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Potential of extracellular microRNAs as biomarkers of acetaminophen toxicity in children

Xi Yang [a,*], William F. Salminen [a,1], Qiang Shi [a], James Greenhaw [a], Pritmohinder S. Gill [b,c], Sudeepa Bhattacharyya [b,c], Richard D. Beger [a], Donna L. Mendrick [a], William B. Mattes [a], Laura P. James [b,c,**]

[a] Division of Systems Biology, National Center for Toxicological Research, Food and Drug Administration, 3900 NCTR Road, Jefferson, AR, USA
[b] Department of Pediatrics, University of Arkansas for Medical Sciences, Little Rock, AR, USA
[c] Clinical Pharmacology and Toxicology Section, Arkansas Children's Hospital, Little Rock, AR, USA

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ABSTRACT

Developing biomarkers for detecting acetaminophen (APAP) toxicity has been widely investigated. Recent studies of adults with APAP-induced liver injury have reported human serum microRNA-122 (miR-122) as a novel biomarker of APAP-induced liver injury. The goal of this study was to examine extracellular microRNAs (miRNAs) as potential biomarkers for APAP liver injury in children. Global levels of serum and urine miRNAs were examined in three pediatric subgroups: 1) healthy children (n = 10), 2) hospitalized children receiving therapeutic doses of APAP (n = 10) and 3) children hospitalized for APAP overdose (n = 8). Out of 147 miRNAs detected in the APAP overdose group, eight showed significantly increased median levels in serum (miR-122, -375, -423-5p, -30d-5p, -125b-5p, -4732-5p, -204-5p, and -574-3p), compared to the other groups. Analysis of urine samples from the same patients had significantly increased median levels of four miRNAs (miR-375, -940, -9-3p and -302a) compared to the other groups. Importantly, correlation of peak serum APAP protein adduct levels (an indicator of the oxidation of APAP to the reactive metabolite N-acetyl-para-quinone imine) with peak miRNA levels showed that the highest correlation was observed for serum miR-122 ($R = 0.94$; $p < 0.01$) followed by miR-375 ($R = 0.70$; $p = 0.05$). Conclusion: Our findings demonstrate that miRNAs are increased in children with APAP toxicity and correlate with APAP protein adducts, suggesting a potential role as biomarkers of APAP toxicity.

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Introduction

Acetaminophen (APAP) is one of the most commonly used drugs for pain and fever in adults and children (James et al., 2008; Algren, 2008). The drug is generally considered to be safe when administered at doses recommended by the manufacturer. However, APAP overdose is a very common cause of acute liver failure (ALF) in adults, and accounts for ~14% of ALF in children (Squires et al., 2006). Since the etiology of ALF for up to 40% of pediatric cases is unknown, it is possible that undiagnosed APAP overdose is responsible for some of these indeterminate ALF cases (James et al., 2008). While liver injury itself is detected by increases in serum transaminase and bilirubin, the current approach for diagnosing APAP poisoning relies on a history of APAP exposure and quantitation of APAP in peripheral blood within the first 24 h after the overdose (i.e., the Rumack nomogram). Limitations of this approach have been well-described and more sensitive, mechanism-based biomarkers are needed (McGill et al., 2012, 2014a, 2014b). Early biomarkers, which correctly identify individuals with toxicity, could be important to detect patients at risk for developing ALF.

MicroRNAs (miRNAs) show promise as possible new biomarkers of disease and injury (Chen et al., 2008). miRNAs are typically 21–23 nucleotides long and regulate gene expression by binding to the 3′ untranslated regions (3′UTR) of their target mRNAs (Ambros, 2004; Krol et al., 2010). Since miRNAs can be detected in body fluids, they are appealing as noninvasive biomarker candidates (Etheridge et al., 2011). Our laboratory previously reported that urinary miRNA profiles were altered in rats after administration of hepatotoxic doses of acetaminophen or carbon tetrachloride (Yang et al., 2012a). Studies in the mouse model of APAP toxicity found that miR-122 and miR-192,

Abbreviations: miRNA, microRNA; DILI, drug induced liver injury; APAP, acetaminophen; ALT, alanine aminotransferase; ALF, acute liver failure.

* Correspondence to: X. Yang, Division of Systems Biology, National Center for Toxicological Research, Food and Drug Administration, 3900 NCTR Road, Jefferson, AR 72079, USA. Fax: +1 870 543 7736.
** Correspondence to: L.P. James, Arkansas Children's Hospital, 1 Children's Way, Little Rock, AR 72202, USA. Fax: +1 501 364 3551.
E-mail addresses: Xi.Yang@fda.hhs.gov (X. Yang), Willie.Salminen@parexel.com (W.F. Salminen), Qiang.Shi@fda.hhs.gov (Q. Shi), James.Greenhaw@fda.hhs.gov (J. Greenhaw), PSGill@uams.edu (P.S. Gill), SBhattacharyya2@uams.edu (S. Bhattacharyya), Richard.Beger@fda.hhs.gov (R.D. Beger), Donna.Mendrick@fda.hhs.gov (D.L. Mendrick), William.Mattes@fda.hhs.gov (W.B. Mattes), jameslaurap@uams.edu (L.P. James).
[1] Current address: PAREXEL International, 7321 Hemlock Lane, Sarasota, FL, USA.

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both found at high levels in liver tissue, are potential liver injury biomarkers (Wang et al., 2009). Compared to alanine aminotransferase (ALT), miR-122 is liver specific and represents over 70% of the total liver miRNAs (Chang et al., 2004). Recent clinical studies support the increase of serum miR-122 under conditions of hepatotoxicity in patients with APAP overdose (Antoine et al., 2013; Starkey Lewis et al., 2011, 2012; Thulin et al., 2013; Krauskopf et al., 2014). Elevations of miR-122 have also been reported in heparin-induced liver necrosis (Harrill et al., 2012), liver steatosis (Cermelli et al., 2011) and hepatitis B and C infections (Su et al., 2013; Arataki et al., 2013; Laterza et al., 2013). It is important to note that these studies did not address the usefulness of measuring serum miR-122 levels in children exposed to high doses of APAP.

In this investigation, global miRNA levels were examined using small RNA sequencing of serum samples and human miRNA PCR array analysis on urine samples from three pediatric subgroups. The subgroups were 1) children with no recent APAP exposure, 2) hospitalized children receiving APAP for treatment of pain and fever, and 3) children with APAP overdose. We evaluated the hypothesis that miRNA expression profiles may have diagnostic potential for APAP toxicity in children.

Method

Study population and design. The study was approved by the institutional review board of the University of Arkansas for Medical Sciences. Following informed consent and assent when age appropriate, blood and urine samples were collected from study subjects (n = 28). There were three subject groups: 1) control group, defined as healthy children with no use of APAP in the preceding 14 days (N = 10); 2) APAP therapeutic group, defined as hospitalized children receiving APAP per standard of care (N = 10); and 3) APAP overdose group, defined as children requiring hospitalization for treatment of APAP overdose (N = 8). Clinical information on study subjects included gender, weight, and dose or doses of APAP received (Group 2) or ingested (Group 3), reported as mg/kg. A single sample was collected in Groups 1 and 2, while multiple samples were collected from some patients in Group 3. Blood samples were centrifuged immediately after collection, and serum and urine samples were stored at −80 °C until further analysis.

Serum ALT and APAP protein adducts quantification. Serum ALT levels were quantified in the clinical laboratory of Arkansas Children's Hospital. Serum APAP protein adduct levels were quantified through a high-performance liquid chromatography with electrochemical detection assay as previously described (Muldrew et al., 2002; James et al., 2003, 2009).

Serum and urine miRNA profiling. Total RNA was isolated from serum (50 μl) using the method described previously (Yang et al., 2012a). The serum miRNA (minimum of 300 ng total RNA) profiling was conducted by Illumina high-throughput small RNA sequencing (HiSeq 2000, Illumina), according to the manufacturer's protocol. Four samples were not included in the analysis due to low RNA volume; therefore, a total of 36 serum RNA samples were sequenced, including: 1) control group, 9 samples from 9 subjects; 2) APAP therapeutic group, 8 samples from 8 subjects; and 3) APAP overdose group, 19 samples from 8 subjects. The Upper Quartile normalization method was used for data analysis (Bullard et al., 2010; Dillies et al., 2013), where the counts were divided by the upper quartile of counts associated with their lane and multiplied by the mean upper quartile of counts across all the samples of the dataset. Absolute fold changes of >2-fold at $p < 0.05$ were considered significant.

Total RNA was isolated from urine (300 μl) by the TRIzol method (Yang et al., 2012a), and 300 ng of RNA was used to generate cDNA. Whole genome profiling of urinary RNA samples was performed using human miRNome miRNA PCR arrays (MAH-3200E, Qiagen, Frederick, MD) covering 752 human miRNAs. These PCR arrays were run per the manufacturer's protocol (Qiagen) on a 7900 real-time PCR system (Applied Biosystems Inc., Foster, CA). Relative miRNA expression levels were determined with the ΔCt method per the manufacturer's recommendations. Three miRNAs (miR-7, miR-671-3p, and miR-943), showing low standard deviation, were selected to normalize the 752 miRNAs from PCR array. Fold changes of >2-fold at $p < 0.05$ were used to select the significantly altered miRNAs. Hierarchical unsupervised clustering analysis (Heatmap) was performed as described previously (Fang et al., 2009); normalized counts (serum) or ΔCt values (urine) were used for these analyses.

Quantitative PCR validation. To validate the miRNA profiling results, TaqMan miRNA qRT-PCR assay (Applied Biosystems) was performed on selected miRNAs (miR-122 and miR-375) for the serum and urine samples and miR-940 levels were confirmed by SYBR Green Qiagen kit as the TaqMan assay was not available. This analysis was limited to miRNAs with a significant correlation ($p < 0.05$) with APAP protein adducts.

Non-human miRNA, ath-miR159a (*Arabidopsis thaliana*), was spiked into RNA samples as a control for extraction and amplification steps. Let-7d was used for normalization of serum samples based on the previous publication (Antoine et al., 2013) and miR-671-3p was used for normalization of urine samples which was the most consistent urinary miRNA.

Statistical analysis. A non-parametric method (Kruskal–Wallis one-way analysis of variance by ranks) was used to determine whether there was a significant difference among the three subgroups. Dunn's method was used for all pairwise multiple comparisons. For correlation analysis between peak miRNA and peak APAP protein adduct, Pearson's correlation test was performed in SigmaPlot (version 11.0, Systat Software Inc.), with $p < 0.05$ considered as statistically significant.

Results

Elevations of ALT and APAP protein adducts

Serum ALT and APAP protein adduct values are summarized in Table 1. Children in Groups 2 and 3 were older than those in Group 1. The median unit dose of APAP in the therapeutic group was 12.6 mg/kg (range: 10–17.5 mg/kg) and the median daily dose was 17 mg/kg (range: 10.2–28.5 mg/kg). The median reported total APAP exposure in overdose patients was 198.4 mg/kg (range: 58.6–559.4 mg/kg). Median values of ALT and APAP adducts were significantly ($p < 0.05$) higher in Group 3 than in the other groups.

Global serum and urine miRNA analysis

Small RNA sequencing (HiSeq 2000, Illumina) detected and quantified a total of 147 miRNAs in all serum samples (n = 36). Comparison of the subgroups revealed that eight serum miRNAs (miR-122, -375, -423-5p, -30d-5p, -125b-5p, -4732-5p, -204-5p, and -574-3p) were increased more than 2-fold in samples from the APAP overdose group (Group 3) compared to the other subgroups (Table 2). Urinary miRNA profiling using the whole genome PCR array found that miR-375, miR-940, miR-9-3p and miR-302a were increased in Group 3 (Table 3) compared to the other two groups. miR-375 was increased in both urine and serum samples in Group 3 patients (Tables 2 and 3). While miR-122 was detected in urine, it was not statistically different among the groups (data not shown). To explore relationships between groups, hierarchical cluster analysis (HCA) was performed on the miRNA levels in individual samples. Since repeated measures were available for the Group 3 subjects (at multiple time points during the hospitalization), only the peak values were selected for HCA analysis. As shown in Fig. 1A, levels of serum miRNAs could separate Group 3 (elevated ALT) from Groups 1 and 2 (normal to low ALT). This panel included eight up-regulated miRNAs (Table 2) and three down-regulated miRNAs (miR-