Journal of Infection (2015) 70, 273—287

BIA
British Infection Association

www.elsevierhealth.com/journals/jinf

Differences in serum microRNA profiles in hepatitis B and C virus infection

Sakura Akamatsu [a,b,c,1], C. Nelson Hayes [a,b,c,1], Masataka Tsuge [c,d], Daiki Miki [a,b,c], Rie Akiyama [a,b,c], Hiromi Abe [a,b,c], Hidenori Ochi [a,b,c], Nobuhiko Hiraga [a,b,c], Michio Imamura [a,b,c], Shoichi Takahashi [e], Hiroshi Aikata [a,c], Tomokazu Kawaoka [a,b,c], Yoshiiku Kawakami [a,b,c], Waka Ohishi [f], Kazuaki Chayama [a,b,c,*]

[a] *Department of Gastroenterology and Metabolism, Applied Life Sciences, Institute of Biomedical & Health Sciences, Hiroshima University, Hiroshima, Japan*
[b] *Laboratory for Digestive Diseases, Center for Genomic Medicine, RIKEN, Hiroshima, Japan*
[c] *Liver Research Project Center, Hiroshima University, Hiroshima, Japan*
[d] *Natural Science Center for Basic Research and Development, Hiroshima University, Hiroshima, Japan*
[e] *Koyo New Town Hospital, Hiroshima, Japan*
[f] *Department of Clinical Studies, Radiation Effects Research Foundation, Hiroshima, Japan*

Accepted 17 October 2014
Available online 5 November 2014

KEYWORDS
Serum biomarkers;
microRNA;
miR-122;
miR-125b;
HBeAg;
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.

* Corresponding author. Department of Gastroenterology and Metabolism, Applied Life Sciences, Institute of Biomedical & Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. Tel.: +81 82 257 5190; fax: +81 82 255 6220.
E-mail address: chayama@hiroshima-u.ac.jp (K. Chayama).
[1] Co-first authors.

http://dx.doi.org/10.1016/j.jinf.2014.10.017

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.

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

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	

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

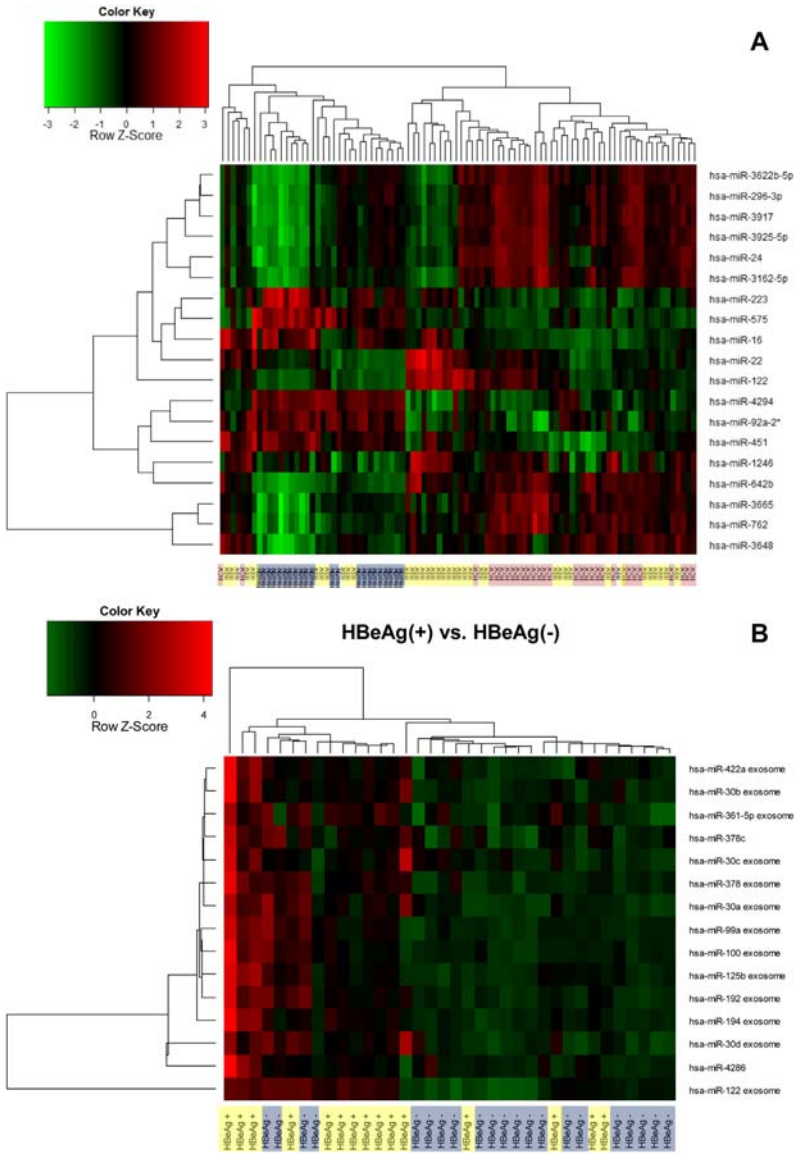

Figure 1 Heatmap of results of serum microRNA microarray analysis. Up-regulated microRNAs are shown in red, and down-regulated microRNAs are shown in green. Hierarchical clustering was performed in R using Euclidean distance and McQuitty clustering. A) Healthy (blue) versus HCV (purple) and HBV (yellow). 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

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*)

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.

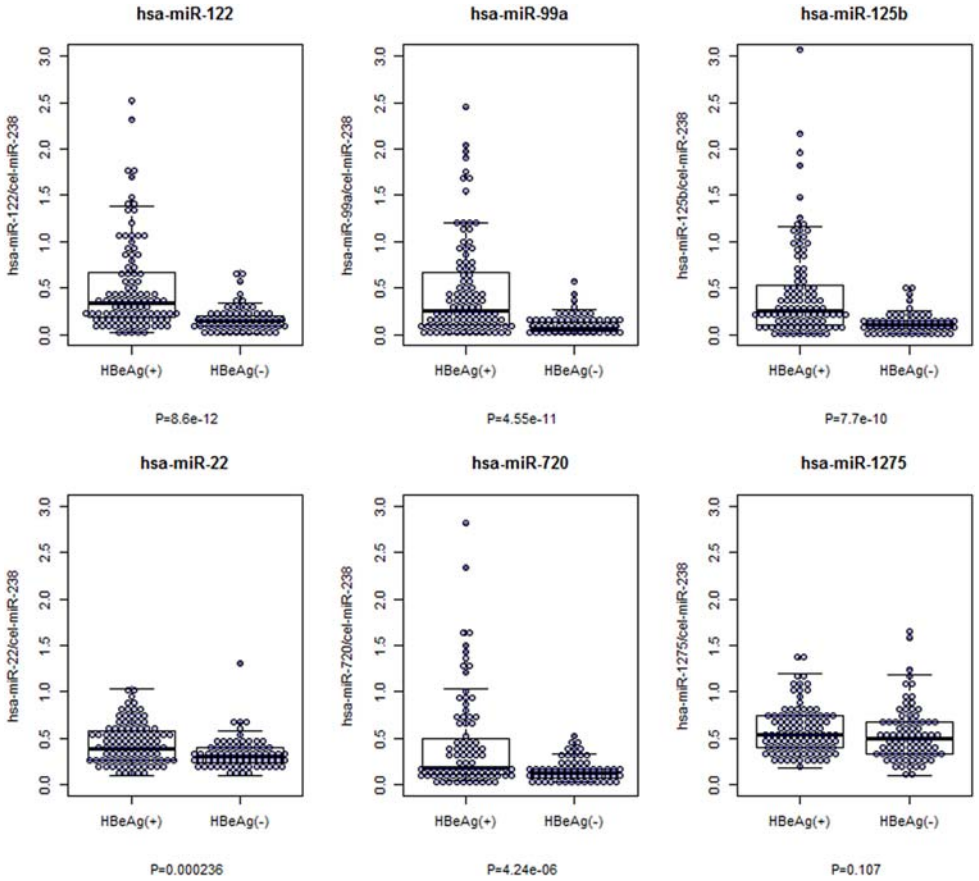

Figure 2 Serum microRNA expression in HBe antigen positive and negative individuals. qRT-PCR microRNA expression levels normalized by cel-miR-238 are shown. *P*-values represent the difference in median values using the non-parametric Kruskal—Wallis rank sum test.

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

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	**

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*)

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			
	g**-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

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

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

Conflicts of interest

None.

Acknowledgments

This work was supported by Grants-in-Aid for scientific research and development from the Ministry of Health, Labor and Welfare and Ministry of Education Culture Sports Science and Technology, Government of Japan. No writing assistance was provided for this manuscript.

Appendix A. Supplementary data

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

References

1. Fields BN, Knipe DM, Howley PM. *Fields virology*. 5th ed. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins; 2007.
2. McMahon BJ. The natural history of chronic hepatitis B virus infection. *Hepatology* 2009 May;**49**(5 Suppl):S45—55 [Consensus Development Conference, NIH Research Support, U.S. Gov't, P.H.S.].
3. Brechot C, Kremsdorf D, Soussan P, Pineau P, Dejean A, Paterlini-Brechot P, et al. Hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC): molecular mechanisms and novel paradigms. *Pathol Biol (Paris)* 2010 Aug;**58**(4):278—87 [Review].
4. Xi Y, Nakajima G, Gavin E, Morris CG, Kudo K, Hayashi K, et al. Systematic analysis of microRNA expression of RNA extracted from fresh frozen and formalin-fixed paraffin-embedded samples. *RNA* 2007 Oct;**13**(10):1668—74 [Research Support, N.I.H., Extramural].
5. Liu AM, Zhang C, Burchard J, Fan ST, Wong KF, Dai H, et al. Global regulation on microRNA in hepatitis B virus-associated hepatocellular carcinoma. *Omics* 2011 Mar;**15**(3):187—91.
6. Bala S, Marcos M, Szabo G. Emerging role of microRNAs in liver diseases. *World J Gastroenterol* 2009 Dec 7;**15**(45):5633—40 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't Review].
7. Ji F, Yang B, Peng X, Ding H, You H, Tien P. Circulating microRNAs in hepatitis B virus-infected patients. *J Viral Hepat* 2011 Jul;**18**(7):e242—51 [Research Support, Non-U.S. Gov't].
8. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U. S. A.* 2008 Jul 29;**105**(30):10513—8 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't].
9. Ura S, Honda M, Yamashita T, Ueda T, Takatori H, Nishino R, et al. Differential microRNA expression between hepatitis B and hepatitis C leading disease progression to hepatocellular carcinoma. *Hepatology* 2009 Apr;**49**(4):1098—112.
10. Hayes CN, Akamatsu S, Tsuge M, Miki D, Akiyama R, Abe H, et al. Hepatitis B virus-specific miRNAs and argonaute2 play a role in the viral life cycle. *PLoS One* 2012;**7**(10):e47490 [Research Support, Non-U.S. Gov't].
11. Shwetha S, Gouthamchandra K, Chandra M, Ravishankar B, Khaja MN, Das S. Circulating miRNA profile in HCV infected serum: novel insight into pathogenesis. *Sci Rep* 2013 Apr;**3**(3):1555 [Research Support, Non-U.S. Gov't].
12. Turchinovich A, Weiz L, Langheinz A, Burwinkel B. Characterization of extracellular circulating microRNA. *Nucleic Acids Res* 2011 Sep 1;**39**(16):7223—33 [Research Support, Non-U.S. Gov't].
13. Arroyo JD, Chevillet JR, Kroh EM, Ruf IK, Pritchard CC, Gibson DF, et al. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc Natl Acad Sci U. S. A* 2011 Mar 22;**108**(12):5003—8 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't].
14. Novellino L, Rossi RL, Bonino F, Cavallone D, Abrignani S, Pagani M, et al. Circulating hepatitis B surface antigen particles carry hepatocellular microRNAs. *PLoS One* 2012;**7**(3):e31952.
15. Gallo A, Tandon M, Alevizos I, Illei GG. The majority of microRNAs detectable in serum and saliva is concentrated in exosomes. *PLoS One* 2012;**7**(3):e30679 [Comparative Study Research Support, N.I.H., Intramural].
16. Huang X, Yuan T, Tschannen M, Sun Z, Jacob H, Du M, et al. Characterization of human plasma-derived exosomal RNAs by deep sequencing. *BMC Genomics* 2013;**14**:319 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't].
17. Conde-Vancells J, Rodriguez-Suarez E, Embade N, Gil D, Matthiesen R, Valle M, et al. Characterization and comprehensive proteome profiling of exosomes secreted by hepatocytes. *J Proteome Res* 2008 Dec;**7**(12):5157—66 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't].
18. Li J, Liu K, Liu Y, Xu Y, Zhang F, Yang H, et al. Exosomes mediate the cell-to-cell transmission of IFN-alpha-induced antiviral activity. *Nat Immunol* 2013 Aug;**14**(8):793—803 [Research Support, Non-U.S. Gov't].
19. Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology* 1994 Jun;**19**(6):1513—20 [Review].
20. Dweep H, Sticht C, Pandey P, Gretz N. miRWalk—database: prediction of possible miRNA binding sites by "walking" the genes of three genomes. *J Biomed Inf* 2011 Oct;**44**(5):839—47 [Research Support, Non-U.S. Gov't].
21. Mathivanan S, Fahner CJ, Reid GE, Simpson RJ. ExoCarta 2012: database of exosomal proteins, RNA and lipids. *Nucleic Acids Res* 2012 Jan;**40**(Database issue):D1241—4 [Research Support, Non-U.S. Gov't].
22. Vartanian JP, Henry M, Marchio A, Suspene R, Aynaud MM, Guetard D, et al. Massive APOBEC3 editing of hepatitis B viral DNA in cirrhosis. *PLoS Pathog* 2010 May;**6**(5):e1000928 [Research Support, Non-U.S. Gov't].
23. Yang JH, Li JH, Jiang S, Zhou H, Qu LH. ChIPBase: a database for decoding the transcriptional regulation of long non-coding RNA and microRNA genes from ChIP-Seq data. *Nucleic Acids Res* 2013 Jan;**41**(Database issue):D177—87 [Research Support, Non-U.S. Gov't].
24. Lum G, Gambino SR. Serum gamma-glutamyl transpeptidase activity as an indicator of disease of liver, pancreas, or bone. *Clin Chem* 1972 Apr;**18**(4):358—62.
25. Bala S, Petrasek J, Mundkur S, Catalano D, Levin I, Ward J, et al. Circulating microRNAs in exosomes indicate hepatocyte injury and inflammation in alcoholic, drug-induced, and inflammatory liver diseases. *Hepatology* 2012 Nov;**56**(5):1946—57 [Research Support, N.I.H., Extramural].
26. Arataki K, Hayes CN, Akamatsu S, Akiyama R, Abe H, Tsuge M, et al. Circulating microRNA-22 correlates with microRNA-122

and represents viral replication and liver injury in patients with chronic hepatitis B. *J Med Virology* 2013 May;**85**(5): 789—98 [Research Support, Non-U.S. Gov't].
27. Wang S, Qiu L, Yan X, Jin W, Wang Y, Chen L, et al. Loss of MiR-122 expression in patients with hepatitis B enhances hepatitis B virus replication through cyclin G1 modulated P53 activity. *Hepatology* 2012 Mar;**55**(3):730—41.
28. Hu J, Xu Y, Hao J, Wang S, Li C, Meng S. MiR-122 in hepatic function and liver diseases. *Protein Cell* 2012 May;**3**(5): 364—71.
29. Chang J, Nicolas E, Marks D, Sander C, Lerro A, Buendia MA, et al. miR-122, a mammalian liver-specific microRNA, is processed from hcr mRNA and may downregulate the high affinity cationic amino acid transporter CAT-1. *RNA Biol* 2004 Jul;**1**(2): 106—13 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't].
30. Chen Y, Shen A, Rider PJ, Yu Y, Wu K, Mu Y, et al. A liver-specific microRNA binds to a highly conserved RNA sequence of hepatitis B virus and negatively regulates viral gene expression and replication. *Faseb J* 2011 Dec;**25**(12):4511—21.
31. Qiu L, Fan H, Jin W, Zhao B, Wang Y, Ju Y, et al. miR-122-induced down-regulation of HO-1 negatively affects miR-122-mediated suppression of HBV. *Biochem Biophys Res Commun* 2010 Aug 6;**398**(4):771—7 [Research Support, Non-U.S. Gov't].
32. Potenza N, Papa U, Mosca N, Zerbini F, Nobile V, Russo A. Human microRNA hsa-miR-125a-5p interferes with expression of hepatitis B virus surface antigen. *Nucleic Acids Res* 2011 Jul; **39**(12):5157—63.
33. Zhang GL, Li YX, Zheng SQ, Liu M, Li X, Tang H. Suppression of hepatitis B virus replication by microRNA-199a-3p and microRNA-210. *Antivir Res* 2010 Nov;**88**(2):169—75 [Research Support, Non-U.S. Gov't].
34. Li D, Liu X, Lin L, Hou J, Li N, Wang C, et al. MicroRNA-99a inhibits hepatocellular carcinoma growth and correlates with prognosis of patients with hepatocellular carcinoma. *J Biol Chem* 2011 Oct 21;**286**(42):36677—85 [Research Support, Non-U.S. Gov't].
35. Takata A, Otsuka M, Kojima K, Yoshikawa T, Kishikawa T, Yoshida H, et al. MicroRNA-22 and microRNA-140 suppress NF-kappaB activity by regulating the expression of NF-kappaB coactivators. *Biochem Biophys Res Commun* 2011 Aug 12;**411**(4):826—31 [Research Support, Non-U.S. Gov't].
36. Xu D, Takeshita F, Hino Y, Fukunaga S, Kudo Y, Tamaki A, et al. miR-22 represses cancer progression by inducing cellular senescence. *J Cell Biol* 2011 Apr 18;**193**(2):409—24 [Research Support, Non-U.S. Gov't].
37. Shi C, Xu X. MicroRNA-22 is down-regulated in hepatitis B virus-related hepatocellular carcinoma. *Biomed Pharmacother* 2013 Jun;**67**(5):375—80 [Research Support, Non-U.S. Gov't].
38. Bandiera S, Ruberg S, Girard M, Cagnard N, Hanein S, Chretien D, et al. Nuclear outsourcing of RNA interference components to human mitochondria. *PLoS One* 2011;**6**(6): e20746.
39. D'Agostino DM, Bernardi P, Chieco-Bianchi L, Ciminale V. Mitochondria as functional targets of proteins coded by human tumor viruses. *Adv Cancer Res* 2005;**94**:87—142.
40. Li LZ, Zhang CZ, Liu LL, Yi C, Lu SX, Zhou X, et al. miR-720 inhibits tumor invasion and migration in breast cancer by targeting TWIST1. *Carcinogenesis* 2014 Feb;**35**(2):469—78 [Comparative Study Research Support, Non-U.S. Gov't].
41. Schopman NC, Heynen S, Haasnoot J, Berkhout B. A miRNA-tRNA mix-up: tRNA origin of proposed miRNA. *RNA Biol* 2010 Sep-Oct;**7**(5):573—6 [Research Support, Non-U.S. Gov't].
42. Ou JH, Laub O, Rutter WJ. Hepatitis B virus gene function: the precore region targets the core antigen to cellular membranes and causes the secretion of the e antigen. *Proc Natl Acad Sci U. S. A* 1986 Mar;**83**(6):1578—82 [Research Support, U.S. Gov't, P.H.S.].
43. Garcia PD, Ou JH, Rutter WJ, Walter P. Targeting of the hepatitis B virus precore protein to the endoplasmic reticulum membrane: after signal peptide cleavage translocation can be aborted and the product released into the cytoplasm. *J Cell Biol* 1988 Apr;**106**(4):1093—104 [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.].
44. Milich D, Liang TJ. Exploring the biological basis of hepatitis B e antigen in hepatitis B virus infection. *Hepatology* 2003 Nov; **38**(5):1075—86 [Review].
45. Lang T, Lo C, Skinner N, Locarnini S, Visvanathan K, Mansell A. The hepatitis B e antigen (HBeAg) targets and suppresses activation of the toll-like receptor signaling pathway. *J Hepatol* 2011 Oct;**55**(4):762—9 [Research Support, Non-U.S. Gov't].
46. Wu S, Kanda T, Imazeki F, Nakamoto S, Tanaka T, Arai M, et al. Hepatitis B virus e antigen physically associates with receptor-interacting serine/threonine protein kinase 2 and regulates IL-6 gene expression. *J Infect Dis* 2012 Aug 1; **206**(3):415—20 [Research Support, Non-U.S. Gov't].
47. Tong S, Kim KH, Chante C, Wands J, Li J. Hepatitis B Virus e Antigen Variants. *Int J Med Sci* 2005;**2**(1):2—7.
48. Nguyen DH, Hu J. Reverse transcriptase- and RNA packaging signal-dependent incorporation of APOBEC3G into hepatitis B virus nucleocapsids. *J Virol* 2008 Jul;**82**(14):6852—61 [Research Support, N.I.H., Extramural].
49. Warner BG, Abbott WG, Rodrigo AG. Frequency-dependent selection drives HBeAg seroconversion in chronic hepatitis B virus infection. *Evol Med Public Health* 2014 Jan;**2014**(1): 1—9.
50. Winther TN, Bang-Berthelsen CH, Heiberg IL, Pociot F, Hogh B. Differential plasma microRNA profiles in HBeAg positive and HBeAg negative children with chronic hepatitis B. *PLoS One* 2013;**8**(3):e58236 [Research Support, Non-U.S. Gov't].
51. Johnston M, Geoffroy MC, Sobala A, Hay R, Hutvagner G. HSP90 protein stabilizes unloaded argonaute complexes and microscopic P-bodies in human cells. *Mol Biol Cell* 2010 May 1;**21**(9):1462—9 [Research Support, Non-U.S. Gov't].
52. Shim HY, Quan X, Yi YS, Jung G. Heat shock protein 90 facilitates formation of the HBV capsid via interacting with the HBV core protein dimers. *Virology* 2011 Feb 5;**410**(1):161—9 [Research Support, Non-U.S. Gov't].
53. Liu C, Zhang X, Huang F, Yang B, Li J, Liu B, et al. APOBEC3G inhibits microRNA-mediated repression of translation by interfering with the interaction between Argonaute-2 and MOV10. *J Biol Chem* 2012 Aug 24;**287**(35):29373—83 [Research Support, N.I.H., Extramural sResearch Support, Non-U.S. Gov't].
54. Zhao D, Wang X, Lou G, Peng G, Li J, Zhu H, et al. APOBEC3G directly binds Hepatitis B virus core protein in cell and cell free systems. *Virus Res* 2010 Aug;**151**(2):213—9 [Research Support, Non-U.S. Gov't].
55. Gibbings DJ, Ciaudo C, Erhardt M, Voinnet O. Multivesicular bodies associate with components of miRNA effector complexes and modulate miRNA activity. *Nat Cell Biol* 2009 Sep; **11**(9):1143—9 [Comment Research Support, Non-U.S. Gov't].
56. Li J, Liu Y, Wang Z, Liu K, Wang Y, Liu J, et al. Subversion of cellular autophagy machinery by hepatitis B virus for viral envelopment. *J Virol* 2011 Jul;**85**(13):6319—33 [Research Support, Non-U.S. Gov't].