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

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KEYWORDS
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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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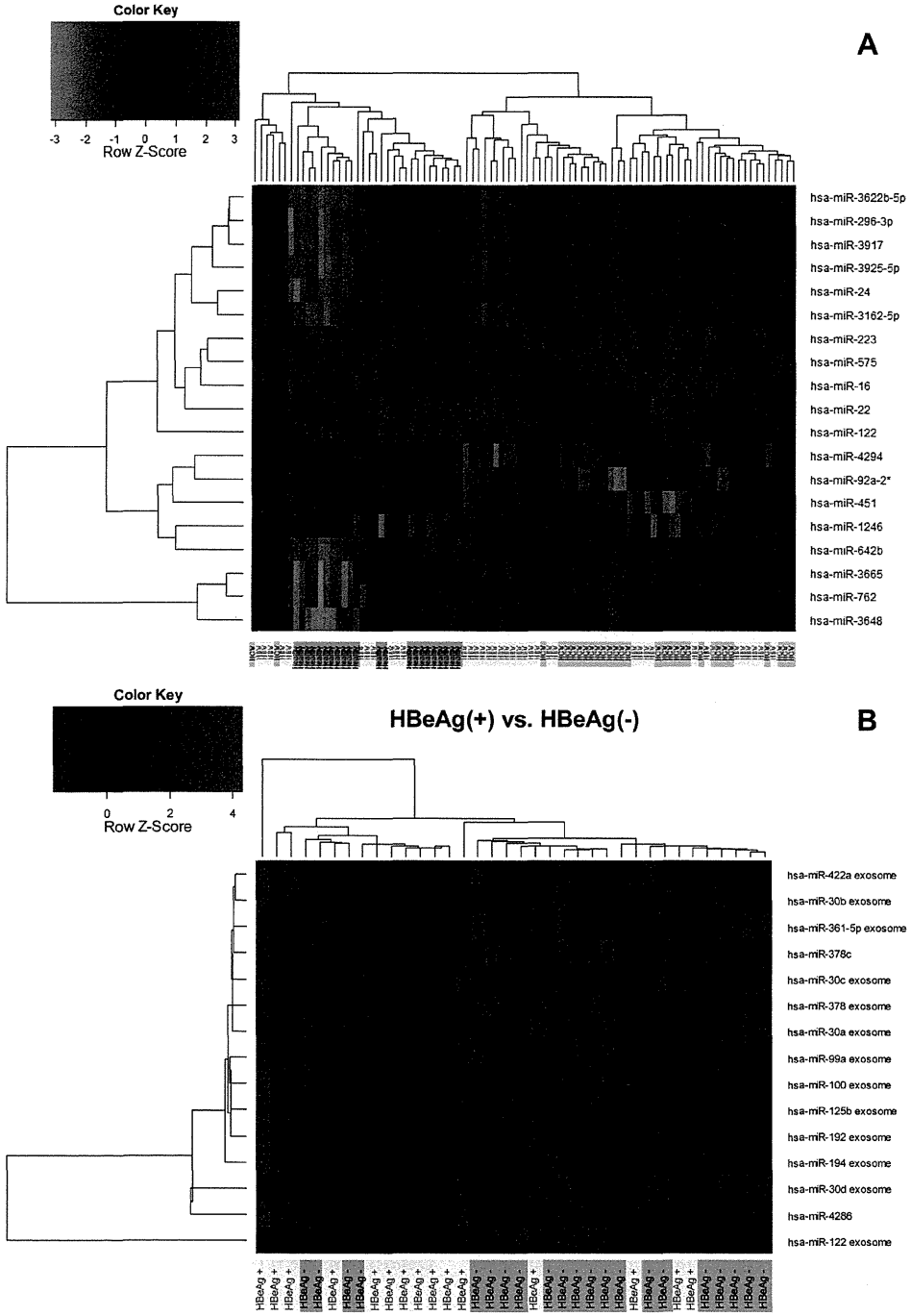

Figure 1 Heatmap of results of serum microRNA microarray analysis. Up-regulated microRNAs are shown in red, and down-regulated microRNAs are shown in green. Hierarchical clustering was performed in R using Euclidean distance and McQuitty clustering. A) Healthy (blue) versus HCV (purple) and HBV (yellow).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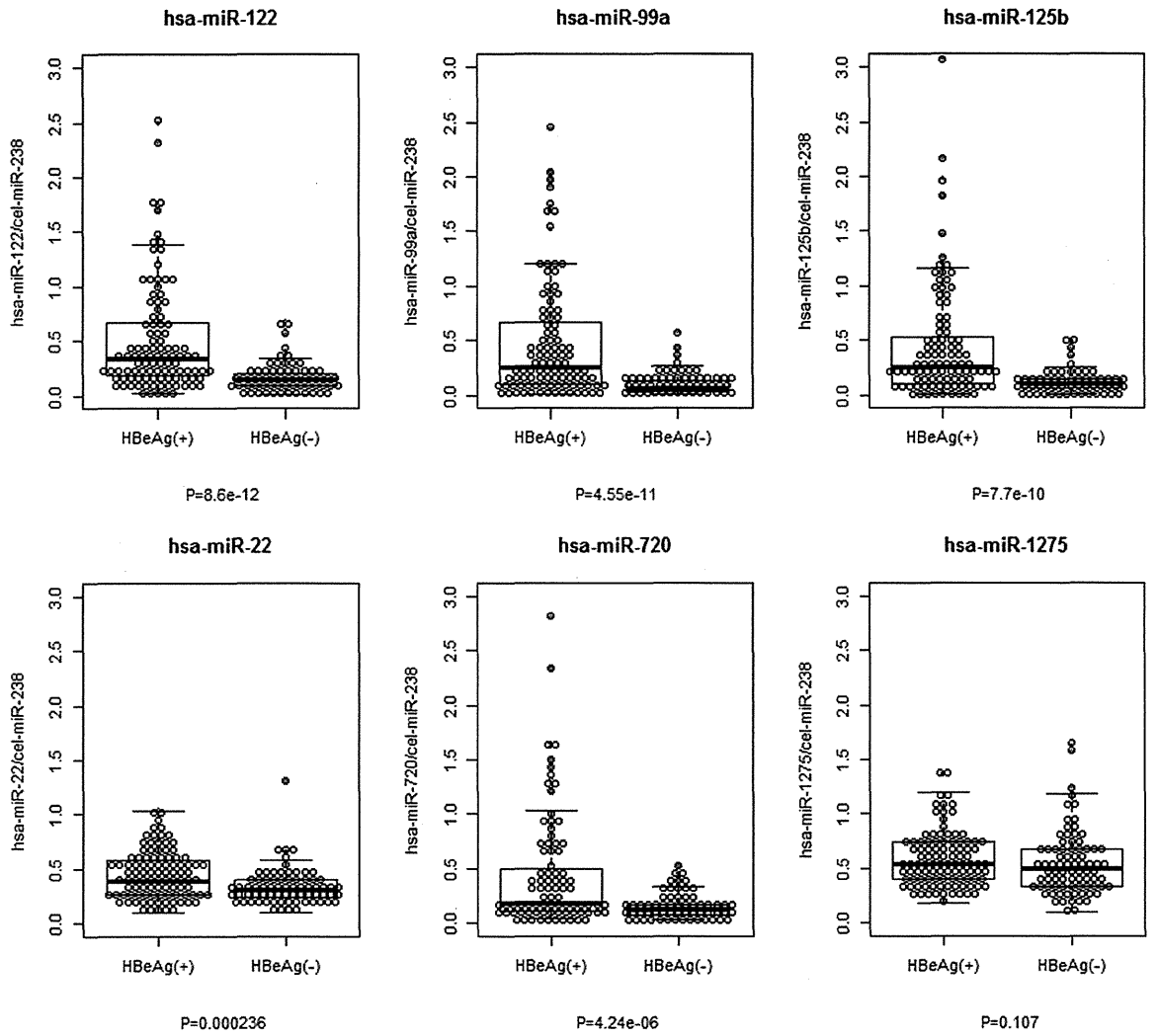

Figure 2 Serum microRNA expression in HBe antigen positive and negative individuals. qRT-PCR microRNA expression levels normalized by cel-miR-238 are shown. *P*-values represent the difference in median values using the non-parametric Kruskal–Wallis rank sum test.

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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