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Profiles of miRNAs in Serum in Severe Acute Drug Induced Liver Injury and their Prognostic Significance

Mark W Russo[1], Nury Steuerwald[1], H. James Norton[1], William E Anderson[1], David Foureau[1], Naga Chalasani,[2] Robert J. Fontana[3], Paul B Watkins[4], Jose Serrano[5], Herbert L Bonkovsky[6].

[1]Carolinas Healthcare System, Charlotte, NC, [2]Indiana University School of Medicine, Indianapolis, IN, [3]University of Michigan School of Medicine, Ann Arbor, MI, [4]University of North Carolina School of Medicine, Chapel Hill, NC, [5]NIDDK, Bethesda, MD, [6]Wake Forest University School of Medicine, Winston-Salem, NC

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Abbreviations: DILI=drug induced liver injury, DILIN=Drug Induced Liver Injury Network, miRNA-micro RNA, RFU=Relative fluorescent unit, RMA=Robust multichip averaging

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Address for Correspondence:
Mark W. Russo, MD MPH
Carolinas HealthCare System, Liver Center, 6th Floor, Morehead Medical Plaza
1025 Morehead Medical Drive, Charlotte, NC, 28204.
Email: mark.russo@carolinashealthcare.org; Ph: 704 355-6649

Abstract

Background and Aims: Drug induced liver injury (DILI) is challenging because of the lack of biomarkers to predict mortality. Our aim was to describe miRNA changes in sera of subjects with acute idiosyncratic DILI and determine if levels of miRNAs were associated with 6 month mortality.

Methods: Clinical data and sera were collected from subjects enrolled in the Drug Induced Liver Injury Network prospective study. miRNAs were isolated from serum obtained from 78 subjects within 2 weeks of acute DILI and followed for 6 months or longer. miRNAs were compared to 40 normal controls and 6 month survivors vs non-survivors. **Results:** The mean age of the DILI cohort was 48 years, and 55% were female. Eleven (14.1%) subjects died, ten within 6 months of DILI onset, 5 (45%) liver related. Lower levels of miRNAs-122, -4463, and -4270 were associated with death within 6 months ($P<0.05$). None of the subjects with miRNA-122 greater than the median value died within 6 months for a sensitivity of 100% and specificity of 57%. In subjects with a serum albumin less than 2.8 g/dL and miR-122 less than 7.89 RFU the sensitivity, specificity, positive and negative predictive values for death within 6 months were 100%, 57%, 38%, and 100%, respectively.

Conclusions: Serum miRNA-122 combined with albumin accurately identified subjects who died within 6 months of drug induced liver injury. If confirmed prospectively, miRNA-122 and albumin may be useful in identifying patients at high risk for mortality or liver transplantation.

Key words: hepatotoxicity, albumin, drugs, herbals, prognosis

KEY POINTS

- There are no reliable biomarkers for mortality from idiosyncratic drug induced liver injury.
- In subjects with evidence of acute liver injury from DILI relatively lower levels of serum miRNAs -122, -4463 and -4270 were associated with mortality.
- Levels of miRNA 122 obtained within 2 weeks of DILI onset that were greater than the median of 8.31 RFU had a sensitivity of 100% for predicting survival.
- A serum albumin <2.8 g/dL and miR-122 less than 7.89 RFU had a sensitivity and specificity of 100% and 57%, respectively, for predicting death within 6 months after acute DILI.

Introduction

Drug induced liver injury (DILI) is an increasingly recognized cause of elevated liver tests (1). Elevations in serum aminotransferases and the potential for hepatotoxicity are among the most common reasons that the development of a drug is abandoned. Acute liver failure from drugs is the third most common etiology for liver transplantation (2). For most drugs, with the notable exception of acetaminophen, DILI from drugs is unpredictable and unrelated to dose or duration of therapy (3).

There are several challenges when diagnosing and managing patients with DILI. Diagnosing DILI can be difficult, and except for a positive rechallenge, there is no clear gold standard for establishing the diagnosis. Common ways to make a diagnosis DILI are by expert opinion or RUCAM, both of which have their limitations (4, 5). After the diagnosis of DILI is established the prognosis and resolution of the injury is challenging to predict with up to 18%-30% of patients developing chronic injury (6). Hy

Zimmerman was perhaps the first to emphasize the grave prognostic implication of hyperbilirubinemia in acute hepatocellular injury due to drugs; the accuracy of what has been termed "Hy's Law", namely that ~ 10% of subjects with such injury will die has been confirmed repeatedly [7-10] Thus, by the time jaundice develops the liver injury is fairly advanced, and better and earlier biomarkers that may predict serious injury earlier in its course are needed.

Changes in miRNAs in serum have been shown to be associated with viral hepatitis, hepatobiliary malignancies, liver fibrosis, and injury from acetaminophen (11-15). A property of miRNAs that make them an attractive biomarker for acute liver injury is that changes in miRNAs may occur earlier than changes seen with traditional liver tests (15). We have previously shown that relatively lower levels of four cytokines (IL-9, IL-17, PDGF-bb, and RANTES, among 27 tested) and serum albumin are highly predictive of death after an acute DILI event (16). The aim of the current study was to define miRNA profiles in sera of subjects with acute DILI and to determine if data from such profiles may also be useful for predicting prognosis in drug induced liver injury.

Methods

Subjects studied

The study population has been previously described (10, 16). Subjects were enrolled in the prospective study of the Drug Induced Liver Injury Network (DILIN) and followed for a minimum of six months after the event. Briefly, serum samples and detailed clinical data were collected from subjects enrolled in the DILIN prospective study (5, 10). Written informed consent was obtained from subjects and the study was approved by the Institutional Review Boards of all participating clinical sites. The full names of the ethics committees who approved the study are provided in the supplementary information. Normal controls were recruited from the Indiana University-Purdue University site. Clinical data were reviewed by the DILIN causality committee, which adjudicated cases as unlikely, possible, probable, very likely, or definite and, in cases involving multiple drugs, assigned each drug a score. Types of liver injury (hepatocellular, mixed, cholestatic) were classified by R values (10).

We studied 78 subjects with acute DILI enrolled between December 2004 and July 2010 who had serum samples obtained within two weeks of clinical onset. Whenever possible subjects were followed for 6 months or longer to determine if they recovered, died, were transplanted, or developed chronic injury. Blood samples were collected and sent to a central DILIN repository for processing and storage at -80 ^{O} C. Sera from 40 healthy controls (volunteer blood donors) with no known history of liver

disease, normal levels of ALT, AST, alkaline phosphatase, and total serum bilirubin and no serologic evidence of active hepatitis A, hepatitis B, hepatitis C or HIV.

miRNAs were isolated from 200 µl of serum using miRNeasy Mini Kit (Qiagen, Valencia, CA). The samples were ligated to biotinylated signal molecules using FlastTag™ Biotin HSR RNA labeling Kit (Genisphere, LLC Hatfield, PA). An enzyme linked oligoabsorbant assay (ELOSA) QC assay was performed to verify labeling prior to array hybridization. Samples were hybridized to GeneChip® 3.0 miRNA microarrays (Affymetrix, Santa Clara, CA). This chip contains 1733 probes for human mature miRNAs and 1658 probes for human pre-miRNAs. Probe level signal intensities, expressed as relative fluorescent units (RFU)/200 uL, were analyzed using Partek Genomics Suite (Partek, St Louis, MO). Robust multichip averaging (RMA) was used for background correction, quantile normalization, and probeset summarization with median polish. Mean miRNA intensities were compared using two-way ANOVA analysis with suitable adjustment for multiple comparisons [Benjamini-Hochberg correction] limiting false discovery rate of 5%.

Descriptive statistics, including means and standard deviations or counts and percentages, were calculated. For interval data, Student's t-test or analysis of variance (ANOVA) was used to compare groups. If the data were not normally distributed, the Wilcoxon rank sum test or the Kruskal-Wallis test was employed. For nominal data, the chi-square or Fisher's exact test was used. The paired t-test or Wilcoxon signed-rank test was used for comparing baseline values and values at 6 months. Spearman's correlations were calculated to assess monotonic relationships between variables measured on an interval scale. Unless otherwise specified, a two-tailed p-value of less than 0.05 was considered statistically significant. SAS® Enterprise Guide® 5.1 was used for all analyses.

The following modeling process was used to select variables among 11 miRNAs and clinical lab test results for prediction of early death (within 6 month of DILI onset). Due to small sample size (e.g. n=10 DILI subjects who died within 6 months of diagnosis) and the relatively large number of variables, the goal was to find a stable model (34) with a small number of variables that is highly predictive of early death. In the first step, univariate analyses were carried out to compare miRNA values between those died within 6 months of DILI onset and those who survived by using the Wilcoxon rank sum test. Data on the miRNAs that were significantly associated with death within 6 months were then examined for a cutoff to use in a prediction algorithm. The miRNA with the best predictive results (measured by overall concordance, sensitivity, specificity, positive predictive value, and negative predictive value) was then combined with serum albumin to determine if the predictive algorithm could be improved. In a previous

analysis of this dataset, serum albumin (≤ 2.8g/dL), a critical value in the Child-Turcotte-Pugh categorization of severity of liver disease was identified as a strong predictor of early death (16).

Results

The study population consisted of 78 subjects with acute DILI and 40 healthy control subjects (Figure 1). Selected characteristics of the study population and controls are shown in Table 1. Six month follow-up visits occurred in 37 DILI subjects among whom sera was obtained from 32 subjects. Five subjects did not provide serum at 6 months. No subject underwent liver transplantation. The mean age of the DILI cohort was 48 years-old and the majority were female (55%) and Caucasian (73%). The majority of subjects (55%) exhibited hepatocellular injury [R > 5], followed by cholestatic injury (22%) [R < 2], which was similar to the entire DILIN cohort (Table 2). Eleven (14%) subjects died, ten within 6 months of DILI onset. Five of these were deemed by the DILIN Committee on causes of death to have died due to liver disease, whereas six from a non-hepatic cause. One subject died 315 days after DILI onset of liver related cause (Table 3).

Acute drug induced liver injury and miRNA levels results

Among 1733 miRNAs and 1658 precursor miRNAs analyzed, 8 were statistically higher in acute DILI cases compared to controls (miR-122, -1246, -4270, -4433, -4463, -4484, -4532, and pre-miR-4767, $p<0.05$) and 3 were significantly decreased in acute DILI cases compared to controls (miR-455-3p, 1281, pre-miR-4274) (Figure 2). Among the DILI cases 4 of the miRNA's were also significantly higher at baseline compared to 6-month follow-up (Figure 3).

miRNA levels and death within 6 months of DILI onset

Three miRNAs were associated with death within 6 months (miR-122, -4463, pre-miR-4270, $P<0.05$) among 11 miRNAs analyzed and five were associated with death at any time (miR-122, -4463, -4270, -4433 and pre-miR-4767, $p<0.05$) *vs.* controls (Figure 4). Among the patients with DILI who survived at least 6 months miR-122 levels were higher compared to controls, 8.18 and 3.92 RFU/200uL, respectively $p=0.001$. Similarly, pre-miR-4270 levels were higher in DILI subjects who survived at least 6 months compared to controls, 3.80 and 2.14, RFU/200uL, respectively, $p=0.007$, as were miR-4463

levels, 5.20 and 2.92, respectively, p=0.003 (Figure 3). Mean levels of miRNA levels were not significantly different between serum samples collected on days 1-7 vs 8-14 after onset of acute injury.

miR-122, Albumin and Death from DILI

None of the subjects with miR-122 greater than the median value for all subjects with DILI (8.31 RFU) died within 6 months for a sensitivity of 100%, specificity of 57%, positive predictive value of 24% and negative predictive value of 100%. Serum albumin was directly associated with 6 month survival, and none of the subjects with a baseline serum albumin greater than 2.8 g/dL died within 6 months. Because lower serum albumin and miR122 were associated with survival, we determined which values provided the best sensitivity for mortality. A value of 7.89 for miR122 was chosen as the cutoff value because it had the best discriminatory value for prediction of outcome. The level of serum albumin < 2.8 g/dL, a value that is used in the Child-Turcotte-Pugh scoring system of severity of liver disease, previously had been shown to be a useful discriminatory value [16]. If, at baseline, the serum albumin was less than 2.8 g/dL and miR-122 was less than 7.89, the sensitivity, specificity, positive and negative predictive values were 100%, 81%, 38%, and 100%, respectively (Table 4). For comparison, a MELD score >17 had a sensitivity, specificity, positive predictive value, negative predictive value, and accuracy for death of 100%, 48%, 20%, 100%, and 54%, respectively. Other MELD scores had lower overall accuracies for predicting death.

Discussion

The major findings of this study are that, 1) Among 3391 miRNAs and pre-miRNAs tested, only 11 were significantly different between subjects with acute DILI and normal controls, with eight significantly higher and three significantly lower; and 2) A model that uses only the relative levels of miR-122, the miRNA most significantly elevated in acute DILI, and level of serum albumin proved to have excellent prognostic value regarding which subjects with acute DILI will live or die within 6 months of the acute event.

Identifying biomarkers that can predict serious acute liver injury from drugs may have practical implications for clinical practice as well as drug development. Currently biomarkers or prognostic models for acute liver failure such as serum alpha-fetoprotein, levels of clotting factors, King's College criteria, serum phosphorus, APACHE score are not specific for non-acetaminophen DILI and suffer from

known limitations (17-19). Thus, novel biomarkers are needed to identify individuals who are at greatest risk for mortality or for chronic hepatitis after an acute drug induced liver injury.

MicroRNA's (miRNA) are small noncoding single stranded RNA molecules that modulate gene expression. These are highly conserved molecules that contain approximately 21-25 nucleotides. Thousands of miRNAs have been identified, and the functions of most of them remain largely unknown. However investigators have reported associations between aberrant expression of miRNAs and disease states (20-22). Micro-RNAs are found not only in tissue but also in serum, plasma, and peripheral blood mononuclear cells making them an attractive target for study.

In the current study, of the 1733 miRNAS analyzed only 11 were significantly different in patients compared to controls; expression of 8 miRNAS were increased compared to controls and the expression of 3 miRNAS were lower compared to controls. We have previously shown that a panel of four immune analytes {IL-9, IL-17, PDGF-bb, and RANTES} and albumin has a high positive predictive value of death within 6 months of DILI onset (16). The current study expands upon on previous work and demonstrates that miRNA combined with albumin is predictive of death within 6 months of DILI onset.

Among the increased miRNAs, miR-122 has been the most extensively studied and more than 70% of miRNA Is from the liver (23). Its level in serum is increased in hepatitis B, hepatitis C, non-alcoholic fatty liver disease and decreased in hepatocellular carcinoma, fibrosis, cirrhosis and metabolic disorders (23). miR-122 has previously been reported to be increased in drug induced liver injury (24, 25). Our results indicate that increased expression miR-122 is also associated with non-acetaminophen acute drug induced liver injury. In a study of 53 subjects, circulating miR-122 was significantly higher in patients with acetaminophen drug induced liver compared to controls, 1,265, $p<0.001$ (20).

miR-122 has been implicated in the regulation of 7-alpha-hydroxlyase translation, an enzyme involved in cholesterol metabolism, the regulation of hepatocyte differentiation, and hepatocyte regeneration (15). It has also been shown to be important for replication of hepatitis C virus in hepatocytes, and the hepatitis C virus has recently been identified to be a 'sponge' that 'soaks up' miR-122 in infected hepatocytes, preventing the action of Xrn2, a host exonuclease that otherwise would lead to breakdown of HCV RNA within the cell (26-28). We now speculate that low miR-122 levels in subjects with acute DILI could be associated with a reduced ability to regenerate.

Although the role of miRNA-122 has been extensively studied, the functions of the other miRNAs associated with mortality from DILI are not as well studied. miR-4270 has been associated with regulating early cellular differentiation and URG4/URGCP oncogene and renal cell carcinoma (29). miRNA-4463 has been associated with insulin resistance and polycystic ovary syndrome (30,31), and mIR-4460 has been associated with prostate cancer (32). A study of miR-1246 demonstrated an association with increased hepatic differentiation in mesenchymal cells (33). Further elucidation of the function of these miRNAs is needed to determine if there is biologic plausibility for their association with mortality from DILI.

Despite analyzing thousands of (pre)miRNAs, we identified only three that were associated with death. There was a consistent association among miRNA-122, pre-miRNA-4270, miR-4463 in controls, DILI subjects who survived, and DILI subjects who died. These miRNA levels were lower among the DILI subjects who died compared to those who survived, yet these same miRNAs were significantly higher in DILI subjects who died than in controls. A possible explanation for the DILI survivors having the higher miRNA levels is that the survivors are able to develop a compensatory response to liver injury that leads to recovery.

We demonstrated that subjects with serum albumin less than 2.8 g/dL, with miRNA-122 < 7.89 accurately identified those at highest risk for death. In fact, our model performed better than MELD score in predicting outcomes. In general, tests that accurately predict death from acute liver failure are lacking and there are no biomarkers that are specific for mortality from drug induced liver injury. Perhaps not coincidentally, serum albumin of 2.8 g/dL is also the cut off for Child-Pugh-Turcotte C classification for cirrhosis. However we assumed low albumin was from hepatic synthetic dysfunction and not other causes of hypoalbuminemia such as nephrotic syndrome. In other recent work, we demonstrated that serum albumin levels less than 2.8 g/dL were useful in combination with levels of four cytokines for prediction of death from acute DILI [16]. The addition of levels of miR-122 to our recent model of cytokines did not significantly increase accuracy any further, so, at this time, we do not have any strong rationale for including levels of cytokines and miRNAs into a model for predicting survival vs death following acute severe DILI. Nevertheless, the model of miRNA-122 and albumin, perhaps, combined with values of other biomarkers, such as immune analytes [16], HMBG1, for example [34], should be prospectively validated in future studies of mortality from drug induced liver injury.

This study has several strengths including the carefully characterized 78 subjects with DILI of known acute onset, the inclusion of a healthy control group as a comparator, and development of a

simple model using albumin and miR-122. In addition, biomarkers for severe non-acetaminophen DILI are limited, which makes our study unique. We were able to combine an easily obtainable liver test, albumin with miRNA-122 to identify a promising model for acute death from DILI. Furthermore, miRNA was obtained from the blood, an easily accessible tissue compared to liver miRNA, which has been the focus of many prior studies.

Limitations of our study include the lack of a validation cohort, a relatively small number of fatal cases, and the identification of miRNAs with significant associations with death but as of yet unknown function. Whether these miRNAs are clinically relevant and have biologic plausibility are unknown and warrant further study. Before our results could be recommended for clinical practice, they need to be prospectively validated in other cohorts and should include a cohort that includes acute liver injury from non-DILI causes as a comparison group. Such further studies are in progress in DILIN. miRNA-122 in combination with albumin appears to be the most promising model for further study.

In conclusion, the serum level of miRNA-122, especially when combined with serum albumin, is a promising early biomarker for identifying individuals at highest risk for mortality within 6 months of developing severe drug induced liver injury. Perhaps, miR-122, combined with other biomarkers, such as selected serum cytokines [16], will increase negative and positive predictive values and accuracy of prediction [34,35]. In addition, study of these markers in acute hepatitis of other etiologies, such as autoimmune, ischemic, or viral hepatitis, is important. Currently we are carrying out such studies to validate and extend these initial findings. The focus should be on further validation of this simple model in prospective studies because of the practical implications it may have in clinical practice.

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Table 1: Selected demographic, clinical, and laboratory features of subjects and controls

	DILI onset (n=78)	6 month follow-up (n=32)	Healthy controls (n=40)
Age, mean (SD) years	48 ± 17.9	51±14.2	49.2±13.1
Female (%)	55	55	28
Self reported race (%)			
White	73	73	95
Black	10	10	5

Other/unknown	17	15	0
Body mass index (kg/m2),mean ± SD	27.1 ± 6.5		30.5±6.9
Absolute eosinophils/uL (mean ± SD)	173 ± 235		
Liver tests (mean, ± SD)			
ALT (U/L)	1065 ± 1382	55±131	17.5±5
AST (U/L)	1003 ± 1249	38±131	24±5
Alkaline phosphatase (U/L)	336 ± 465	89±33	63±14
Total bilirubin (mg/dL)	8.1 ± 7.4	0.9±0.7	0.6±0.2
INR	1.8 ± 1.2	1.1±0.4	
MELD≤9*	10		
10-19	34		
20-29	17		
30-39	5		
≥40	1		

*MELD score not available for 11 subjects

Table 2:Selected characteristics and outcomes among DILI subjects studied (n=78)

Pattern of injury (%)	
Hepatocellular	46 (59)
Cholestatic	17 (22)
Mixed	12 (15)
Unknown	3 (4)
Severity of injury (%)	
Mild	9 (11)
Moderate	38 (49)
Severe/fatal	20 (26)
Unknown	11 (14)

Liver related mortality (%)	5 (45)
Chronic DILI (%)	4 (5)

Table 3: Characteristics of subjects who died

Subject	Implicated drug(s)	Days to death	Cause of death
1	oxacillin	102	Drug overdose
2	ciprofloxacin	27	Liver related
3	XL-999 (experimental tyrosine kinase inhibitor for acute myeloid leukemia)	4	AML, sepsis
4	amlodipine	42	Subdural hemorrhage, sepsis
5	Vincristine, L-asparaginase	136	Acute lymphoblastic leukemia
6	nitrofurantoin	6	Liver related
7	ciprofloxacin, niacin	6	Liver related
8	antithymocyte globulin	6	Aplastic anemia
9	carbamezepine	21	Thymoma
10	amiodarone, nafcillin	40	Liver related
11	phenytoin	315	Liver related

Table 4: Outcome of subjects with serum albumin*2.8 g/dL and miRNA122<7.89

	Death within 6 months	Alive
# predicted to die within 6 months	8	13

# Predicted to live >6 months	0	54

*1 patient did not have a serum sample for albumin and was excluded from the analysis

Figure Titles and Legends

Figure 1. Flow diagram of the study subjects

Sera from 78 subjects with acute DILI and 40 healthy controls were analyzed as described in Materials and Methods.

Figure 2. miRNA Levels in Serum for Subjects with Acute DILI, expressed as Fold-Difference from Those for Control Subjects. Y axis is fold change

Only the 11 miRNAs that were significantly different after adjustment for multiple comparisons with false discovery rate limited to 5% are shown. Values for 8 were significantly increased; values for three were significantly decreased.

Figure 3. miRNA Concentrations differentially distributed among DILI onset and 6 month follow-up. Y axis is fold change

Results are shown as box and whisker plots, with median values shown as central horizontal bars, interquartile ranges [25-75%] as boxes, and arror bars denoting 5-95%ile values. * denote $p<0.05$ by Wilcoxon rank sum test. Controls n=40, Onset N=78, 6 month N=32

Figure 4. Values for miR-122 among those who survived (n=68) vs died (n=10) within 6 months of DILI onset. Results are expressed as in Fig. 3. Y axis is fold change.

Figure 1 Flow diagram of the study subjects

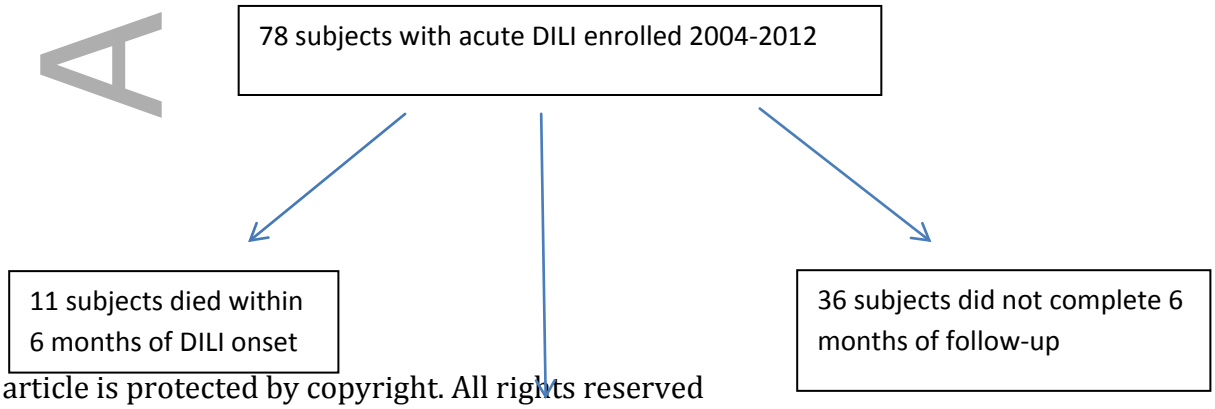
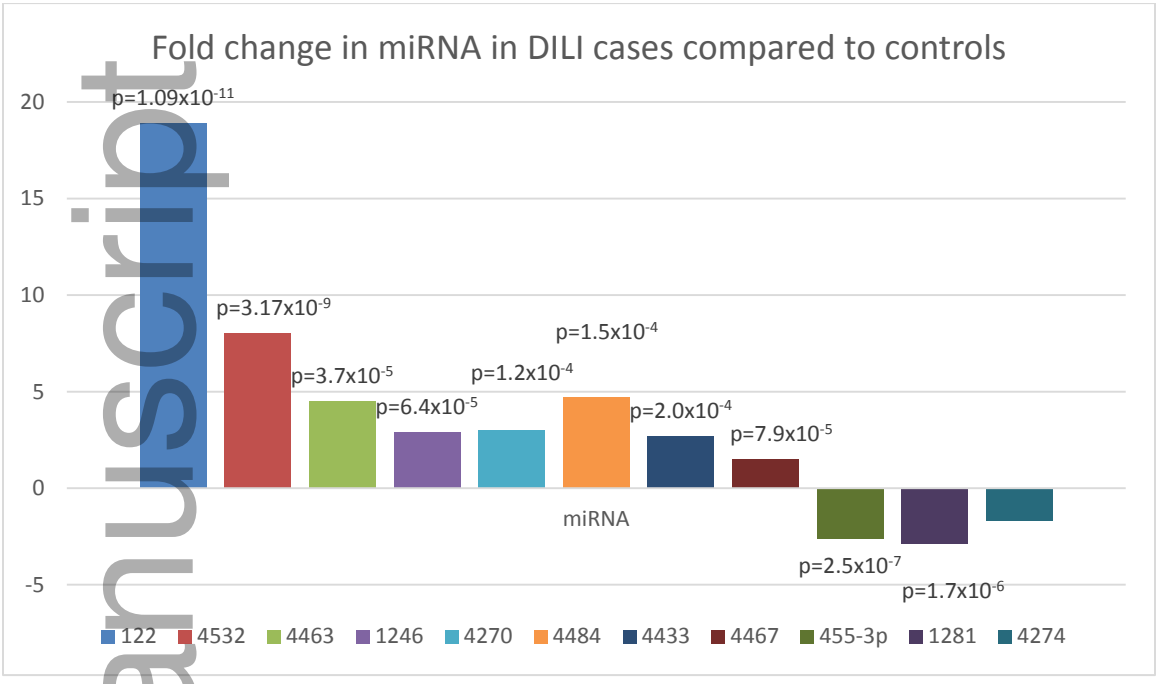


Figure 2: miRNA Levels in Serum for Subjects with Acute DILI, expressed as Fold-Difference from Those for Control Subjects.



Only the 11 miRNAs that were significantly different after adjustment for multiple comparisons with false discovery rate limited to 5% are shown. Values for 8 were significantly increased; values for three were significantly decreased.

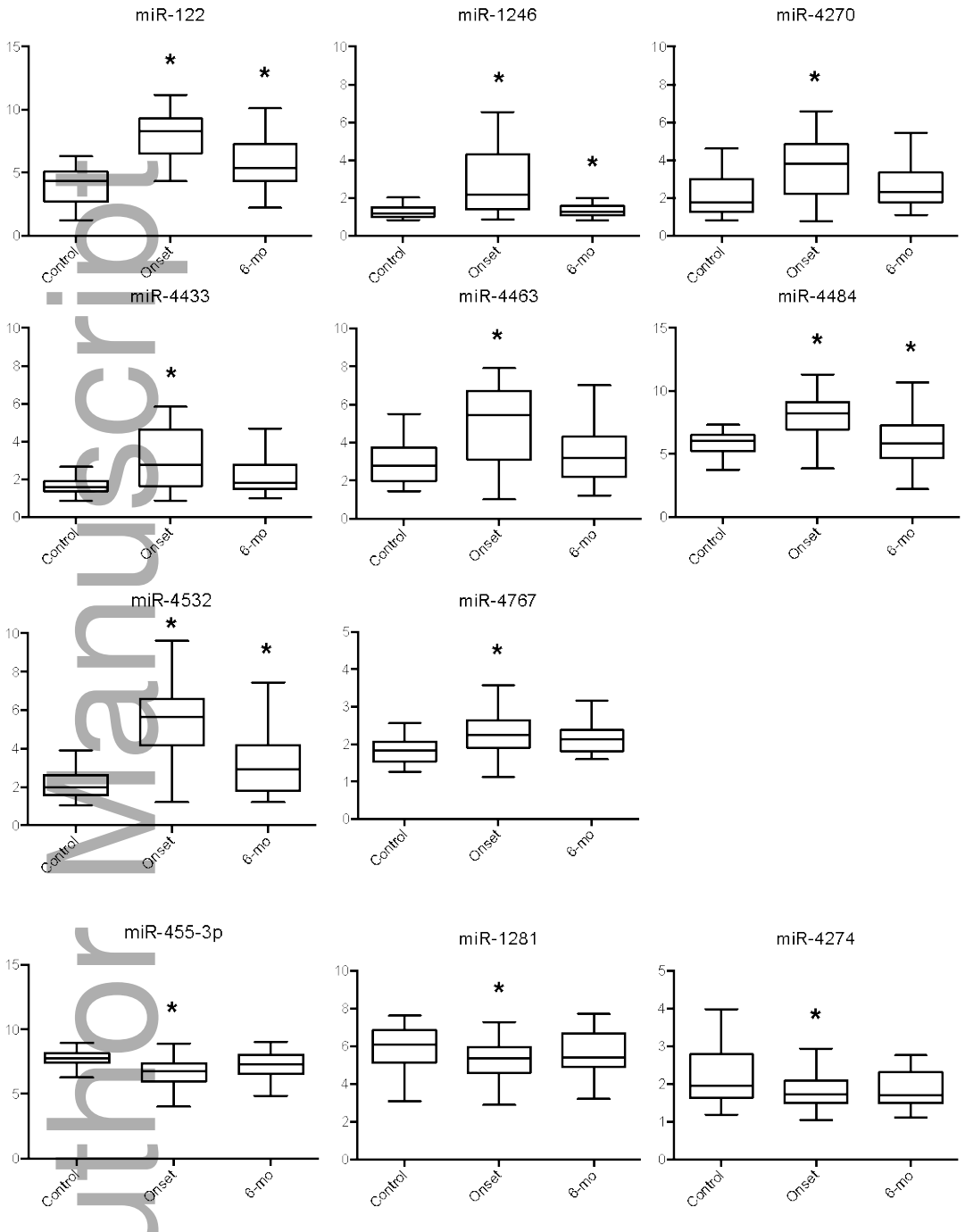


Figure 3: miRNA Concentrations differentially distributed among DILI onset and 6 month follow-up. Y-axis is fold change.

Figure 4. Values for miR-122 among those who survived (n=68) vs died (n=10) within 6 months of DILI onset. Results are expressed as in Fig. 3. Y axis is fold change.