Plasma MicroRNA-122 as a Biomarker for Viral-, Alcohol-, and Chemical-Related Hepatic Diseases

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BACKGROUND: The liver is frequently subject to insult because of viral infection, alcohol abuse, or toxic chemical exposure. Extensive research has been conducted to identify blood markers that can better discern liver damage, but little progress has been achieved in clinical practice. Recently, circulating microRNAs (miRNAs) have been reported as potential biomarkers for the noninvasive diagnosis of cancer. In this study, we investigated whether plasma miRNAs have diagnostic utility in identifying liver disease.

METHODS: The study was divided into 2 phases: marker selection by real-time quantitative PCR analysis of a small set of plasma samples, and marker validation with a large set of plasma samples from 83 patients with chronic hepatitis B viral infections, 15 patients with skeletal muscle disease, and 40 healthy controls. Two mouse model systems, D-galactosamine- and alcohol-induced liver injury, were also developed to evaluate whether differences in miRNA concentration were associated with various liver diseases.

RESULTS: Among the miRNA candidates identified, miR-122 presented a disease severity–dependent change in plasma concentration in the patients and animal models. Compared with an increase in aminotransferase activity in the blood, the change in miR-122 concentration appeared earlier. Furthermore, this change was more specific for liver injury than for other organ damage and was more reliable, because the change was correlated with liver histologic stage.

CONCLUSIONS: Our findings suggest that circulating miR-122 has potential as a novel, predictive, and reliable blood marker for viral-, alcohol-, and chemical-induced liver injury. Liver diseases are an important health issue. Severe liver disease can lead to persistent inflammation and necrosis. The 2 primary adverse outcomes of this chronic phase are cirrhosis and hepatocellular carcinoma, both of which can cause liver-related death. The main etiology of many liver diseases is either a viral infection or alcohol abuse. The simultaneous existence of other factors, such as infection by more than 1 virus or exposure to toxic chemicals, may exacerbate the liver disease.

Currently, the enzymatic activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in blood are the most widely used biochemical markers for hepatocellular damage; however, serum ALT and AST activities are known to be increased in some other clinical disorders besides liver disease (1–3). Additionally, these 2 biomarkers do not always correlate well with histomorphologic data in clinical application (4). Studies have attempted to identify a more specific and reliable marker of liver injury to supplement the information provided by ALT or AST activity (4, 5); however, progress has been slow and has not led to the development of any markers that are better than ALT or AST.

MicroRNAs (miRNAs) are an emerging class of highly conserved, noncoding small RNAs that function by regulating the activity of mRNA targets and thereby play important roles in a wide range of physiological and pathologic processes (6, 7). Some miRNAs are produced in cell- or tissue-specific manners. Consequently, miRNA changes within a tissue type might correlate with certain disease states. The current miRNA literature has focused on tumors and miRNAs as a new kind of tissue-based marker for cancer classification and prognostication (8–10). Moreover, miRNAs are present in the peripheral blood in a remarkably stable form; thus, they might serve as potential blood-based biomarkers (11–14). Recently, Laterza et al. (15) and
Wang et al. (16) found circulating miRNAs to be useful as potential biomarkers of drug-induced liver injury in animal models; however, the diagnostic value of circulating miRNAs in patients remains undefined. We explored whether the differential production of tissue-specific miRNA in the blood is applicable for detecting various types of liver injury and the associated pathologic development of liver disease.

**Materials and Methods**

**PATIENT ENROLLMENT**

Patients with chronic hepatitis B virus (HBV) infections were recruited from Changhai Hospital (Shanghai, China) and the First Affiliated Hospital of Bengbu Medical College (Bengbu, China). Our study of these patients was divided into 2 phases: marker selection with a small set of plasma samples from 20 patients and 15 sex- and age-matched healthy blood donors, and an independent validation with a large set of plasma samples from 83 patients [59 males, 24 females; mean (SD) age, 40.19 (13.12) years] and 40 healthy controls [28 males, 12 females; mean age, 39.13 (13.37) years]. Chronic HBV infection was defined as positivity for HBV surface antigen for at least 6 months, positivity for HBV DNA by PCR analysis, and HBV infection–compatible results in a liver biopsy. Liver biopsy samples were obtained at the time of blood sampling. Patients who were coinfected with HIV or another concomitant chronic liver disease were excluded from this study. An ultrasound scan was performed at baseline to exclude hepatocellular carcinoma.

We also recruited patients who had muscle disorders as the apparent sole cause of abnormal liver enzyme results (n = 15) from Changhai and Changzheng Hospital (Shanghai, China). Six of these patients [4 males, 2 females; mean (SD) age, 22.4 (2.1) years] presented soon after extreme exercise (such as long-distance running during military training). The other 9 patients [3 males, 6 females; mean age, 36.4 (3.7) years] had a previous diagnosis of polymyositis, which was based on muscle biopsies and/or serum aldolase activities. Patients who had identifiable liver disease, used potentially hepatotoxic drugs, or experienced recent episodes of hypotension, heart failure, or renal insufficiency were excluded from this study.

The study protocol was approved by the institutional review board of each participating institution, and informed consent was obtained from each patient and healthy control individual before enrollment in the study. No patients received treatment before blood sampling and tissue biopsy.

**ANIMALS AND ANIMAL MODELS**

Five-week-old male BALB/c mice weighing 20–25 g were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. The mice were acclimated for approximately 1 week and were fasted overnight before any experiments. All experimental protocols were in compliance with the guidelines established by the institution and were approved by the Institutional Animal Care and Use Committee.

For the model of drug-induced liver injury, mice were injected intraperitoneally (0.2 mL/mouse) with 700 mg/kg body weight d-galactosamine hydrochloride (D-GalN) (Sigma-Aldrich), followed immediately by injection of 5 µg/kg body weight lipopolysaccharides (LPS) (Sigma-Aldrich), as described previously (17). Plasma and liver tissue samples were collected (details described below) at 0.5, 1, 2, 4, and 8 h after administration of either D-GalN/LPS (treatment group, n = 10 per time point) or 9 g/L NaCl (control group, n = 5 per time point).

For the model of alcohol-induced liver injury, mice were administered 8 g/kg body weight ethanol (Sigma-Aldrich) via gastric perfusion, as described previously (18). Samples of plasma and liver tissue were collected at 0.5, 1, 2, 3, and 6 h after the administration of either ethanol (treatment group, n = 10 per time point) or 9 g/L NaCl (control group, n = 5 per time point).

**SAMPLE COLLECTION, PROCESSING, AND RNA EXTRACTION**

Blood was collected from patients, healthy donors, and mice into tubes containing EDTA (BD Biosciences) and processed for plasma isolation within 2 h of collection. To obtain plasma, we centrifuged samples as described previously (19). After the first centrifugation at 1600g for 10 min, plasma supernatants were carefully transferred to a new tube and centrifuged again at 16 000g for 10 min. Total RNA (including small RNA molecules) was extracted from 500 µL plasma with TRIzol reagent (Invitrogen) and the mirVana miRNA Isolation Kit (Applied Biosystems). TRIzol reagent was added to the plasma sample at a volume ratio of 1:0.8, and the samples were mixed completely. After chloroform addition and phase separation, the aqueous layer was mixed with 1.25 volumes of absolute ethanol and loaded onto the cartridge provided in the mirVana miRNA Isolation Kit and eluted according to the manufacturer’s instructions. The final elution volume was 30 µL. The concentration and purity of the small RNAs were measured with an ultraviolet spectrophotometer (Eppendorf). The concentration of RNA extracted from plasma ranged from 6.4 to 26.7 ng/µL.
REAL-TIME QUANTITATIVE REVERSE-TRANSCRIPTION PCR

Approximately 50 ng of small RNA from each sample was reverse-transcribed with a miRNA-specific stem-loop reverse-transcriptase primer (Applied Biosystems); U6 RNA was used as a miRNA internal control. The reactions were run in a GeneAmp PCR 9700 Thermocycler (Applied Biosystems). miRNA concentrations were then confirmed by SYBR Green I–based quantitative PCR with individual miRNA-specific primers (Applied Biosystems). miRNA production was monitored with the StepOnePlus Real-Time PCR System (Applied Biosystems). We used approximately 2.5 ng cDNA for this assay. All reactions were run in triplicate, and results were normalized to those for U6 RNA. Relative miRNA production was determined with the $\Delta\Delta Ct$ method and reported as $2^{-\Delta Ct}$, where Ct is the threshold cycle. Differences in miRNA concentration in the disease group compared with the control group were expressed as -fold changes.

CLINICAL CHEMISTRY ANALYSIS

Plasma samples were assayed for ALT activity with the Olympus AU1000 (Olympus) automated biochemical analyzer according to the manufacturer’s instructions.

HISTOPATHOLOGY ASSESSMENT

Liver biopsy samples from patients and liver tissue samples from autopsied mice were excised quickly and stored in a 37 g/L formaldehyde solution. Fixed tissue sections were embedded in paraffin, sectioned into 5-$\mu$m slices, mounted on poly(t-lysine)-treated slides, stained with hematoxylin and eosin (American Histo Labs), and examined by light microscopy. The necrotic and inflammation grade of the liver biopsy samples from patients were assessed in a blinded fashion according to the classification proposed by Desmet et al. (20) and Ishak et al. (21). Necroinflammatory activity was graded from A0 to A3 (A0, no histologic activity; A1, mild activity; A2, moderate activity; A3, severe activity).

STATISTICS

Values are expressed as the mean (SD). Differences between 3 or more groups were analyzed with the Kruskal–Wallis test for multiple comparisons. Between-group comparisons were made with the Mann–Whitney U-test, the Pearson $\chi^2$ test, the Student t-test, and Spearman correlation analysis as appropriate. Multivariate logistic regression analysis and ROC curves were used to examine the accuracy of using miR-122 for diagnosing liver injury. Two-tailed $P$ values <0.05 were considered statistically significant. All statistical analyses were performed with SAS software (version 9.1.3; SAS Institute) and SPSS software (version 17.0).

Results

PLASMA miRNA SELECTION FOR PATIENTS WITH CHRONIC HBV INFECTIONS

The goal of the present study was to explore the potential use of miRNAs as biomarkers for liver disease. According to published data on profiling miRNA production, we selected 8 miRNAs with high production in liver tissue (miR-194, miR-192, miR-148, miR-122, miR-21, miR-22, miR-16, and miR-15a) (22–25). We chose 20 patients with chronic HBV infections, measured the concentrations of these 8 miRNAs in plasma by quantitative PCR, and compared the concentrations with those of 15 healthy control individuals. We observed no significant differences between the 2 groups in the concentrations of miR-194 ($P = 0.141$), miR-148 ($P = 0.168$), miR-21 ($P = 0.233$), miR-22 ($P = 0.109$), miR-16 ($P = 0.304$), and miR-15a ($P = 0.187$) (Mann–Whitney U-test). The concentrations of the remaining 2 miRNAs, miR-192 and miR-122, were increased significantly ($P = 0.013$, and $P < 0.001$, respectively) in patient plasma compared with the controls. Given that miR-122 is the miRNA most frequently isolated from adult liver and, more importantly, exhibits a tissue-specific production pattern (23, 25), we chose miR-122 as our target miRNA for the remaining experiments.

INCREASED PLASMA miR-122 IN PATIENTS WITH CHRONIC HBV INFECTIONS

In this marker-validation phase, we collected plasma from an independent group of 83 patients with chronic HBV infections and 40 healthy control individuals. There were no significant differences between the patients and healthy controls with respect to age ($P = 0.675$, t-test) or sex ($P = 0.901$, Pearson $\chi^2$ test). All quantitative PCR values were normalized to those for U6 RNA and calculated with the $2^{-\Delta Ct}$ method. The mean concentration of miR-122 in the disease group was expressed as a -fold change compared with the control group. ALT activity, a biomarker commonly used for the diagnosis and assessment of liver disease, was also measured in all of the plasma samples. To investigate changes in miR-122 concentration, we divided the patients into 4 groups (group I, 5 U/L < ALT $\leq$ 50 U/L; group II, 50 U/L < ALT $\leq$ 250 U/L; group III, 250 U/L < ALT $\leq$ 500 U/L; and group IV, ALT $>$ 500 U/L). miR-122 concentrations changed significantly across the 83 patients in the 4 groups ($P < 0.001$, Kruskal–Wallis test). In addition, the mean miR-122 concentration of each of these groups was significantly different from that of the control group (group I, $P < 0.001$; group II, $P < 0.001$; group III, $P < 0.001$; group IV, $P < 0.001$) (Fig. 1A). miR-122 con-
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Concentration and ALT activity were significantly correlated (Spearman $r = 0.896; P < 0.001$; Fig. 1B). The miR-122 concentration and ALT activity of each study participant are shown in Fig. 2. For patient samples 1–23, ALT activity was within the reference interval (5–50 U/L), whereas the mean miR-122 concentration was 3.43-fold higher in these individuals than in the control group. To determine whether the plasma miR-122 concentration was correlated with the extent of pathologic changes in the liver, we evaluated 83 patients with liver injuries (hepatocellular inflammation/necrosis of grades A1 to A3) and 40 healthy controls and compared the results with the corresponding ALT results (Fig. 3A). The results are presented for individual samples and are expressed as the -fold difference in concentration for a given marker compared with the upper limit of the reference interval (95% CI). We observed either no or a slight ALT increase in the patients when we observed mild (A1) necroinflammatory activities in their liver tissues. Increases in miR-122 concentration were observed in 82 of 83 patients with histopathologic changes, however, and the increase in miR-122 was reflected in an increase in the severity of the liver disease. To investigate the characteristics of miR-122 as a potential biomarker of liver injury, we performed ROC curve analyses on data from all 123 samples (patients and controls). The area under the ROC curve (AUC) for miR-122 was 0.989 (95% CI, 0.975–1.000) (Fig. 3B), and the AUC for ALT was 0.933 (95% CI, 0.892–0.971) (Fig. 3C). The AUCs for miR-122 and ALT were significantly different ($P = 0.007$, SAS macro %ROC). A multivariate logistic regression analysis with adjustment for sex, age, and ALT showed miR-122 to be a useful biomarker ($P < 0.001$) for detecting liver injury.

UNCHANGED miR-122 IN PATIENTS WITH MUSCLE INJURY

To identify whether plasma miR-122 concentrations change in patients without liver disease, we collected plasma samples from 15 patients with muscle injuries that caused an increase in ALT activity and from 15 healthy individuals and assayed the samples by quantitative PCR. The miR-122 and ALT data were expressed as the -fold difference in the mean concentration of the disease group compared with that of the control group. As shown in Fig. 4, the mean increases in miR-122 concentration and ALT activity in the patients with muscle injuries were 1.08-fold and 7.8-fold, respectively. The difference in miR-122 concentration between the patients with muscle injuries and the control individuals was not significantly different ($P = 0.149$, Mann–Whitney U-test), whereas ALT activity was markedly higher in the plasma of the patients with muscle injuries ($P < 0.001$).

PLASMA miR-122 IN A MODEL OF D-GalN/LPS-INDUCED LIVER INJURY

To further assess whether differential miR-122 production was associated with other kinds of liver injury, we used a murine model of D-GalN/LPS-induced liver injury. Although both miR-122 concentration and ALT activity in plasma showed a time-dependent change, the miR-122 concentration increased much more rapidly than ALT activity at every time point (Fig. 5A). A 3.0-fold increase in miR-122 was first observed as early as 0.5 h, whereas a 3.3-fold increase in ALT was seen at 1 h. ALT activity reached a relative maximum of a 92-fold increase at 8 h; however, the miR-122 concentration was increased by approximately 1000-fold at 8 h, an order of magnitude higher than observed for ALT. The liver histopathologic evaluation revealed features
of early morphologic changes, including hepatic steatosis, as early as 0.5 h. The severity of hepatocyte necrosis increased in a time-dependent manner. Spotty necrosis appeared in the livers of the mice by 2 h after administration, and bridging and massive liver necrosis appeared by 8 h (Fig. 5B). The resulting changes in liver histology appeared to correlate with the steady time-dependent increase in plasma miR-122 concentration.

### PLASMA miR-122 IN A MODEL OF ALCOHOL-INDUCED LIVER INJURY

After 0.5, 1, 2, 3, and 6 h of ethanol or saline treatment, plasma samples and liver tissue were collected for ALT, miRNA, quantitative PCR, and histopathologic analysis. In this model of alcohol-induced liver injury, miR-122 concentration and ALT activity in the plasma showed similar time-dependent increases in the mice (Fig. 6A). A 2.1-fold increase in miR-122 concentra-
tion was seen as early as 0.5 h after ethanol ingestion; a 1.9-fold increase in ALT activity was observed at 1 h. At 6 h, the increase in miR-122 plasma concentration was 6.5-fold higher than that for ALT activity in plasma. Typical photomicrographs of the liver histopathology sections are presented in Fig. 6B. Interestingly, increases in miR-122 concentration were observed in the plasma at 0.5 h, but histopathologic changes in the liver were not observed at this time point. Hepatocyte necrosis increased with the development of alcohol-induced liver injury. Prominent hepatic steatosis was present at 1 h, and massive necrosis associated with intralobular hemorrhage appeared in the livers of these mice by 6 h after ethanol gavage.

Discussion

The search for noninvasive biomarkers for the diagnosis of diseases has become a rapidly growing area of clinical research (4, 5, 26–28). The discovery of nucleic acids circulating in the peripheral blood has created a new approach for the noninvasive clinical diagnosis of disease (29–31). Circulating mRNA molecules have been investigated as potential markers of disease (32–34), and recent studies of miRNAs have offered the possibility of yet another class of molecular markers (11–14, 19). Unlike screening for large numbers of mRNAs, a modest number of miRNAs or even 1 specific miRNA might be sufficient to differentiate patients from healthy individuals.

In this study, we have characterized the role of miR-122 in the plasma as a noninvasive biomarker for diagnosing liver injury in patients. miRNA profiles for liver diseases were initially investigated with a small set of human plasma samples. In this phase of the study, miR-122 exhibited the most statistically significant difference between HBV patients and control individuals among the miRNA candidates. miR-122 not only is evolutionary conserved across species and but also is a liver-specific miRNA (23). In the next phase, a large-scale validation study, miR-122 was further assessed with an independent group of 83 patients and 40 healthy control individuals; ALT activity was measured in the same samples. Our results show a positive correlation between ALT activity and miR-122 concentration in the plasma, and the increases in miR-122 were higher than those observed for ALT. Importantly, increases in miR-122 coincided with histopathologic changes in the same samples, whereas ALT activity remained within the reference interval. ROC curve and multivariate logistic regression analyses further confirmed the superiority of miR-122 for reliably diagnosing patients with HBV-induced liver injury.

ALT activity is about 3000-fold higher in the liver than in serum. When liver cells are injured, ALT is released from damaged cells into the circulation. miR-122 concentrations in the liver are several thousand times higher than in other tissues (23, 24). Similarly, cellular damage in the liver causes the transport or release of cellular miRNAs into the plasma (16). The specific transport mechanism, however, remains unknown.

Although ALT is fairly abundant in the liver, it is also found in the kidney, the heart, and skeletal muscle cells. Increases in serum ALT activity can also arise from extrahepatic injury, particularly in response to skeletal muscle injury due to inflammation-, trauma-, or overuse-related conditions (2). This increase in ALT due to muscle injury may make both the accuracy of an ALT-based differential diagnosis and follow-up treatment more difficult. A patient with a primary diagnosis of polymyositis also has increased liver function values (ALT and AST), but before the patient can receive the recommended methotrexate treatment, the possibility of liver disease must be ruled out (3). Liver disease can be verified by liver biopsy, but this examination is invasive and may not be routinely performed. Thus, a marker with an increased specificity for the liver would be helpful for evaluating for the presence of liver disease. In the present study, we selected 15 patients with muscle disorders as the sole cause of the increased ALT activity to determine whether changes in miR-122 concentration could be seen in the plasma of these patients.
Compared with the healthy controls, plasma ALT activity was significantly increased in these patients, whereas the miR-122 concentration remained unchanged. These data indicate that miR-122 is highly restricted to the liver and may be a specific blood-based biomarker of liver disease. The sample size for the patients with muscle disorders was small, however, and further validation of miR-122 as a biomarker in a larger sample set is therefore necessary.

Two model systems of liver injury in mice, D-GalN/LPS-induced injury and alcohol-induced injury, were established to clarify whether a change in circulating miR-122 concentration could actually reflect a general liver injury. We observed a time-dependent increase in plasma miR-122 concentration in animals treated with these liver toxins and correlated them with histopathologic features. The magnitude of the change in miR-122 was greater and occurred earlier than the changes seen for ALT. Of note is that we found no obvious histopathology findings of liver damage in the alcohol-treated animals at a time when the plasma miR-122 concentration had already increased significantly. These findings suggest that miR-122 concentration may be more diagnostically sensitive for detecting certain kinds of liver injury than ALT activity, the conventional marker. Furthermore, changes in miR-122 concentration may even precede the onset of microscopically detectable changes in liver cells.

In conclusion, we report that increased concentrations of miR-122 are found in the plasma of patients and animal models with viral-, alcohol-, and chemical-induced liver diseases. miR-122 has reasonable sensitivity and specificity for the diagnosis of liver injury and is easily detectable in blood samples. Thus, miR-122 has potential for use as a liver

Fig. 5. Plasma miR-122 concentrations, ALT activities, and associated liver histopathology after D-GalN/LPS injection.

(A), Change in plasma miR-122 concentration and ALT activity 0.5–8 h after treatment with D-GalN/LPS (treated, 10 animals/group) or with 9 g/L NaCl (control, 5 animals/group). The -fold change in miR-122 concentration and ALT activity is the mean value of samples at each time point, compared with the control group. Data are presented as the mean and SD (error bars). (B), Representative photomicrographs of hematoxylin/eosin-stained liver sections from control and D-GalN/LPS-treated mice for several time points (original magnification ×200).
injury biomarker for diagnosing and monitoring diseases.

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