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Circulating MicroRNA is a Biomarker of Biliary Atresia

Adam M. Zahm, Nicholas J. Hand, LaTasha A. Boateng, and Joshua R. Friedman

Division of Gastroenterology and Nutrition, Department of Pediatrics, University of Pennsylvania School of Medicine, Children's Hospital of Philadelphia Research Institute, Philadelphia, PA 19104

Abstract

The lack of reliable noninvasive diagnostic biomarkers of biliary atresia (BA) results in delayed diagnosis and worsened patient outcome. Circulating microRNAs (miRNAs) are a new class of noninvasive biomarkers with encouraging diagnostic utility. In this study we examined the ability of serum miRNAs to distinguish BA from other forms of neonatal hyperbilirubinemia. BA-specific serum miRNAs were identified using a microfluidic array platform and validated in a larger, independent sample set. The miR-200b/429 cluster was significantly increased in the sera of BA patients and has promising diagnostic clinical performance.

INTRODUCTION

Biliary atresia (BA) is an idiopathic neonatal liver disease characterized by inflammatory and fibrotic obliteration of the extrahepatic bile ducts, leading to severe cholestasis and biliary cirrhosis. The only therapies are Kasai portoenterostomy (KPE) and liver transplantation. The success rate of KPE decreases significantly with increased age at the time of surgery (1, 2). A definitive diagnosis of BA is often delayed, and currently requires invasive procedures including liver biopsy and intra-operative cholangiography. New, noninvasive diagnostic indicators may speed diagnosis, thereby improving patient outcome following KPE.

MicroRNAs (miRNAs) are short, noncoding RNAs that regulate target genes via transcript degradation or translational repression. Cell- and tissue-specific miRNA expression are essential for vertebrate organ development (3–5) and miRNAs are dysregulated in numerous pathologies, including experimental BA (6). Recent studies have identified stable populations of miRNAs present in cell-free plasma and serum preparations (7–10). These circulating miRNAs can reflect altered expression or release from injured and diseased tissues and can distinguish similar disorders (11–13).

We hypothesize that tissue injury associated with BA results in a unique circulating miRNA profile that can be used as a diagnostic biomarker. Here we describe a cluster of miRNAs

Address correspondence and reprint requests to Joshua R. Friedman, ARC 902G, 3615 Civic Center Blvd, Philadelphia, PA 19104 (friedmaj@mail.med.upenn.edu).

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elevated in BA sera compared to cholestatic control samples that possess promising diagnostic characteristics.

MATERIALS AND METHODS

Sample collection and RNA isolation

Human serum samples were obtained from the Childhood Liver Disease Research and Education Network's (ChiLDREN) prospective longitudinal study of cholestasis in infancy (Prospective Database of Infants With Cholestasis (PROBE), clinicaltrials.gov NCT00061828). All BA diagnoses were confirmed by cholangiography, operative exploration, and/or histology. All mice were housed, handled, and euthanized in accordance with federal and institutional guidelines under the supervision of the Children's Hospital of Philadelphia Institutional Animal Care and Use Committee. Following euthanasia, liver and extrahepatic bile duct (EHBD) tissue were excised, flash frozen and pulverized. Total RNA was isolated from 60 μ L of human serum and from mouse tissue samples using the mirVana miRNA Isolation Kit (Ambion, Austin, TX). The *C.elegans* miRNAs cel-miR-54 and cel-miR-238 were added to serum samples as exogenous normalizing controls immediately following serum denaturation. RNA was eluted in 100 μ L elution solution (95°C) and stored at -80°C.

miRNA analysis by low density array (LDA)

TaqMan® Array Human MicroRNA A Cards (Applied Biosystems Inc., Foster City, CA) were used to quantify serum miRNA content according to manufacturer's instructions. Briefly, 3 μ L of total RNA from each sample was reverse transcribed. RT products were pre-amplified and diluted in 0.1X TE buffer, pH 8.0. Arrays were processed and analyzed by the ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems). Detector threshold cycle (Ct) values were normalized to the mean Ct of each array.

qRT-PCR

For serum samples, total RNA volumes of 1.33 μ L were reverse transcribed using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems Inc.) and miRNA-specific RT primers from the appropriate TaqMan® miRNA Assay Kit (Applied Biosystems). For tissue samples, 10 ng total RNA was reverse transcribed as above. Real-time quantitative polymerase chain reaction (qRT-PCR) was performed on a Stratagene MX3000P using TaqMan® miRNA assay kits and TaqMan® Universal PCR Master Mix (Applied Biosystems). Serum miRNA levels were normalized to two exogenous *C.elegans* miRNAs (cel-miR-54 and cel-miR-238) and tissue miRNA levels were normalized to endogenous sno-202 and sno-234 levels.

Statistical analysis

Statistical calculations were performed with Stata 11.0 (StataCorp, College Station, TX). Differences in miRNA levels between sample populations were assessed by the Wilcoxon rank-sum test. Correlation was determined using Spearman's rank correlation test. Null hypotheses were rejected at $p < 0.05$. Hierarchical cluster analysis using complete-linkage clustering with Euclidean (L2) distance was performed using the 104 miRNAs detected in all 10 LDA samples.

RESULTS

Identification of BA-associated circulating miRNA

Serum miRNA profiles of BA patients and age- and sex-matched controls with indeterminate cholestasis (n=5 each) were determined using the TaqMan® Array Human MicroRNA A Card platform (Applied Biosystems). Hierarchical cluster analysis showed BA samples possess an altered serum miRNA profile compared to cholestatic controls (Fig. 1A). 134 of 375 miRNAs were detected in at least 3 out of 5 samples in the case or control group. A total of 11 miRNAs were selected for further analysis by applying the following criteria: a fold change >1.5, mean Ct <30 in at least one group, and p-value <0.05. Individual qRT-PCR reactions for these miRNAs were performed on the same RNA samples used in the LDA experiment. Of the 11 candidate miRNAs, 9 were confirmed by qRT-PCR as significantly altered in BA sera (online-only supplemental Table 1, <http://links.lww.com/MPG/A153>). MiRNA levels measured by qRT-PCR were significantly correlated to those by LDA (Fig. 1B).

Validation of BA-associated circulating miRNA

To validate the candidate BA-specific circulating miRNAs, qRT-PCR was performed on serum RNA from an independent set of BA and cholestatic controls (n=24 each) (online-only supplemental Table 2, <http://links.lww.com/MPG/A154>). The two patient groups displayed similar demographic and clinical characteristics, including total and direct serum bilirubin levels (Table 1). Of the nine candidate miRNAs, only miR-200b was significantly altered in BA sera compared to controls. To confirm the elevation of miR-200b, we measured levels of miR-200a and miR-429, which are co-transcribed with miR-200b from the miR-200b/429 locus. Both miR-200a and miR-429 were significantly elevated in BA sera (Fig. 2A). Serum levels of each miR-200b/429 member were significantly correlated with the other cluster members at $p < 0.0001$ (data not shown). Members of the paralogous miR-200c/141 cluster, miR-200c and miR-141, were unaltered in BA sera (Fig. 2B).

Diagnostic utility and source of BA-specific circulating miRNA

The diagnostic utility of the miR-200b/429 cluster was examined using receiver operating characteristic (ROC) analysis. All three miRNAs of the cluster displayed good diagnostic properties, with area under the ROC curve (AUC) values greater than 0.80 (Table 2 and Fig. 2C). At optimal thresholds, the miR-200b/429 cluster correctly classified up to 85% of patient samples, with sensitivity and specificity values ranging from 71% to 92% (Table 2). As shown in Table 1 the total protein and γ -glutamyl transpeptidase (GGTP) levels were elevated in the sera of BA patients compared to controls, so we also performed ROC analysis on these parameters. The potential diagnostic utility of the total protein was low (AUC 0.706), while the serum GGTP displayed a comparable AUC value (0.893) to those of the miR-200b/429 cluster.

To identify the tissue source of circulating miR-200b/429 cluster miRNAs, we isolated RNA from liver and extrahepatic bile duct (EHBD) tissue of 8-week-old Balb/c mice and measured miRNA levels by qRT-PCR. Interestingly, miR-200b/429 levels were up to 29-fold higher in EHBD tissue compared to liver (Fig. 2D), suggesting that cholangiocytes are the source of elevated miR-200b/429 cluster levels in BA.

DISCUSSION

A timely diagnosis of BA is critical for optimal patient outcome following KPE, yet current non-invasive tests are unable to reliably distinguish BA from other forms of neonatal hyperbilirubinemia. Earlier reports had suggested GGTP as a biomarker of BA (14, 15), and

this is supported by our results. However, subsequent studies have found that serum GGTP levels are frequently elevated in other forms of neonatal cholestasis (16, 17). Wang et al. used a proteomic approach to identify 11 serum proteins significantly altered in BA compared to non-BA cholestasis (18). A classifier derived from the proteins correctly identified nine of ten BA and non-BA cholestatic control samples. Other proposed serum biomarkers, including interleukins, hyaluronic acid, insulin-like growth factor-1, and α -enolase have not been validated as clinically useful indicators of BA (19–22). In this report, we have identified a BA-specific circulating miRNA signature that may serve as a novel biomarker. A screen of serum miRNA by TaqMan® array identified nine miRNAs that are significantly altered in BA versus cholestatic controls. Subsequent validation in a larger, independent sample set confirmed a single miRNA cluster, miR-200b/429, as significantly increased in BA sera.

The miR-200b/429 cluster performed well diagnostically, correctly classifying up to 85% of patients. Although not all samples were properly distinguished, elevated circulating miR-200b/429 levels may prove a reliable indicator for an accelerated diagnostic workup in the jaundiced infant or in population-based screening tests. Future studies with large sample sets are needed to confirm our findings, establish reliable diagnostic thresholds for BA, and to compare or combine miRNA with other markers.

The high miR-200b/429 expression we observed in EHBD implicates this tissue as a source of the circulating fraction in BA. We propose that the elevation of miR-200b/429 in BA sera results from the destruction of EHBD, a feature unique to BA compared to the control patients. If serum miR-200b/429 levels correlate with EHBD damage, the cluster may also have prognostic value by predicting native liver survival in patients undergoing KPE. High expression may also occur in the intrahepatic bile ducts (IHBD); this would likely be masked in our liver RNA samples, as IHBD tissue represents a small fraction of total liver mass. Serum levels of the hepatocyte-specific miR-122 were not altered between groups (Fig. 2B), suggesting that hepatocyte injury was similar between BA and cholestatic controls.

Circulating miRNAs with prognostic potential have previously been reported in a variety of pathological contexts (23–25), and this study suggests that circulating miRNAs are of diagnostic value in BA. Future work will include the development and testing of diagnostic and prognostic logistical regression models using serum miR-200b/429 levels in larger populations and in combination with other biochemical parameters in an effort to facilitate early detection and improved patient outcome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Low-density array (LDA) analysis of serum miRNA in human biliary atresia (BA). A, Dendrogram of unsupervised hierarchical analysis using 104 miRNAs detected in all samples by LDA. B, Scatterplot of relative serum miRNA levels of 9 candidate miRNAs as determined by LDA and individual qRT-PCR. Control sample means are scaled to 1. Open circles, cholestatic control samples; filled circles, BA samples; ρ , Spearman rank correlation coefficient.

FIGURE 2.

Validation of circulating miRNAs in biliary atresia (BA). A,B Box-whisker plots of serum levels of miR-200b/429 cluster members (A) and miR-200c/141 and miR-122 (B) in an independent set of cholestatic controls and BA cases (n=24 each). Box, 25% to 75%; whisker, upper and lower adjacent values; line, median; points, outside values. Cholestatic control sample means are scaled to 1. * $p < 0.05$ vs. cholestatic control. C, Receiver-operating characteristic curve of miR-200a in cholestatic control and BA sera (n=24 each). AUC = area under the curve. D, Box-whisker plot of relative miRNA expression in liver and extrahepatic bile duct (EHBD) tissue of 8-week-old Balb/c mice (n=6). Box, 25% to 75%; whisker, upper and lower adjacent values; line, median; points, outside values. * $p < 0.05$ vs. liver.

Table 1

Validation set patient characteristics

	Cholestatic controls (n=24)	Biliary atresia (n=24)
Gender, male/female	16/8	12/12
Age at collection, days	51.00 ± 4.12	55.75 ± 3.33
Total bilirubin, mg/dL	8.51 ± 0.56	8.28 ± 0.46
Direct bilirubin, mg/dL	5.32 ± 0.38	5.08 ± 0.35
AST, IU/L	165.54 ± 21.47	180.55 ± 16.52
ALT, IU/L	118.13 ± 15.26	122.79 ± 13.34
Albumin, g/dL	3.40 ± 0.09	3.62 ± 0.10
Total protein, g/dL	5.60 ± 0.11	6.07 ± 0.13 *
GGTP, U/L	243.24 ± 70.38	571.33 ± 72.97 *
INR	0.98 ± 0.02	1.32 ± 0.34
PTT, seconds	32.11 ± 0.96	39.24 ± 5.91
Hemoglobin, g/dL	10.73 ± 0.46	10.82 ± 0.30
WBCs, 1000/mcl	10.93 ± 0.93	12.86 ± 0.67
Birth weight, kg	3.85 ± 0.18	4.25 ± 0.14

AST=aspartate aminotransferase; ALT=alanine aminotransferase; GGTP= γ -glutamyl transpeptidase; INR=international normalized ratio; PTT=partial thromboplastin time; WBCs=white blood cells.

*
P<0.05.

Table 2

Diagnostic properties of BA-specific serum miRNAs

miRNA	Sensitivity, %	Specificity, %	Correctly classified, %	AUC	95% CI
miR-200b	79.17	79.17	79.17	0.807	0.675–0.939
miR-200a	83.33	83.33	85.42	0.862	0.750–0.974
miR-429	70.83	91.67	81.25	0.806	0.677–0.934

AUC=area under the curve; CI=confidence interval