World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
wjg@wjgnet.com
doi:10.3748/wjg.v18.i37.5188

World J Gastroenterol 2012 October 7; 18(37): 5188-5196
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Serum levels of microRNAs can specifically predict liver injury of chronic hepatitis B

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Supported by National Science and Technology Major Project of China, No. 2012ZX10005001-004; Leading Academic Discipline Project of Shanghai Municipal Education Commission, No. J50301; Doctoral Fund of Ministry of Education of China, No. 20093107120010; and E-institutes of Shanghai Municipal Education Commission, No. E 03008
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Received: December 16, 2011 Revised: July 26, 2012
Accepted: July 29, 2012
Published online: October 7, 2012

Abstract

AIM: To investigate whether circulating microRNAs (miRNAs) can serve as molecular markers to predict liver injury resulted from chronic hepatitis B (CHB).

METHODS: The profiles of serum miRNA expression were first generated with serum samples collected from 10 patients with CHB and 10 healthy donors (Ctrls) by microarray analysis. The levels of several miRNAs were further quantitated by real-time reverse transcription polymerase chain reaction with serum samples from another 24 CHB patients and 24 Ctrls. Serum samples of 20 patients with nonalcohlic steatohepatitis (NASH) were also included for comparison. The comparison in the levels of miRNAs between groups (CHB, NASH and Ctrl) was analyzed with Mann-Whitney *U*-test. The correlation between miRNAs and clinical pathoparameters was analyzed using Spearman correlation analysis or canonical correlation analysis. The receiver-operator characteristic (ROC) curves were also generated to determine the specificity and sensitivity of each individual miRNA in distinguishing patients with CHB from Ctrls.

RESULTS: miRNA profile analysis showed that 34 miRNAs were differentially expressed between CHB and Ctrl subjects, in which 12 were up-regulated and 22 down-regulated in CHB subject (fold change > 2.0 and $P < 0.01$). The median levels of miR-122, -572, -575 and -638 were significantly higher ($P < 1.00 \times 10^{-5}$) while miR-744 significantly lower ($P < 1.00 \times 10^{-6}$) in CHB compared with the Ctrl. The levels of miR-122, -572 and -638 were also higher ($P < 1.00 \times 10^{-3}$) while the level of miR-744 lower in CHB ($P < 0.05$) than in NASH, although the difference between them was not as significant as that between CHB and Ctrl. ROC curve analysis revealed that the levels of miR-122, -572, -575, -638 and -744 in serum were sensitive and specific enough to distinguish CHB, NASH and Ctrl. Multivariate analysis further showed that the levels of these miRNAs were correlated with the liver function parameters. Most significantly, it was the scatter plot of principal component with the levels of these miRNAs, but not the parameters of liver function, which clearly distinguished CHB, NASH and Ctrl subjects.

CONCLUSION: Serum levels of miR-122, -572, -575, -638 and -744 are deregulated in patients with CHB or NASH. The levels of these miRNAs may serve as potential biomarkers for liver injury caused by CHB and NASH.

Key words: Chronic hepatitis B; Nonalcohlic steatohepatitis; Serum microRNAs; Liver injury

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Zhang H, Li QY, Guo ZZ, Guan Y, Du J, Lu YY, Hu YY, Liu P, Huang S, Su SB. Serum levels of microRNAs can specifically predict liver injury of chronic hepatitis B. *World J Gastroenterol* 2012; 18(37): 5188-5196 Available from: URL: http://www.wjgnet.com/1007-9327/full/v18/i37/5188.htm DOI: http://dx.doi.org/10.3748/wjg.v18.i37.5188

INTRODUCTION

Hepatitis B virus (HBV) infection is one of the major health problems in China[1]. Of the 350 million individuals worldwide infected with the HBV, one-third are from China[2]. HBV infection results in chronic hepatitis B (CHB) and patients with CHB exhibit a high risk of developing liver cirrhosis and hepatocellular carcinoma[3]. Although HBV itself is noncytopathic, host immune response often causes liver damage in patients with HBV infection. Currently, the most commonly used markers of liver injury are the enzymatic activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in blood; however, these markers are devoid of sufficient sensitivity and specificity to diagnose virus-induced liver damages[4,5]. Therefore, assessing the severity of HBV-induced damages and monitoring the progression of CHB are major clinical challenges.

microRNAs (miRNAs) are evolutionarily conserved, and are small (typically ~22 nt in size) regulatory RNA molecules that modulate the levels of specific targets, and are thus actively involved in a wide range of physiologic and pathologic processes[6,7]. Interestingly, miRNAs are very stable in circulation systems, and tissue or organ-specific intracellular miRNAs can often be detected in blood under pathological conditions[8-12]. The elevated levels of these miRNAs in blood are most likely caused by their release into the circulation system in the processes accompanied with cell death, such as cell turnover, cell destruction and pathological injury[13-16]. For example, the levels of miR-1, a muscle and heart-specific miRNA, is elevated in blood during acute myocardial infarction[13]. miR-141, a miRNA highly expressed in prostate cancer cells, is present at a significantly higher level in prostate cancer patients than healthy donors[14]. In addition, the levels of serum miRNAs can also be associated with different physiological conditions. For instance, miRNAs of presumed placental origin were detected at high levels in the plasma of pregnant women[17]. CHB is an infectious illness, and the host immune response to HBV infection is thus expected to cause both hepatocellular damage and viral clearance[18]. In fact, CHB progresses with significant apoptosis and necrosis of hepatocytes[19]. It is of interest to determine whether particular miRNAs are released to blood of CHB patients and can serve as predictor for CHB liver injury.

The goal of this study is to investigate whether the circulating miRNAs can be used as molecular biomarkers to monitor the pathological development of CHB. As liver cells are also damaged along the progression of nonalcoholic steatohepatitis (NASH) and caused by the build-up of fat cell in the liver[20], we included samples of NASH patients in this study. Through the comparison of serum miRNA expression profiles among CHB and NASH patients and healthy donors, we found that the expression of miR-122, -572, -575, -638 and -744 was deregulated in both CHB and NASH patients. The expression of these five miRNAs was significantly correlated with pathological parameters of liver. We, therefore, conclude that these five miRNAs may serve as potential biomarkers for CHB and NASH-induced liver injury.

MATERIALS AND METHODS

Study subjects and clinical parameters

Sera collected from 34 CHB, 20 NASH patients and 34 healthy donors (Ctrls) were included in this study. Samples from 10 CHB patients and 10 Ctrls were subjected to miRNA microarray analysis to obtain serum miRNA profiles. Those miRNAs with altered levels were further measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR) with the samples from the remaining 24 CHB patients and 24 Ctrls. To determine the specificity of miRNA level change, serum samples from 20 NASH patients were also included for qRT-PCR analysis. Serum samples of Ctrls were randomly selected from a collection of 120 individuals who had annual physical examination at Shanghai Shuguang Hospital, Shanghai, China. Samples of CHB and NASH were from patients seeking treatment in Shanghai Shuguang Hospital. The diagnostic criteria for CHB followed the guidelines that defined by the Chinese Society of Hepatology and Chinese Society of Infectious Diseases in 2005[21]. The diagnosis of NASH was based on the guidelines for diagnosis and treatment of nonalcoholic fatty liver diseases that issued by Fatty Liver and Alcoholic Liver Disease Study Group of the Chinese Liver Disease Association in 2006[22]. The clinical parameters of these patients are listed in Table 1. This study was approved by the Institutional Review Board of Shanghai Shuguang Hospital.

Serum sample collection and RNA isolation

All serum samples were derived from freshly-drawn blood and stored at -80 ℃. RNA in serum was isolated using a miRVana PARIS kit (Ambion, Austin, TX, United States) according to the manufacturer's protocol followed by the treatment of RNase-free DNase I (Promega, Madison, WI, United States) to eliminate DNA contamination. The concentration of RNAs extracted from serum ranged from 1.5 to 12 ng/μL.

Table 1 Clinical characteristics of participants in validation

Group	CHB	NASH	Ctrl
Individuals (*n*)	24	20	24
Gender (*n*)			
Male	21	17	19
Female	3	3	5
Age (yr)	37.6 ± 9.0	39.4 ± 9.6	35.6 ± 10.2
ALT (IU/L)	82.6 (14-412)	51.7 (18-203)	21.5 (14-43)
AST (IU/L)	62.6 (9-206)	31.55 (17-62)	21.4 (16-49)
GGT (IU/L)	69.4 (10-580)	45.8 (13-124)	20.8 (12-33)
ALP (IU/L)	95.8 (51-211)	66.0 (41-90)	62.3 (42-96)
TBIL (μmol/L)	19.2 (10.2-50.7)	19.5 (13.1-31.7)	15.9 (6.9-27.1)
HBV DNA	7 377 395 (0-94 800 000)	0	0
Bile acid (μmol/L)	6.9 (1.4-13)	17.9 (3-118.7)	8.4 (4.1-12)
HBV status (*n*)			
$HBsAg^+$	24	0	0
$HBsAg^-$	0	20	24

Bile acid were given as medians (range). ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: γ-glutamyltransferase; ALP: Alkaline phosphatase; TBIL: Total bilirubinand; CHB: Chronic hepatitis B; NASH: Nonalcohlic steatohepatitis; Ctrl: Healthy donor; HBsAg: Hepatitis B surface antigen.

Serum miRNA profiling and data analysis

The profiles of serum miRNAs of 10 CHB patients and 10 Ctrls were generated using Agilent Human miRNA microarray V3 (Agilent Technologies Inc, Santa Clara, CA, United States). The microarray chip is comprised of 2371 different probes for a total of 851 human miRNAs. One hundred nanograms of serum RNA was used for each array. The arrays were read using the Agilent microarray scanner and the data were extracted using Feature Extraction V10.7 (Agilent Technologies, CA, United States). All data were transformed to log base 2. The differences between samples were calculated using unsupervised analysis (SAS system, Shanghai Biochip, Shanghai, China). Only the miRNAs with the fold difference > 2.0 and $P < 0.01$ were considered significant.

Validation of internal reference for serum miRNA quantification

There has been no consensus on the reference genes for qRT-PCR analysis of serum miRNAs. However, 6 miRNAs, RNU6B[23], miR-24[14,24], miR-16[15], miR-181a[25], miR-454[26] and miR-638[27], have been reported to be consistently present in human serum. Therefore, these miRNAs were empirically analyzed by qRT-PCR in samples from all patients and Ctrls. The cycle threshold (Ct) values were converted into relative quantities for analysis with geNorm software[28], which selects the optimal number of the most stable genes for normalization. To calculate the expression stability of a given gene (gene stability measure *M*), the program uses an algorithm based on the mean of the pairwise variation of a given reference gene compared to all other control genes. The higher the value of *M* is, the more the expression variability of the corresponding reference gene is. The least stable gene, i.e., the gene with the highest *M* value, was excluded from the subsequent analysis. The remaining genes was recalculated for *M* values and the gene with highest *M* was again excluded until the two most stable genes were left.

Quantification of serum miRNAs

qRT-PCR-based quantification of miRNAs (200 μL of serum from each participant) was performed with Bulge-Loop™ miRNA qPCR Primer Set (Guangzhou Ribobio, Guangzhou, China) and SYBR Green PCR Master Mixture (TOYOBO, LTD, Japan) according to the manufacturer's instructions using a Rotor-Gene 6000 Real-time PCR machine (Corbett Life Science, Sydney, Australia). The specificity of each PCR products was validated by melting curve analysis at the end of PCR cycles. All samples were analyzed in triplicate and the Ct was defined as the number of cycles required for the fluorescent signal to reach the threshold. The levels of miRNAs in serum were calculated using the formula $2^{\Delta Ct}$ where ΔCt = Ct of internal reference -Ct of target miRNA.

Establishment of receiver-operator characteristic curves

Receiver-operator characteristic (ROC) curves were established to evaluate the difference in the levels of serum miRNAs among CHB, NASH and Ctrls. Statistical significance for correlations was calculated using Spearman's non-parametric rank test and the correlation coefficient R generated by Spearman correlation formula.

Statistical analysis

Comparisons between groups were analyzed using Mann-Whitney *U*-test, Pearson χ^2 test, canonical correlation analysis or Spearman correlation analysis wherever appropriate. All tests were two-tailed and $P < 0.05$ was considered statistically significant.

RESULTS

Serum miRNA profiles of CHB patients are distinct from those of Ctrls or NASH patients

To determine whether there was difference in serum miRNA profiles between people with or without CHB, we performed miRNA microarray with RNAs isolated from the sera of 10 CHB patients and 10 Ctrls. Among a total of 851 miRNAs analyzed, 34 of them were differentially expressed between CHB patients and Ctrls (fold change > 2.0 and $P < 0.01$) (Table 2).

In order to validate the serum miRNA profiles generated from microarray, we initially turned our attention to identifying a particular serum miRNA that can be used as an internal control. As the levels of RNU6B, miR-24, -16, -181a, -454 and -638 were previously reported be relatively consistent[14,15,23-26], we measured their levels in 16 serum samples (4 CHB, 4 NASH and 8 Ctrls). We employed GeNorm to calculate the stability values (*M*-values) for these candidate miRNAs and excluded the candidates with the lowest stability (the highest *M* value). The stability value was recalculated until the two most stable miRNAs were predicted. Defining *M*-values below 1.5 as the critical limit, GeNorm data analysis showed that miR-24

Table 2 Differentially expressed miRNAs in chronic hepatitis B patients and healthy donors

miRNA	Fold change	*P* value	CHB (log_2) mean	Ctrls (log_2) mean
hsa-miR-122	8.29	2.99E-03	8.14	5.09
hsa-miR-138	4.23	5.69E-03	2.68	0.60
hsa-miR-638	4.18	2.43E-03	12.62	10.55
hsv1-miR-H1	3.93	7.92E-03	7.67	5.70
hsa-miR-575	3.67	5.69E-03	9.59	7.71
hsa-miR-572	3.36	3.98E-03	7.58	5.83
kshv-miR-K12-3	3.34	2.60E-03	10.19	8.45
hsa-miR-1915	3.12	5.22E-03	11.30	9.66
hsa-miR-623	3.07	4.69E-03	6.85	5.23
hsa-miR-1268	2.81	6.43E-03	9.94	8.45
hsa-miR-939	2.63	3.94E-03	8.81	7.42
hsa-miR-498	2.29	4.30E-03	6.07	4.87
hsa-miR-421	0.37	4.05E-03	0.93	2.38
hsa-miR-598	0.35	5.24E-04	0.67	2.20
hsa-miR-155	0.34	6.40E-03	2.40	3.94
hsa-miR-424	0.33	9.76E-03	3.67	5.26
hsa-miR-23b	0.28	8.42E-03	5.44	7.29
hsa-miR-195	0.27	1.17E-03	1.20	3.10
hsa-miR-487b	0.26	5.46E-03	1.49	3.44
hsa-miR-224	0.25	3.71E-03	1.45	3.45
hsa-miR-495	0.24	2.50E-03	1.16	3.21
hsa-miR-181c	0.22	6.78E-03	1.88	4.03
hsa-miR-654-3p	0.21	8.27E-03	1.99	4.22
hsa-let-7e	0.21	2.52E-03	0.75	2.99
hsa-miR-382	0.21	9.11E-03	1.78	4.02
hsa-miR-17[1]	0.19	9.66E-03	2.47	4.89
hsa-miR-128	0.18	6.17E-03	2.38	4.82
hsa-miR-625	0.18	2.70E-04	2.14	4.61
hsa-miR-30e[1]	0.16	2.94E-03	1.89	4.51
hsa-miR-139-5p	0.16	3.10E-03	2.39	5.03
hsa-miR-30c	0.16	8.92E-03	3.66	6.32
hsa-miR-744	0.15	9.63E-03	2.40	5.10
hsa-miR-374b	0.12	3.05E-03	2.35	5.44
hsa-miR-376c	0.11	4.32E-03	2.91	6.04

[1]MicroRNA (miRNA) cloning studies sometimes identify two about 22 nt sequences miRNAs which originate from the same predicted precursor. When the relative abundancies clearly indicate which is the predominantly expressed miRNA, the mature sequences are assigned names of the form miRNA (the predominant product) and miRNA* (from the opposite arm of the precursor). For example, miR-123 and miR-123* would share a pre-miRNA hairpin, but more miR-123 would be found in the cell. In the past, this distinction was also made with "s" (sense) and "as" (antisense). CHB: Chronic hepatitis B; Ctrl: Healthy donor.

and -181a had the least *M* value (0.656) among these candidate miRNAs, implicating that they were the most stable ones. We thus selected miR-24 as the internal control to standardize differentially presented serum miRNAs in qRT-PCR quantification.

Microarray analysis with 10 CHB and 10 Ctrl samples showed that the median levels of serum miR-122, -572, -575 and -638 were higher while median levels of miR-30c and -744 were lower in CHB patients than those in Ctrls (Table 2). In order to validate these microarray-generated results, RNA was prepared from serum samples of another 24 CHB patients and 24 Ctrls and was subsequently subjected to qRT-PCR to measure the levels of miR-122, -572, -575, -638 and -744. Identical to what we observed with microarray analysis, the levels of miR-122, -572, -575 and -638 were 83.40-, 43.17-, 15.24- and 12.95-fold higher in the sera of CHB patients than those of Ctrls ($P = 1.61 \times 10^{-8}$, $P = 1.20 \times 10^{-6}$, $P = 8.27 \times 10^{-8}$, $P = 2.88 \times 10^{-9}$, respectively) (Figure 1A-D) while the level of miR-744 was 5.11-fold lower in CHB patients than that in Ctrls ($P = 1.04 \times 10^{-7}$) (Figure 1E). The level of miR-30c was a little higher in CHB patients than that in Ctrls although it was not statistically significant (Figure 1F).

To determine how specific these altered serum miRNAs were to CHB, we next examined the levels of these miRNA in serum samples of NASH patients. qRT-PCR showed that the levels of serum miR-122, -638, -575 and -572 were 3.04-, 16.32-, 4.27- and 5.62-fold higher ($P = 6.89 \times 10^{-4}$, $P = 7.50 \times 10^{-5}$, $P = 6.72 \times 10^{-6}$ and $P = 1.14 \times 10^{-7}$, respectively) while the level of miR-744 was 3.75-fold lower in NASH patients than in Ctrls ($P = 2.15 \times 10^{-7}$) (Figure 1A-E). When comparing the levels of these serum miRNAs between CHB and NASH samples, we found that the levels of miR-122, -572 and miR-638 were 27.42-, 2.65- and 3.57-fold higher ($P = 5.83 \times 10^{-7}$, $P = 4.18 \times 10^{-3}$, $P = 8.89 \times 10^{-4}$, respectively) while the level of miR-744 was 1.36-fold lower in CHB patients than in NASH patients ($P = 4.8 \times 10^{-2}$) (Figure 1A-C, E). In contrast, no significant difference was found in the levels of miR-575 and -30c between the serum samples of CHB and NASH patients (Figure 1D and F). These results demonstrated that a subset of miRNAs was differentially present in the sera of CHB patients.

Levels of a subset of serum miRNAs can be used to distinguish CHB patients from NASH patients or Ctrls

To determine whether the levels of serum miRNAs can be used to distinguish patients with CHB from those with NASH or Ctrls, we established ROC curves to analyze the difference in the levels of serum miR-122, -638, -575, -572 and -744 between groups. Comparing CHB subjects with Ctrls, ROC curve areas of miR-122, -638, -575, -572 and -744 were found to be 0.98 (95% CI: 0.88-1.00), 1.00 (95% CI: 0.93-1.00), 0.91 (95% CI: 0.79-0.97), 0.95 (95% CI: 0.85-0.99) and 0.95 (95% CI: 0.84-0.99), respectively. The sensitivity and the specificity of each of these miRNAs were 87.5% and 100%, 100% and 100%, 83.3% and 83.3%, 79.2% and 100%, 91.7% and 95.8%, respectively in the CHB subjects and Ctrls (Figure 2A). These results clearly showed that the levels of serum miR-122, -638, -575, -572 and -744 can distinguish patients with CHB from Ctrls.

We next compared the levels of these serum miRNAs between NASH subjects and Ctrls. ROC curve areas of miR-122, -638, -575, -572 and -744 were 0.80 (95% CI: 0.65-0.91), 0.97 (95% CI: 0.87-0.99), 0.90 (95% CI: 0.77-0.97), 0.85 (95% CI: 0.71-0.94), and 0.96 (95% CI: 0.85-0.99). The sensitivity and the specificity were 95.0% and 62.5%, 95.0% and 95.8%, 90.0% and 79.2%, 100.0% and 66.7%, 100.0% and 95.8%, respectively in NASH subjects and Ctrls (Figure 2B). These results also demonstrated that the levels of these five miRNAs can distinguish patients with NASH from Ctrls. Interestingly, comparison of CHB subjects with NASH subjects implicated that the levels of miR-122, -638, -572 and -744 were use-

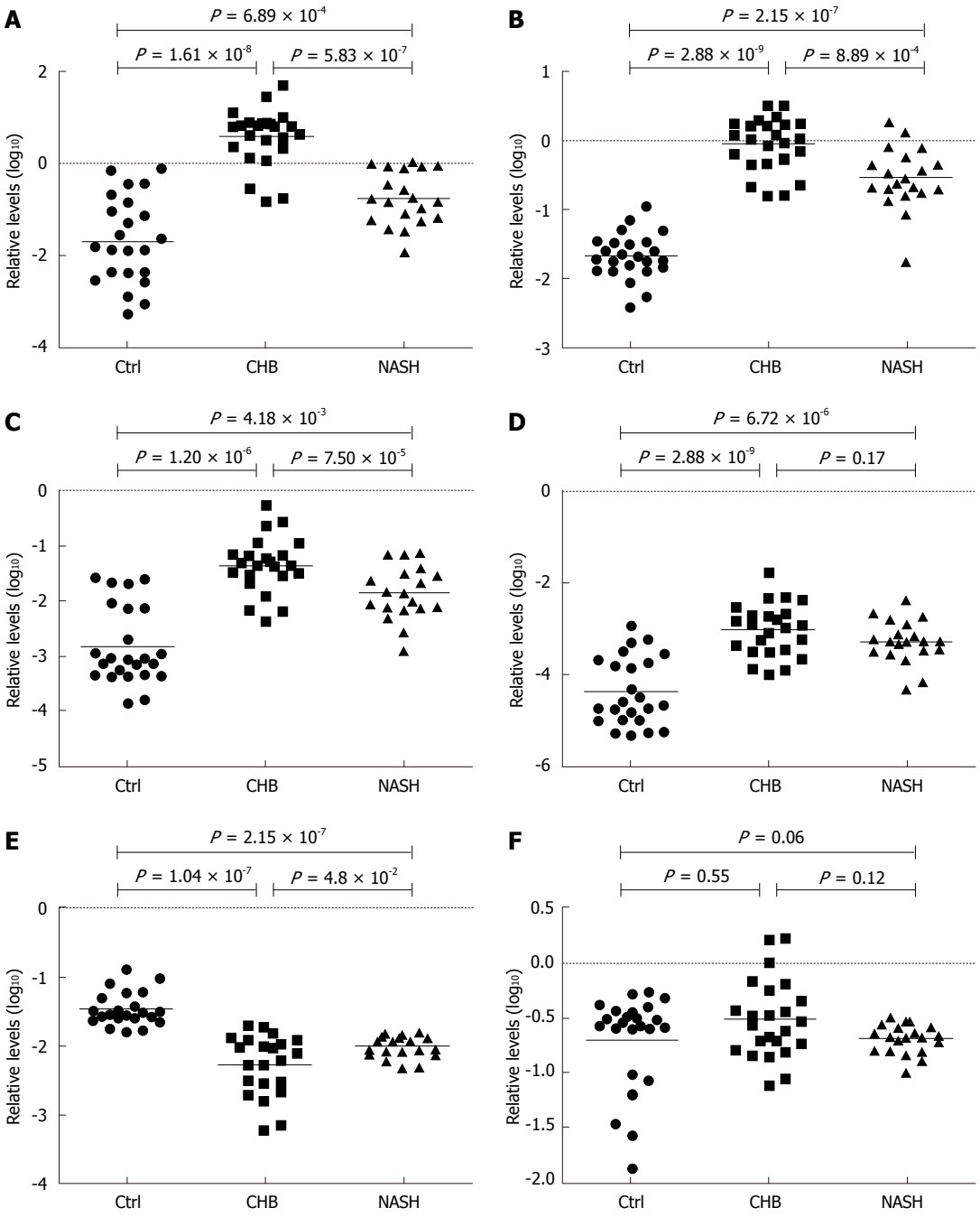

Figure 1 Serum levels of microRNAs in chronic hepatitis B, nonalcohlic steatohepatitis and healthy donors. The levels of serum miR-122 (A), miR-638 (B), miR-572 (C), miR-575 (D), miR-744 (E) and miR-30c (F) in patients with chronic hepatitis B (CHB) (n = 24), with nonalcohlic steatohepatitis (NASH) (n = 20) and healthy donors (Ctrl) (n = 24) were measured by quantitative reverse transcription polymerase chain reaction. The line at each group represents the median value of indicated miRNA. The values are normalized to miR-24 and shown in $\log_{10}$ scale at y-axis. P values on the top are NASH *vs* Ctrl, on the left are CHB *vs* Ctrl and on the right are NASH *vs* Ctrl.

ful markers for discriminating patients with CHB from those with NASH because ROC curve area of miR-122, -638, -572 and -744 were 0.94 (95% CI: 0.83-0.99), 0.79 (95% CI: 0.65-0.90), 0.75 (95% CI: 0.60-0.87) and 0.68 (95% CI: 0.52-0.81) and the sensitivity and the specificity were 87.5% and 100.0%, 83.3% and 70.0%, 75.0% and 75.0%, 50.0% and 85%, respectively, in the two groups (Figure 2C). Together, these results demonstrated that the levels of miR-122, -638, -572 and -744 in serum can be used to distinguish CHB, NASH and Ctrls.

Aberrant levels of serum miR-122, -572, -575, -638 and -744 correlate with the liver pathological parameters

To investigate whether the levels of serum miR-122, -572, -575, -638 and -744 can be used as independent molecular indicators of CHB- or NASH-induced liver injury, we first determined the potential correlation of these five miRNAs in themselves among serum samples from patients with CHB, NASH and Ctrls. Spearman correlation analysis showed that the levels of these five miRNAs in sera were highly correlated among them-

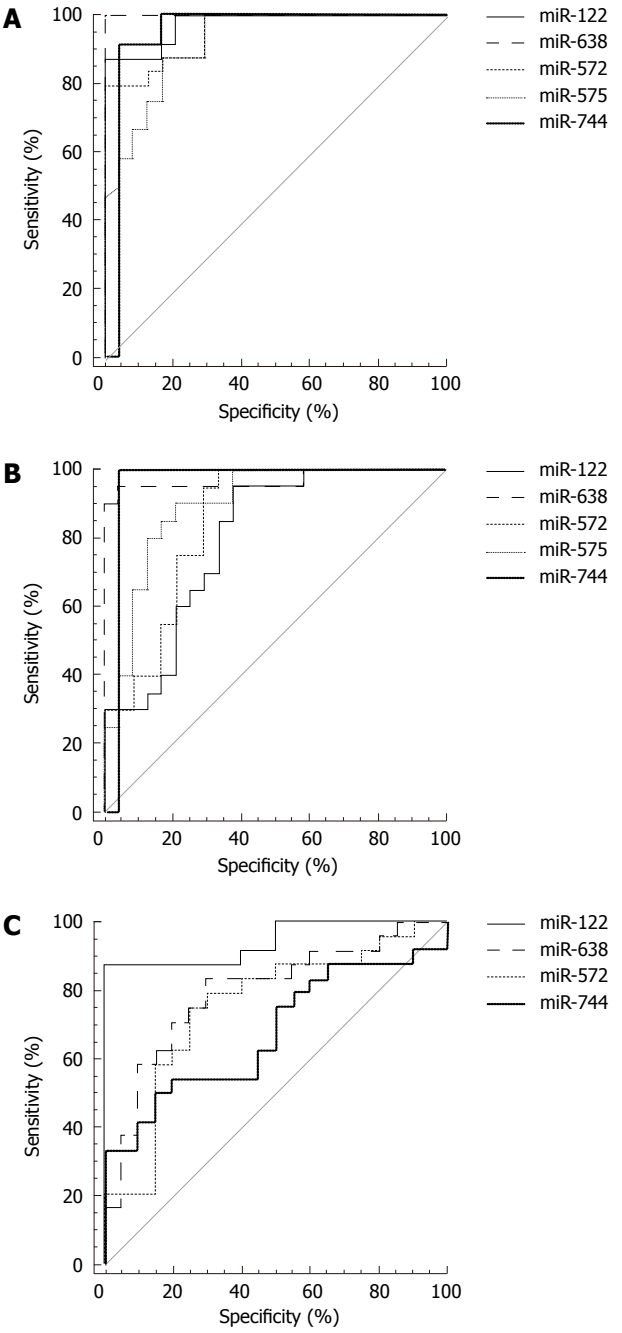

Figure 2 Receiver-operator characteristic curve analyses. Receiver-operator characteristic curves of the miR-122, -638, -572, -575 and -744 were established to discriminate chronic hepatitis B (CHB) from healthy donor (Ctrl) (A), nonalcohlic steatohepatitis (NASH) from Ctrl (B) and CHB from NASH (C).

selves ($r \geqslant 0.57$, $P = 1.00 \times 10^{-4}$; Table 3). We next analyzed the potential correlations between each of these five miRNAs and each of the clinical liver pathological parameters. Spearman correlation analysis showed that correlation only existed between selected miRNAs and selected liver function parameters (Table 3). For example, miR-122 was significantly correlated with both ALT ($r = 0.559$, $P = 1.00 \times 10^{-6}$) and AST ($r = 0.692$, $P = 1.00 \times 10^{-6}$; Table 3). However, none of these miRNAs was correlated with markers of hepatitis viruses including HBsAg, HBeAg and HBV DNA (data not shown).

Table 3 Coefficient of Spearman correlation between microRNA variables and liver function parameter variables (all 68 samples)

Variables	miR-122	miR-638	miR-572	miR-575	miR-744
miR-122	1.000	0.757[b]	0.780[b]	0.614[b]	-0.669[b]
miR-638	0.757[b]	1.000	0.876[b]	0.822[b]	-0.733[b]
miR-572	0.780[b]	0.876[b]	1.000	0.794[b]	-0.639[b]
miR-575	0.614[b]	0.822[b]	0.794[b]	1.000	-0.570[b]
miR-744	-0.669[b]	-0.733[b]	-0.639[b]	-0.570[b]	1.000
ALT	0.559[d]	0.431[d]	0.375[d]	0.299[c]	-0.413[d]
AST	0.692[d]	0.474[d]	0.465[d]	0.324[d]	-0.434[d]
GGT	0.421[d]	0.371[d]	0.280[c]	0.214	-0.355[d]
ALP	0.358[d]	0.312[d]	0.320[d]	0.306[c]	-0.180
TBIL	0.034	0.041	-0.114	-0.074	-0.068
Bile acid	0.068	-0.020	0.008	-0.023	0.090

[b]$P < 0.01$ microRNA (miRNA) *vs* miRNA without superscript b in the same column (2-tailed test); [c]$P < 0.05$ level miRNA *vs* liver function parameter (2-tailed test); [d]$P < 0.01$ level miRNA *vs* liver function parameter (2-tailed test). ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: γ-glutamyltransferase; ALP: Alkaline phosphatase; TBIL: Total bilirubin and bile.

In the subsequent studies, we considered both levels of miRNAs and liver pathological parameters as multiple factors and analyzed their correlation using multivariate analysis (canonical correlation analysis). As shown in Figure 3A, the levels of miR-122, -638, -572, -575 and -744 were apparently correlated with liver functional parameters (ALT, AST, γ-glutamyltransferase, ALP, TBIL and bile acid) ($r = 0.74$, $P < 1.00 \times 10^{-4}$). Moreover, the changes in the levels of these miRNAs were greater than those in the values of ALT or AST in the CHB and NASH subjects (Figure 3B). Most significantly, the scatter plot of principal component with the levels of these miRNAs clearly distinguished CHB, NASH and Ctrl subjects (Figure 3C). In contrast, identical analysis with the values of liver functional parameters was unable to distinguish the three groups of subjects (Figure 3D). These data indicated that the profile of miR-122, -572, -575, -638 and -744 in the serum was a better indicator than those well-established liver functional markers for liver injury caused by CHB or NASH.

DISCUSSION

miRNAs can be released into circulating system through damaged cells and tissues. Circulating miRNAs are very stable in plasma and can be found in lipid or lipoprotein complexes[29], apoptotic bodies[30], microvesicles[31] or exosomes[32]. Recent studies have shown that the levels of circulating miRNAs can alter significantly at different physiological stages and pathological conditions. For example, the level of miR-122 (liver specific), miR-133a (muscle specific), and miR-124 (brain specific) are respectively elevated in blood of patients with liver, muscle, and brain injury[9]. Moreover, the level of miR-141 is significantly higher in patients with prostate cancer than in healthy controls[14]. These observations suggest that circulating miRNAs may represent a new class of

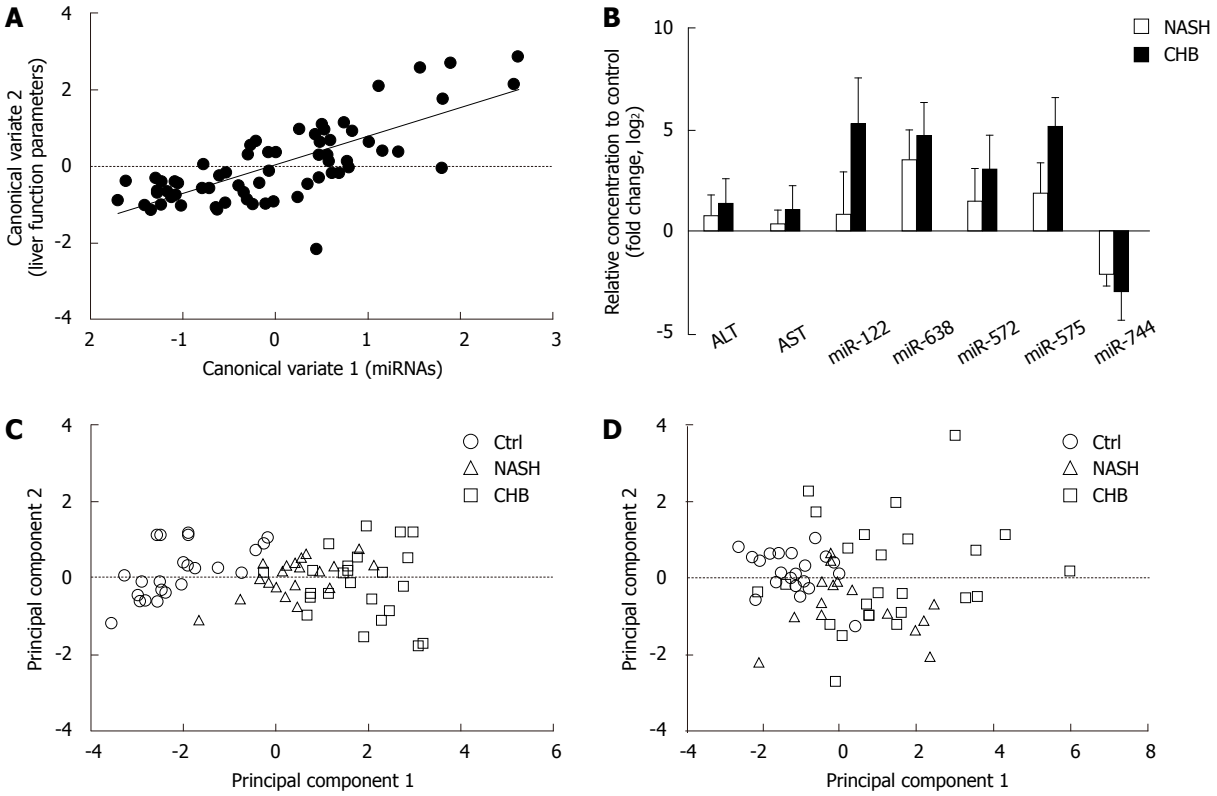

Figure 3 Aberrant levels of serum miR-122, -572, -575, -638 and -744 correlate with the liver pathological parameters. A: Canonical correlation analysis. The correlation between microRNA (miRNA) variables and liver function parameter variables were calculated by canonical correlation analysis. All the data were $\log_{10}$ transformed. Correlation coefficient $r = 0.74$ and $P < 1.00 \times 10^{-4}$. miRNAs: miR-122, -572, -575, -638 and -744; Liver function parameters: Alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyltransferase (GGT), alkaline phosphatase (ALP), total bilirubin (TBIL) and bile acid; B: Comparison of serum miRNAs, ALT and AST in chronic hepatitis B (CHB) and nonalcohlic steatohepatitis (NASH). The comparison among the levels of ALT, AST, miR-122, -572, -575, -638 and -744 in serum samples collected from patients with NASH and CHB (indicated on x-axis). The relative change of ALT, AST and miRNA expression levels were expressed ratio in $\log_2$ compared with healthy control (Ctrl) (indicated on y-axis). The values of ALT, AST and miRNA fold change are the average of samples from CHB (n = 24), NASH (n = 20) and Ctrls (n = 24), and the SD is shown as an error bar; C, D: Scatter plot of principal components analysis. All the data were $\log_{10}$ transformed to carry out analysis. Scatter plot of first two principal component of miRNAs variables including miR-122, -572, -575, -638 and -744 (C), and liver function parameters including ALT, AST, GGT, ALP, TBIL and bile acid (D) in CHB (n = 24), NASH (n = 20) and Ctrls (n = 24).

biomarkers for monitoring the progress of certain diseases[8-17]. This possibility is supported by several recent reports which demonstrate the potential of miR-9 as a novel non-invasive molecular marker for traumatic spinal cord injury[10], miR-208a for early detection of acute myocardial infarction[11], miR-146a/223 for sepsis[12], miR571 and miR-652 for liver cirrhosis-induced alcoholic hepatitis or hepatitis C[33] and a plasma miRNA panel (miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a and miR-801) for HBV-related HCC[34]. In this study, we further validated that the levels of a subset of miRNAs possess the value as novel and sensitive biomarkers for predicting liver diseases.

miR-122 was previously reported as a liver-specific miRNA[35]. In rodents, liver injury induced by alcohol or chemicals leads to the increased level of plasma miR-122 which occurs earlier than the increase in commonly-used marker ALT[9,36]. Moreover, the level of plasma miR-122 exhibits an excellent correlation with the necroinflammatory activity of HBV[23] and HCV infection[37]. In addition to miR-122, the levels of miR-575, -572, -638 and -744 in serum were also altered in patients with CHB or NASH compared with the Ctrls (Figure 1 and Table 2). Importantly, the alteration of these miRNAs correlated well with well-established liver functional parameters (Figure 3 and Table 3). Our study supports a notion that serum miRNA profile may be used to envisage the occurrence of liver injury caused by CHB and NASH.

CHB and NASH have different histological features in necro-inflammation and fibrosis[18-20]. In this study, the median levels of ALT and AST were significantly higher in CHB than in NASH (ALT: 82.6 U/L *vs* 51.7 U/L, $P < 0.05$; AST: 62.6 U/L *vs* 31.55 U/L, $P < 0.05$) (Table 1). Patients with CHB generally exhibited more severe liver damage than those with NASH, thus explaining the detection of higher levels of serum miR-122, -638 and -572, but lower level of miR-744 in CHB than those in NASH (Figure 1). Current laboratory testing with the established liver function parameters do not reliably predict the type and severity of liver injury[4,5]. It is supported by our observation that scatter plot of principal components analysis with liver function parameters was unable to distinguish CHB from NASH (Figure 3D). However, the changes in the levels of these five miRNAs were significantly greater than those in the values of ALT or AST in the CHB and NASH patients (Figure 3B). Scatter

plot of principal components analysis with the levels of serum miRNAs clearly distinguished CHB, NASH and Ctrls (Figure 3C). In hepatitis, hepatocytes die through the mechanisms of both apoptosis (programmed cell death) and necrosis. Hepatocytes presumably synthesize less AST and ALT in the process of apoptosis[38]. In contrast, either apoptosis or necrosis of hepatocytes will release cellular miRNAs directly into the circulating system. This may explain the reason why the sensitivity of serum miRNAs was superior to ALT or AST in diagnosing liver damages. Although investigations with a larger number of samples may be necessary to fully validate our findings, our results do suggest that the alteration of serum miRNA profile may be a more precise molecular biomarker for predicting the seriousness of liver injury.

This study demonstrated that miR-122, -638, -572 and -575 were presented at higher levels while miR-744 was at lower levels in the sera of patients with CHB and NASH. The levels of these miRNAs were not only correlated with liver pathological parameters, but also were more precise indicators for the type and severity of liver diseases than commonly-used markers such as ALT and AST. In conclusion, analyzing the alteration of serum miR-122, -638, -572, -575 and -744 levels may represent a powerful strategy to diagnose liver injury caused by liver inflammation.

ACKNOWLEDGMENTS

We thank Kun Wang for his assistance with statistical analysis.

COMMENTS

Background

microRNAs (miRNAs) are small, non-protein coding transcripts involved in many cellular and physiological mechanisms. Recently, a new class of miRNA called "circulating miRNAs" was found in cell-free body fluids such as serum and urine. Circulating miRNAs have been shown to be very stable, specific, and sensitive biomarkers. In this paper, the authors investigated miRNAs with altered levels in the serum of patients with chronic hepatitis B (CHB).

Research frontiers

Currently, the most commonly used markers of liver injury are the enzymatic activities of alanine aminotransferase and aspartate aminotransferase in blood; however, these markers are devoid of sufficient sensitivity and specificity to diagnose virus-induced liver damages. Therefore, assessing the severity of hepatitis B virus-induced damages and monitoring the progression of CHB are major clinical challenges. Circulating miRNA as a biomarker is a new frontier in diagnostics. The goal of this study was to investigate whether the circulating miRNAs can be used as molecular biomarkers to monitor the pathological development of CHB.

Innovations and breakthroughs

Through comparison of serum miRNA expression profiles among CHB, nonalcohlic steatohepatitis (NASH) patients and healthy donors, the authors found that the expression of miR-122, -572, -575, -638 and -744 was deregulated in both CHB and NASH patients. The authors further showed that the expression of these five miRNAs was significantly correlated with pathological parameters of liver. The authors concluded that these five miRNAs may serve as potential biomarkers for CHB and NASH-caused liver injury.

Applications

The study results suggest that serum levels of miR-122, -572, -575, -638 and -744 are deregulated in patients with CHB or NASH. The levels of these miRNAs may serve as potential biomarkers for liver injury caused by CHB and NASH.

Peer review

The authors compared and analyzed the miRNAs profile of CHB, NASH and healthy control. They found that the level of some miRNAs varied in patients with different clinical diagnosis. For instance, the levels of miR-122, -572, -575, -638 and -744 were significantly correlated with the liver function parameters and they concluded that serum levels of these miRNAs may serve as potential biomarkers for liver injury caused by CHB and NASH. The study is quite interesting and useful for clinical practices, although the results have to be validated by using a larger number of samples.

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