[48] While hypermutation is deleterious to the virus, a small fraction may acquire mutations conferring a selective advantage.[22] Warner et al. proposed a frequency-dependent selection model positing that while HBeAg suppresses the immune response, HBeAg-negative strains may have an initial competitive advantage by benefitting from HBeAg-mediated immune suppression conferred by HBeAg-positive strains while expending fewer of its resources.[49] However, as the frequency of the HBeAg-positive strain falls, the immune system begins to mount a defense against HBeAg-negative viruses, leading to seroconversion.

It is not clear why serum microRNA levels of several microRNAs, including miR-122, miR-22, miR-125, and miR-99a, tended to be higher in HBeAg-positive individuals compared to HBeAg-negative individuals and are higher in HBV-infected individuals compared to healthy subjects. However, Winther et al. reported similar results in children with chronic hepatitis B and found that plasma levels of a subset of microRNAs decreased significantly in one child before and after HBe seroconversion.[50] We have previously shown that both HBc and HBs proteins co-localize and physically interact with AGO2 in hepatocytes and that siRNA ablation of AGO2 suppressed HBV DNA and HBsAg production,[10] suggesting that components of the RNA silencing machinery are recruited during HBV replication. HSP90 has been reported to act as a chaperone during RNA loading of Argonaute proteins[51] and is also essential in catalyzing HBV reverse transcription and capsid formation by interacting with the pregenomic RNA encapsidation signal, reverse transcriptase, and the core protein.[52] Interestingly, APOBEC3G has been shown to interfere with microRNA regulation by disrupting assembly of the miRNA-inducing silencing complex (miRISC).[53] APOBEC3G itself is also incorporated into nucleocapsids by directly binding to the core protein.[54] While microRNA-mediated gene silencing is associated with accumulation in P-bodies, microRNAs may also be sorted into multivesicular bodies by ESCRT proteins and secreted as exosomes.[55] MiR-122, miR-125b, miR-199a, miR-210, and possibly other microRNAs bind directly to targets within the HBV genome. MiR-199a and miR-210 have been shown to suppress HBsAg production in cell culture. However, HBV has been shown to enhance autophagy without a corresponding increase in protein degradation by HBsAg-mediated activation of the unfolded protein response, and disruption of autophagy inhibits HBV production.[56] Although it is not clear how or if HBeAg is involved in this process, it is possible that the loss of non-secreted intracellular HBeAg or a conformational change in precore RNA resulting from precore mutations interferes with viral control of autophagy or suppression of innate immune signaling. This loss of control over the intracellular environment might result in suppressed viral replication and decreased secretion of exosome-associated microRNAs.

The millions of people chronically infected with HBV or HCV pose a serious public health challenge. While cirrhosis and HCC may develop over a span of decades, HCC is often not detected until late in development, resulting in poor prognosis and leaving few treatment options. Sensitive, non-invasive methods able to detect subtle changes in disease state are needed for early identification of individuals at increased risk. Serum microRNAs may improve

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early detection by providing an indirect means to monitor changes in gene and microRNA expression in the liver.

Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at doi:10.1016/j.jinf.2014.10.017.

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