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Plasma MicroRNAs as Sensitive and Specific Biomarkers of Tissue Injury

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BACKGROUND: MicroRNAs (miRNAs) are endogenous, small noncoding RNAs. Because of their size, abundance, tissue specificity, and relative stability in plasma, miRNAs hold promise as unique accessible biomarkers to monitor tissue injury.

METHODS: We investigated the use of liver-, muscle- and brain-specific miRNAs as circulating biomarkers of tissue injury. We used a highly sensitive quantitative PCR assay to measure specific miRNAs (miR-122, miR-133a, and miR-124) in plasma samples from rats treated with liver or muscle toxicants and from a rat surgical model of stroke.

RESULTS: We observed increases in plasma concentrations of miR-122, miR-133a, and miR-124 corresponding to injuries in liver, muscle, and brain, respectively. miR-122 and miR-133a illustrated specificity for liver and muscle toxicity, respectively, because they were not detectable in the plasma of animals with toxicity to the other organ. This result contrasted with the results for alanine aminotransferase (ALT) and aspartate aminotransferase, which were both increased with either organ toxicity. Furthermore, miR-122 exhibited a diagnostic sensitivity superior to that of ALT when the results were correlated to the liver histopathologic results. The miR-124 concentration increased in the plasma of rats 8 h after surgery to produce brain injury and peaked at 24 h, while the miR-122 and miR-133a concentrations remained at baseline values.

CONCLUSIONS: These results demonstrate that tissue-specific miRNAs may serve as diagnostically sensitive plasma biomarkers of tissue injury.

An ideal biomarker of tissue injury should be abundant, be preferentially (or exclusively) produced in the tissue of interest, and be typically present at low concentrations in the blood and other body fluids. Upon tissue injury, such biomarkers should be released into the systemic circulation or other body fluid, where they can be detected in a blood-based assay or assay of another accessible body fluid. By keeping these general principles in mind, one can test the hypothesis that microRNAs (miRNAs)[6] can serve as accessible biomarkers of tissue injury. miRNAs are endogenous, small (approximately 22 nucleotides) noncoding RNAs that downregulate gene expression *(1)*. Some miRNAs are produced at high concentrations within cells in a tissue-specific manner *(2, 3)*, and such miRNAs have recently been reported to be remarkably stable in plasma *(4, 5)*. More importantly for biomarker purposes, a prostate tumor–derived miRNA, miR-141, has been shown to distinguish patients with prostate cancer from healthy control individuals *(4)*, and miR-122 and miR-192 have quite recently been used to detect liver injury in mice after acetaminophen treatment *(5)*. Taken together, the data on the abundance of miRNAs, their tissue specificities, the differential increases in populations with different phenotypes, and the fact that miRNAs are amplifiable in vitro with rapidly deployable PCR-based methods make miRNAs interesting candidates for biomarkers of tissue injury.

We evaluated the hypothesis that miRNAs can serve as accessible biomarkers of tissue injury. We identified both highly and differentially produced tissue-specific miRNAs from analyses of compiled human and rat databases, both from our own internal studies and from the literature *(2–4, 6)*. Candidate miRNA biomarkers revealed by these analyses included miR-122 (liver specific), miR-133a (muscle specific), and miR-124 (brain specific). We used rat models of

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[6] Nonstandard abbreviations: miRNA, microRNA; TMPD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; HMG-CoA, hydroxymethylglutaryl-CoA; $CBrCl_3$, trichlorobromomethane; CCl_4, carbon tetrachloride; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK, creatine kinase; MCAO, middle cerebral artery occlusion; VILIP-1, visinin-like protein 1.

compound-induced end-organ toxicity for both liver and muscle evaluations and explored a rat surgical model of brain injury.

Materials and Methods

ANIMAL STUDIES

Male and female Sprague Dawley rats were obtained from Charles River Laboratories. The rats were approximately 7 to 8 weeks of age and weighed 125–325 g at the start of the study. The animals were acclimated for approximately 1 week and randomized into treatment and control groups. The exploratory toxicity studies targeting liver or skeletal muscle included administration of 2 or 4 mg/kg 2,3,5,6-tetramethyl-*p*-phenylenediamine (TMPD) (a skeletal muscle toxicant) by subcutaneous injection twice daily for 2 or 3 days, 1.0 mg/kg hydroxymethylglutaryl-CoA (HMG-CoA) reductase inhibitor (skeletal muscle toxicant) per day by oral gavage for 14 days, 0.03 or 0.1 mg/kg trichlorobromomethane ($CBrCl_3$) (liver toxicant) by oral gavage for 2 or 4 days, and 0.1 or 0.3 mg/kg carbon tetrachloride (CCl_4) (liver toxicant) by oral gavage for 2 or 4 days. Studies were conducted in compliance with all applicable state and national guidelines and were approved by the Institutional Animal Care and Use Committee. Doses for each model compound tested were calculated from the animal's body weight. The animals were maintained on a calorie-restricted diet during the study and were fasted overnight before necropsy.

BLOOD SAMPLES AND CLINICAL CHEMISTRY ANALYSES

Approximately 2 mL of blood was collected from the vena cava at necropsy into a serum separator tube. We used a Roche/Hitachi Modular Analytics Clinical Chemistry P 800 Module (Roche Diagnostics) to measure serum biochemical parameters for each animal. These parameters included alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatine kinase (CK). To separate the plasma, we collected additional aliquots of blood into EDTA-containing tubes. These tubes were inverted several times and centrifuged at approximately 2000*g* for 20 min at 4 °C. Plasma samples were removed, aliquoted, and stored at −70 °C.

HISTOPATHOLOGY ASSESSMENT

At the scheduled necropsy, we performed a macroscopic evaluation and collected skeletal muscle, heart, kidney, and liver tissues for histomorphologic evaluation. Tissues were fixed in 10% neutral buffered formalin (100 mL 37% aqueous solution of formaldehyde, 4.0 g NaH_2PO_4, and 6.5 g Na_2HPO_4 per liter), embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined by microscopy. The severity of treatment-related lesions was evaluated on a grading scale of 0 to 5 [i.e., no (0), very slight (1), slight (2), moderate (3), marked (4), and severe (5) observable pathology]. In our studies conducted to support biomarker qualification, we followed guidelines set by the Society of Toxicologic Pathology on the performance of histopathologic evaluations *(7)*. These guidelines included internal peer review.

RAT MIDDLE CEREBRAL ARTERY OCCLUSION (MCAO) STROKE MODEL

We used a surgical model of stroke that involved occluding the middle cerebral artery. Ischemia was induced in Sprague Dawley rats by cannulating the femoral vein by means of the MCAO intraluminal filament method, as previously described *(8)*. Transient (60–90 min; $n = 5$ animals) and permanent occlusions ($n = 3$ animals) were performed to obtain different levels of injury. Control animals ($n = 4$ animals) underwent sham operations. Plasma samples containing EDTA were collected as described above at 0, 2, 4, 8, and 24 h after surgery. The unpaired Student *t*-test was used with GraphPad Prism software (version 4.00; GraphPad Software) to test the statistical significance of differences between experimental groups of rats subjected to transient and permanent ischemia (24-h time point).

ISOLATION OF RNA FROM PLASMA

We isolated RNA from plasma samples with the TRIzol® reagent (Invitrogen) and then precipitated the nucleic acids. We used 250 μL of pooled plasma samples and 100 μL of individual plasma samples. In brief, 1 mL TRIzol reagent was added to 100 μL plasma in a 1.5-mL microcentrifuge tube. Samples were vortex-mixed and allowed to stand for 5 min at room temperature; 200 μL of chloroform was then added to remove proteins. We centrifuged the sample, transferred the supernatant to a separate vial, and precipitated the RNA with 0.75 volumes of 2-propanol. The RNA pellet was rinsed, air dried, and then resuspended in 25 μL of Tris buffer (10 mmol/L Tris, 1 mmol/L EDTA, pH 7.4). Samples of purified RNA samples were quantified with a NanoDrop spectrophotometer ND-1000 (Thermo Scientific) at 260 nm, and either 250 ng (initial evaluation with pooled samples) or 100 ng (subsequent studies with individual animals) was used in subsequent miRNA quantitative PCRs.

QUANTITATIVE PCR OF miRNA

miRNA was quantified with samples of total RNA from 9 adult rat tissues (brain, heart, kidney, liver, lung, ovary, spleen, testes, thymus) purchased from Ambion, according to a previously described method *(9)*. Specific miRNAs were quantified by means of a hairpin-loop reverse-transcription reaction. We used TaqMan

Table 1. miR-122, miR-133a, and miR-124 concentrations in 9 rat tissues.

	Tissues evaluated, miRNA copies/cell[a]								
miRNA	Brain	Heart	Kidney	Liver	Lung	Ovary	Spleen	Testes	Thymus
miR-122	0.5	0.6	3.0	49 000	9.7	6.1	1.9	0.5	5.0
miR-124	120 000	19	17	5.0	18	53	14	450	96
miR-133a	220	170 000	55	14	1700	430	49	81	890

[a] As estimated by copies per 20 pg total RNA.

Reverse Transcription Reagents (Applied Biosystems) for cDNA synthesis. Total RNA was reverse-transcribed with hairpin primers designed to target the specific miRNA at a concentration of 5 ng/μL. TaqMan PCR was performed with 20 ng cDNA in a 20 μL volume on the ABI Prism Sequence Detection System 7900 HT (Applied Biosystems). Duplicate measurements were obtained for each sample on a 384-well plate. On the same plates, we also ran calibration curves of control miRNA diluted into total yeast RNA. Slopes and *y* intercepts obtained from the calibration curves were used to estimate the miRNA concentrations in the samples.

Results

Several miRNAs have previously been shown to exhibit tissue-specific patterns in human and rats *(2–4, 6, 10)*. miR-122, miR-124, and miR-133a are known to be highly specific for liver, brain, and muscle, respectively. To verify that these 3 miRNAs are indeed produced specifically and abundantly and therefore are good biomarker candidates, we measured the concentrations of these miRNAs in 9 rat tissue RNA samples purchased commercially (Table 1). As expected, liver tissue had high miR-122 concentrations (approximately 50 000 copies/10 pg total RNA), several thousand times higher than in the tissue with next highest concentration. The estimated concentration of miR-124 in brain was approximately 120 000 copies/10 pg total RNA, approximately 260-fold higher than in tissue from the organ with next highest concentration, the testes. Although rat skeletal muscle samples were not assayed in this early evaluation, previous work has shown that miR-133a occurs at high concentrations in both heart and muscle *(9)*; therefore, the heart data were considered comparable to muscle data. In the present study, the estimated miR-133a concentration in heart was approximately 170 000 copies/10 pg, 100-fold higher than in lung, the tissue with the next highest concentration.

In the first set of "proof-of-concept" experiments, we generated pools of plasma samples from rats that had been treated with TMPD, HMG-CoA reductase inhibitor, or $CBrCl_3$. Each plasma pool consisted of equal volumes of plasma from 5 animals. For the control pools, time-matched and vehicle-treated animals were selected, whereas the pools from the treated animals were from animals with histopathology findings of skeletal muscle degeneration/necrosis of grades 2–5 for TMPD and HMG-CoA reductase inhibitor, and findings of hepatocellular degeneration/necrosis of grades 2 and 3 for $CBrCl_3$. In these samples, quantitative PCR was used to measure the miRNAs previously identified as being liver, muscle, and brain specific (miR-122, miR-133a, and miR-124, respectively). Increases in miR-122 and miR-133a were observed in these samples (Fig. 1), whereas miR-124 values remained at baseline in all samples (data not shown). Data are expressed as the -fold increase in concentration above that of the mean of the control pool and are plotted for comparison with the data for AST and ALT, the traditional biomarkers of skeletal muscle and liver injury.

The largest increases in ALT (100-fold) occurred in animals treated with HMG-CoA reductase inhibitor, followed by those treated with $CBrCl_3$ (75-fold) and TMPD (25-fold), although hepatocellular degeneration/necrosis of grades 2 and 3 was observed only with $CBrCl_3$ treatment. Remarkably, the miR-122 concentration in animals treated with $CBrCl_3$ was approximately 6000-fold higher than the baseline values for the control group. On the other hand, the fact that the increases in miR-122 were not observed in animals treated with skeletal muscle toxicants (HMG-CoA reductase inhibitor or TMPD) demonstrated the liver-injury specificity. There was an approximate 60-fold increase in AST in TMPD-treated rats, whereas lesser increases (approximately 30-fold) were seen in rats treated with HMG-CoA reductase inhibitor or $CBrCl_3$. Increases of 550- and 800-fold in miR-133a concentration were seen in animals treated with the muscle toxicants TMPD and HMG-CoA reductase inhibitor, respectively. In contrast, only a minimal increase in miR-133a (approximately 10-fold) was seen in animals

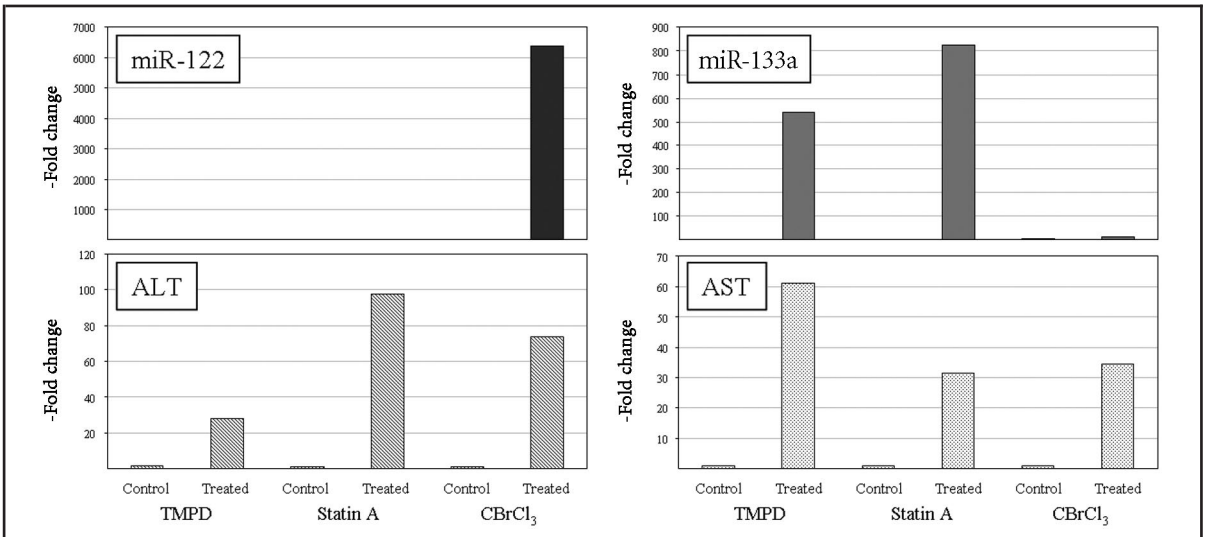

Fig. 1. Fold change in miR-122, miR-133a, ALT, and AST concentrations in pooled rat plasma.

Each plasma pool consisted of equal aliquots of plasma from each of 5 animals. Control pools contained plasma from time-matched, vehicle-treated animals, and treated pools contained plasma from animals with substantial histopathologic findings.

treated with the liver toxicant $CBrCl_3$. Taken together, the data for these pooled plasma samples demonstrate not only the utility of miR-122 and miR-133a as tissue-specific accessible biomarkers of liver and skeletal muscle toxicity, respectively, but also indicate the specificity relative to AST and ALT.

In a separate set of experiments, we assessed individual animals with less severe muscle and liver injuries (hepatocellular degeneration/necrosis of grades 1–2 and skeletal muscle degeneration/necrosis of grades 1–2) to evaluate the diagnostic sensitivities of miR-122 and miR-133a as biomarkers of tissue injury. Plasma samples from individual animals were from rat studies that used CCl_4, $CBrCl_3$, and TMPD as toxicants. Both miR-122 and miR-133a were analyzed, and the results were compared with those for AST and ALT (and CK for TMPD) (Fig. 2). Results are shown by individual animal and are presented as the -fold change in marker concentration relative to that of the untreated control group (vehicle only) for that study. In rats treated with CCl_4 or $CBrCl_3$, we observed slight increases in AST or ALT in most animals when we noted hepatocellular degeneration/necrosis in the liver; however, a few animals with liver histopathology showed no change in these conventional biomarkers. In contrast, increases in miR-122 were observed in all animals with histopathology, and the magnitude of the change was greater than that seen with ALT and AST ($\geq$100-fold). Furthermore, increases in miR-122 were seen in individual treated animals (filled circles, Fig. 2) that did not have histopathology findings for the liver but were from dosed groups in which liver toxicity was observed histologically in other animals. No increases in miR-133a were observed in animals with liver toxicity. Animals treated with the muscle toxicant TMPD showed similar increases in AST and ALT and approximately 100-fold increases in miR-133a. In this treatment, we likewise found that skeletal muscle of treated animals that we did not score as histologically positive exhibited increased miR-133a concentrations, whereas we did not detect miR-122 in the plasma. These findings indicate that miR-122 and miR-133a may be very specific and sensitive accessible biomarkers for detecting liver and muscle toxicity, respectively, compared with the conventional biomarkers AST and ALT, and increases in these miRNA biomarkers may even precede the onset of microscopically detectable changes.

In the MCAO model of stroke for brain injury, we assessed plasma samples for the presence of the brain-specific miR-124 according to the time after occlusion (Fig. 3), as well as the liver- and muscle-specific miRNAs miR-122 and miR-133a. Concentrations of miR-124 were increased in plasma beginning at 8 h, but only in the transient occlusion/ischemia samples, whereas baseline values were observed for both miR-122 and miR-133a in all samples (data not shown). Interestingly, this early increase in miR-124 was not seen in rats that had undergone permanent occlusion. Larger increases in miR-124 concentrations (up to 150-fold compared with sham-operated rats) were seen

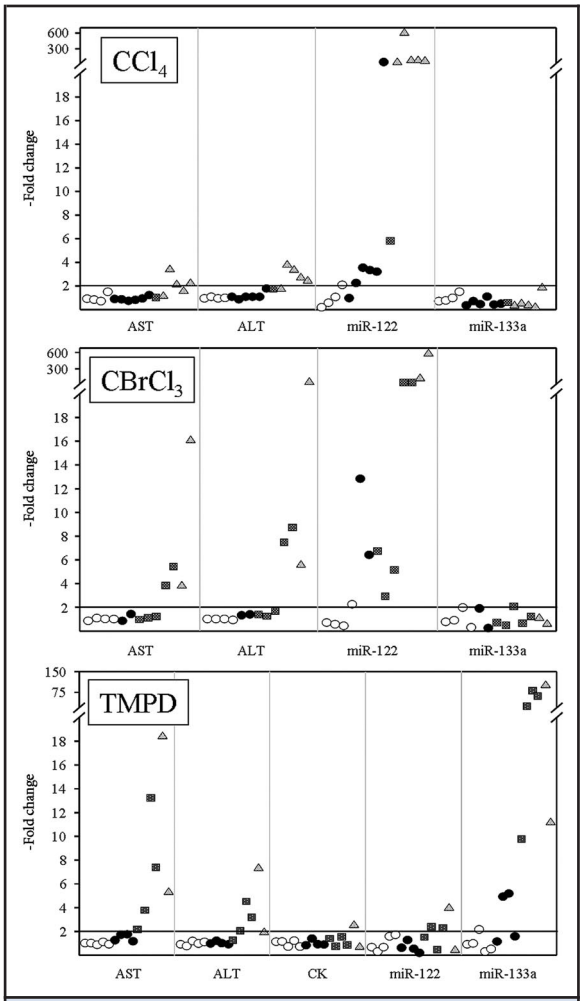

Fig. 2. Fold change in miR-122, miR-133a, AST, ALT, and CK concentrations in plasma from individual animals with liver or skeletal muscle injury.

Results are presented as the -fold change in concentration for a given marker relative to that of the untreated control group (vehicle only) from that study. Results for untreated control animals and treated animals without histopathology are represented as open and filled circles, respectively. Histopathology grading (defined as either muscle or hepatocellular degeneration/necrosis) is indicated by textured boxes for grade 1 and by triangles for grade 2. See Materials and Methods for dose groups and time points for each toxicant.

24 h after stroke for both the transient and permanent occlusions. There were no statistically significant differences between rats subjected to transient vs permanent ischemia ($P = 0.95$, 24-h time point). The increases we have observed further illustrate the potential utility of circulating tissue-specific miRNAs for monitoring tissue injury.

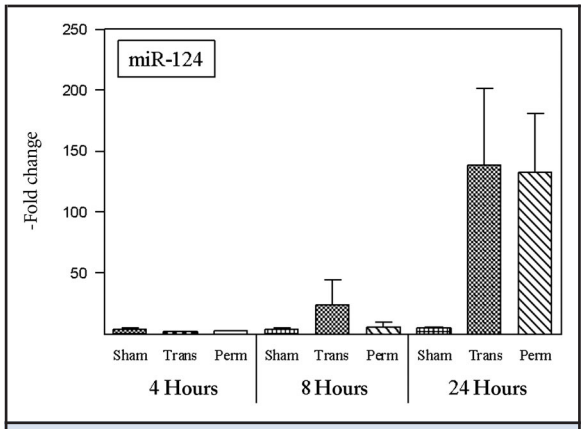

Fig. 3. Fold change in plasma miR-124 concentrations in the rat MCAO stroke model.

Results are presented as group mean (SD) for the -fold change in concentration relative to that for sham surgery at a given time point (4, 8, or 24 h). Trans, transient ischemia; Perm, permanent ischemia.

Discussion

The first biomarkers of tissue injury were enzymes that are released into the circulation after injury. The detection and/or relative quantification of such enzymes were carried out with assays designed to measure enzyme activity. The subsequent development of immunoassays that quantify proteins on the basis of mass rather than activity expanded the repertoire of proteins with potential clinical utility. Enzymatic assays are more readily adaptable across species; however, antibody-based approaches target epitopes that are rarely conserved across species, and also such assays are often challenging to develop quickly.

Biomarkers of muscle and liver injury are among those often deployed and highly relied upon in clinical practice and during drug development. Among the biomarkers of liver injury that have found utility in the clinical laboratory, 2 that are commonly and consistently measured are ALT and AST *(11, 12)*, which are increased in cases of drug-induced toxicities, such as those produced by acetaminophen and ticrynafen *(13–15)*, as well as in cases of viral hepatitis, metastatic carcinoma, and alcohol consumption *(12)*. Both enzymes, however, have multiple isoforms distributed among a wide range of tissues *(16)*. Thus, these markers lack diagnostic specificity. ALT, AST, and CK are increased in cases of muscle injury, such as statin-induced myopathy *(17)*. Given the tissue distributions of ALT and AST, increases in ALT that are greater than for AST are generally considered to indicate liver toxicity, whereas

increases in AST that are greater than for ALT generally suggest muscle toxicity *(16)*. There are occasions, however, when this general rule does not hold true, including for the HMG-CoA reductase inhibitor study we have described, which produced larger increases in ALT relative to AST without an associated liver toxicity.

We used 2 different muscle-damaging agents, TMPD and HMG-CoA reductase inhibitor, to evaluate the presence of miR-133a in the plasma after treatment. Although the molecular mechanisms are not completely understood, both compounds induce skeletal muscle toxicity, which presents as degeneration and necrosis of the quadriceps and soleus skeletal muscles. Increases in miR-133a in the plasma that correlated with the severity of the histopathology were observed in both studies (Figs. 1 and 2), and these increases were an order of magnitude higher than the increases observed for AST in the same samples. For the TMPD treatment, we observed a slight CK increase in only 1 animal that sustained a grade 2 injury, suggesting that miR-133a identified the induced toxicity more clearly than CK. It is noteworthy that increases in miR-133a were not observed with liver toxicants, demonstrating that the 2 markers have potentially superior specificities for diagnosing and differentiating muscle and liver toxicities.

We evaluated 2 model liver toxicants, CCl_4 and $CBrCl_3$, to assess miR-122 as a biomarker of liver toxicity. Both toxicants induced hepatocellular degeneration and necrosis in the liver. We observed consistent increases in plasma miR-122 concentrations in animals treated with these liver toxicants and correlated them with histopathologic findings. Increases in miR-122 were also orders of magnitude higher than those observed for ALT, and such increases were observed even in toxicant-treated animals that did not present with ALT increases or notable liver histopathology. Because higher doses and longer periods of exposure induced histopathology, we speculate that miR-122 is a more diagnostically sensitive marker for detecting liver injury at either end point. We expect that additional investigations with both lower-grade histopathologies (i.e., grade 1 and 0 from same treatment groups) and additional negative samples from nonliver toxicants will confirm this observation. In samples from TMPD-treated rats, plasma miR-122 concentrations were not increased, providing supporting evidence for the liver injury–specific nature of the miR-122 increases in plasma.

We used the rat MCAO model of stroke to study brain damage. Biomarkers of brain injury that are most commonly cited in the literature include S-100B, neuron-specific enolase, glial fibrillary acidic protein, myelin basic protein, and fatty acid–binding protein *(18–24)*. Most of these biomarkers, however, are considered to lack specificity for brain injury *(18–24)*. More recently, a new biomarker of brain injury [visinin-like protein 1 (VILIP-1)] with an apparently better tissue diagnostic specificity was identified via gene array analysis *(25)*. The brain-specific miR-124 data we have presented suggest that miR-124 can be used to monitor ischemia-related brain injury starting at 8 h and peaking at 24 h after occlusion (an approximately 150-fold increase in plasma relative to the sham surgery control group). In a similar set of experiments, VILIP-1 was not detected in the plasma of MCAO rats but was detected in the cerebrospinal fluid *(25)*. The observed increase in brain-specific miR-124 further demonstrates the utility of miRNAs as accessible biomarkers for monitoring tissue injury originating from a specific organ.

In summary, these data for liver-, muscle- and brain-specific miRNAs support the hypothesis that tissue-specific miRNAs can be exploited as circulating accessible biomarkers of tissue injury. Furthermore, the very low miRNAs concentrations detected in the control animals compared with the extremely high concentrations seen in toxicant-treated animals with histologically documented toxicity highlight the large signal-to-noise ratios seen with miRNAs, suggesting that miRNAs are attractive and accessible biomarkers for monitoring not only injury to liver, muscle, and brain but also injury to other tissues. The ease of designing and optimizing an assay with such analyte specificity and of adapting it across species makes miRNA assays a very attractive platform for the rapid deployment of improved safety biomarkers for drug development. Such a panel of diagnostic miRNAs could serve to detect induced toxicity in multiple tissues simultaneously.

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