Published in final edited form as:

J Pediatr Gastroenterol Nutr. 2010 April ; 50(4): 411–416. doi:10.1097/MPG.0b013e3181cb42ee.

Serum markers may distinguish biliary atresia from other forms of neonatal cholestasis

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Abstract

Background—Biliary atresia (BA) is the most serious liver disease in infants. Diagnosis currently depends on surgical exploration of the biliary tree. Non-invasive tests that distinguish BA from other types of neonatal liver disease are not available.

Methods—To identify potential serum biomarkers that classify children with neonatal cholestasis, we performed 2-dimensional difference gel electrophoresis, statistical analysis, and tandem mass spectrometry using serum samples from 19 infants with BA and 19 infants with non-BA neonatal cholestasis.

Results—11 potential serum biomarkers were found that could in combination classify children with neonatal cholestasis.

Conclusions—Although no single biomarker or imaging test adequately distinguishes BA from other types of neonatal cholestasis, combinations of biomarkers, imaging tests and non-invasive clinical criteria should be further explored as potential tests for rapid and accurate diagnosis of BA.

Keywords

Proteomics; biliary atresia; neonatal cholestasis; biomarker

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Supplemental digital content is available for this article. Direct URL citations appear in the printed text, and links to the digital files are provided in the HTML text of this article on the journal's Web site (www.jpagn.org).

Introduction

Biliary atresia is a severe neonatal liver disease resulting from sclerosing cholangiopathy of unknown etiology (1–4). It causes one third of all cholestatic neonatal disease and is the most common indication for liver transplantation in children. Unfortunately, there are currently no non-invasive diagnostic methods that clearly identify children with BA. Definitive diagnosis requires surgery, cholangiogram, and liver biopsy. Early diagnosis is particularly important for BA because early surgical intervention by Kasai portoenterostomy correlates with good long-term outcome (5). Thus, there is real need for novel blood tests that facilitate differentiation between BA and other neonatal cholestatic diseases.

Previous studies examining the potential for changes in individual serum or hepatic markers to distinguish BA from other neonatal liver diseases identified differences in extracellular matrix proteins and modifying enzymes, cell adhesion molecules, cytoskeletal proteins, cell proliferation and death markers, immunologic markers, growth factors and their receptors that might be useful in BA diagnosis or to evaluate prognosis. While some of these approaches appear promising, a more global approach is still needed to identify BA-specific changes in serum protein abundance. New proteomic technology including sensitive and accurate techniques of two dimensional difference gel electrophoresis (2D DIGE) and tandem mass spectrometry (MS/MS) significantly facilitate identification new disease biomarkers (6). Although this approach has been used for cancer biomarker identification (7), to our knowledge, there are no reports using this technique to identify BA biomarkers. For this work, We used high throughput proteomic technology and advanced data mining software to identify 11 proteins whose relative abundance distinguishes children with BA from infants with non-BA cholestasis in our cohort. This work provides new hope that a blood test for BA can be developed.

Materials and Methods

Patient population and serum samples

Serum samples from infants with newly recognized cholestatic disease were obtained from the Biliary Atresia Research Consortium (BARC), a multi-center collaborative study group supported by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) and established to investigate BA and neonatal cholestasis (8). Children enrolled are followed by expert hepatologists from 10 large pediatric medical centers for as long as they remain cholestatic. We are confident that all children with BA were correctly identified based on operative cholangiogram and liver pathology. Entry criteria for the BARC study include age less than 180 days, serum direct or conjugated bilirubin greater than 20% of total and greater than 2 mg/dL. Children with liver failure, malignancy, hypoxia, shock, ischemic hepatopathy within the preceding two weeks, or ECMO-associated cholestasis were excluded as were children with prior hepatobiliary surgery. Children with primary hemolytic disease, drug or TPN-associated cholestasis, bacterial or fungal sepsis, or birth weight less than 1500 gm are also excluded unless they are definitively diagnosed with biliary atresia or another cholestatic disease being studied by the network. Two children in the non-BA group had alpha-1 antitrypsin (1AT) deficiency (ZZ Pi Type). One child had a positive CMV IgM antibody. The etiology of the liver disease in other non-BA infants is unknown reflecting the ongoing challenge of diagnosing cholestatic infants. These children are called “indeterminate intrahepatic cholestasis” (IIC), a term now preferred by BARC investigators and accounting for 33% of children enrolled in BARC.

Blood collected in BD VacutainerR SST Serum Separation Tubes (Becton Dickinson), was allowed to clot (30–45 minutes, room temperature) before centrifugation ($1,100 \times g$; 10 minutes). Serum was stored at -80°C before analysis. Detailed de-identified clinical data from

BARC was available for analysis. The protocol was approved by Washington University's Human Research Protection Office.

Proteomic analysis

Detailed methods are provided in online supplemental material. Briefly, serum samples thawed in the presence of protease inhibitors were depleted of the 12 most abundant serum proteins and subjected to two-dimensional difference gel electrophoresis (DIGE) (9) after labeling one percent of each protein sample with Cy2, Cy3 or Cy5 fluorescent dye. Each gel contained one BA sample, one non-BA sample, and one sample that was a mixture of all proteins (i.e. pooled sample to facilitate cross-gel alignments). Selected proteins were identified using tandem mass spectrometry. The Protein MASCOT score for the proteins shown in Table 2 ranged from 36 to 1360. Detailed protein identification data are provided in Supplemental Table 1 (<http://links.lww.com/MPG/A15>).

Data analysis and statistics

GE Healthcare's DeCyder Differential In-gel Analysis (DIA) software was used to determine spot boundaries and normalize differences between images from each gel. Normalization eliminated non-sample differences due to fluorescent response of different Cy dyes and sample loading. Pooled samples were used by DeCyder Biological Variation Analysis (BVA) software (GE Healthcare) for cross-gel alignment. DeCyder Extended Data Analysis (EDA) module (GE Healthcare) was used for statistical comparisons and to perform predictive modeling. To generate the classifier we used EDA's Regularized Discriminant Analysis (RDA) algorithm, a statistical approach specifically designed for this type of analysis (10). Standard clinical parameters (Table 1) were evaluated by Mann-Whitney rank sum or t-test.

Results

Patient selection criteria

Our goal was to identify novel BA biomarkers that might be useful when children were first recognized to have cholestasis. Therefore, we evaluated serum samples from children enrolled in the BARC study whose serum was obtained within a few days of enrollment. Clinical data are in Table 1. Work flow is outlined in Figure 1. Final diagnosis for each child was BA (n = 19), alpha-1 anti-trypsin deficiency (n = 2), or indeterminate intrahepatic cholestasis (IIC; n = 16). One additional child had positive CMV IgM antibody. The critical importance of distinguishing children with BA from those with non-BA cholestatic disease is emphasized by the difference in outcome between BA and non-BA groups. All children with BA had a Kasai portoenterostomy. 8/19 children with BA were alive with a normal bilirubin at follow up. 10/19 children with BA had a liver transplant and two infants with BA died. In contrast, none of the non-BA children had a liver transplant or died of liver disease. There were no significant differences in any routinely measured laboratory values between BA and non-BA groups although elevated GGT and elevated direct/total bilirubin ratio in the BA group almost reached statistical significance.

Proteomic analysis of serum from BA and non-BA cholestatic infants

We analyzed 38 serum samples using 19 large format DIGE gels. Samples were depleted of abundant proteins, labeled with fluorescent dyes, and then separated by two dimensional gel electrophoresis (Figure 2). Using DeCyder BVA, 491±69 spots were manually matched to provide gel alignment "landmarks". Automated matching produced an additional 584±78 spots. All matches were manually verified across each gel to generate a list of 1110±181 properly matched spots. Some protein spots were absent on individual gels. Protein spots matched across at least 16/19 gels were used for EDA statistical analyses. 571 spots meeting

these criteria were analyzed to identify potential biomarkers that might distinguish infants with BA from non-BA liver disease.

Identification of potential biomarkers

From our 38 serum samples we randomly selected 28 samples to use as a “training set” to identify biomarkers (14 BA and 14 non-BA). The remaining 10 samples were used as a “test set” to determine if the classifier algorithm generated could correctly distinguish between BA and non-BA liver disease. Average serum protein abundance differences between BA and non-BA groups were compared using t-tests. 138 spots had more than 1.2-fold difference between the groups ($P < 0.1$), but only 47 spots were completely resolved from neighboring peptides. Of these spots, 42 (90%) were present on at least 18/19 gels; 28 spots (67%) were identified by tandem mass spectrometry and used for classifier generation and testing. Interestingly, many identified potential protein biomarkers were found in multiple spots suggesting protein isoforms or post-translational processing.

Generation of a classifier to distinguish BA from non-BA cholestasis

No identified protein individually distinguished BA from non-BA serum. We hypothesized that combinations of protein abundance ratios might, however, distinguish these groups. To generate a classifier, we analyzed peptide abundance data of the training set using EDA's Regularized Discriminant Analysis (RDA) (10), a statistical approach well suited to large numbers of observations and small sample sizes (i.e. spot number $\gg$ patient number). This method generates classifiers with non-linear boundaries between groups where gamma and lambda parameters can be varied to change the shape of decision borders and generate stable boundaries. Using this approach, we identified 11 spots whose abundance correctly identified 9/10 “test set” serum samples randomly selected before quantitative image analysis (Table 2, Figure 3A). A heat map shows relative abundance of these proteins in serum of children with BA or non-BA cholestasis (Figure 4). To evaluate specificity and sensitivity of the classifier, a ROC curve was generated (Figure 3B) by varying gamma and lambda parameters for the RDA classifier. The C-statistic (area under the curve) for these data was 0.8. In combination with other clinical or diagnostic criteria, this C-statistic suggests that serum proteomic analysis might become a useful adjunct to medical decision making for cholestatic infants (11).

Discussion

Biliary atresia is a devastating disease for which current diagnostic and treatment methods are inadequate. Because early diagnosis is essential for effective drainage via Kasai portoenterostomy, better non-invasive diagnostic techniques are needed. This is especially important since many neonatal diseases can be confused with BA, and diagnosis currently requires operative cholangiogram and liver biopsy (1,2). Thus, although children with BA have a more severe form of liver disease than children with many other types of cholestasis, this may not be initially apparent based on clinical presentation or routinely investigated laboratory parameters as supported by data in Table 1. For this study we hypothesized that liver injury in infants with BA is distinct enough from other types of neonatal cholestasis that combinations of serum biomarkers might distinguish infants with BA from other cholestatic children. For these studies we started with serum from 38 infants, aligned more than 1100 spots across 19 large format DIGE gels, and used bioinformatics techniques to identify 11 proteins whose abundance could classify serum from cholestatic infants into BA or non-BA groups. We recognize that this work will need replication with independent serum samples. Nonetheless, these promising data suggest that non-invasive tests for BA are achievable, especially if serum biomarkers are combined with additional imaging and clinical parameters. Test methods, however, will need to be simplified before these biomarkers could be used clinically.

Before discussing the potential significance of proteins in our classifier, we note that a recent analysis demonstrated changes in serum apolipoprotein C-II and transthyretin in infants with BA at early and late stages of disease (12). Although these proteins were among our diagnostic biomarkers, comparing their data with ours is difficult because of few control samples in their study ($n = 2$) and their emphasis on changes in serum proteins with disease progression in BA, versus our emphasis on differential diagnosis between BA and other cholestatic disease. In both studies, changes identified are “relative” protein abundance levels, but diagnostic tests will require alternative approaches to determine absolute serum biomarker levels. Nonetheless, their data suggest that biomarkers for BA could change as disease progresses. Our data only apply to children at initial contact with pediatric gastroenterologists (i.e., at the time of initial diagnosis).

In addition to potential diagnostic significance, identified biomarkers can be tied to prior observations about BA and cholestatic disease. While we need to avoid over-interpretation, it is worth considering potential biological significance of our findings. Both apolipoprotein C-II and E were more abundant in serum from BA than non-BA infants consistent with previously reported apolipoprotein E elevations in individuals with biliary tract obstruction (13–15). These proteins are linked on chromosome 19 and their expression is regulated by two hepatic control region *cis*-acting liver enhancers (16) that are activated by the farnesoid X-activated receptor (FXR; NR1H4), a nuclear hormone receptor that induces gene expression in response to several bile acids (17). Remarkably, expression of complement component 3, another protein that was more abundant in BA than non-BA serum is also activated by FXR (18). Collectively, these data suggest that FXR activity is higher in liver of infants with BA than in non-BA cholestasis, but there are other possible explanations.

Elevated apolipoprotein C-II in infants with BA correlates with old observations about dyslipoproteinemia in cholestatic disease. In particular, lipoprotein-X (LP-X) was previously suggested to help distinguish BA from other types of neonatal cholestasis (19). LP-X is an unusual lamellar particle with high free cholesterol and phospholipid levels that accumulates in serum during biliary tract obstruction (20,21). LP-X is also found in individuals with deficiency in lecithin cholesterol acyl transferase (LCAT), an enzyme that produces cholesterol ester from free cholesterol and lecithin. Interestingly apolipoprotein C-II, inhibits LCAT (22) consistent with the idea that LP-X may be elevated in children with BA compared to other neonatal cholestatic diseases because of increased apolipoprotein C-II.

The identified potential biomarkers also suggest a more significant pro-inflammatory state in BA than other neonatal cholestatic diseases consistent with prior observations (23–25). For example, complement C3 and factor B are acute phase reactant proteins that were more abundant in BA versus non-BA serum. These same proteins are elevated in adults with large bile duct obstruction or viral hepatitis (26–28) and in primary biliary cirrhosis (29,30). In contrast, serum levels of C3 and factor B were reduced in chronic active hepatitis and cryptogenic cirrhosis suggesting that their abundance may be useful as part of a diagnostic fingerprint that distinguishes BA from other neonatal liver diseases. Note, however that although recent data suggest that complement may be activated in BA (31), our data do not provide insight into complement activation.

Low serum levels of transthyretin (prealbumin), apolipoprotein H and alpha2-HS glycoprotein are also consistent with a pro-inflammatory state in infants with BA since these proteins are negative acute phase reactants (32–35). Furthermore, although low serum transthyretin is often assumed to reflect malnutrition, transthyretin falls rapidly in rats after biliary tract obstruction (36) and inflammation is among the most potent transthyretin regulators (35,37). Interestingly, mannose binding lectin (MBL2) is a component of the innate immune system (38,39) but amyloid P, an acute phase reactant in mice closely related to C-reactive protein, is not a human

acute phase reactant (40). Collectively, elevated levels of positive acute phase proteins and reduced levels of negative acute phase proteins in serum of infants with BA is consistent with a pro-inflammatory state in BA compared to other forms of neonatal cholestasis. Caution must be used interpreting these data however, since FXR mRNA is repressed in mouse liver during the acute phase response (41), at least after treatment with some inflammatory mediators. While this might seem to contradict the hypothesis outlined above that FXR activity is elevated in liver of infants with BA, FXR mRNA levels and FXR activity need not be related since FXR is activated by bile acids that accumulate more significantly in cholestatic than inflammatory liver disease.

Conclusions

Developing rapid non-invasive diagnostic tests that clearly distinguish BA from other forms of neonatal cholestasis is important. Our studies suggest that although no single marker reliably separates BA from non-BA serum samples, combinations of markers could be valuable. In particular, application of modern statistical methods to complex data sets provides a new opportunity to distinguish BA from other types of liver disease. Interpretation of the biological significance of identified potential biomarkers, however, is challenging. First, since we compared serum from infants with BA to serum from non-BA cholestatic infants, these data provide no insight into how these protein levels might compare to healthy infants. In addition, these analyses provide limited insight into hepatic function, liver pathology, or the extent of hepatic injury. Comparable proteomic analyses of liver tissue might provide additional valuable information, but would likely require analysis of relatively large biopsy samples to avoid the complex issues that arise in sampling heterogeneous tissue. Finally, while it is tempting to speculate that these biomarkers reflect specific disease processes as we have done, the complexity of BA and non-BA liver disease makes it possible to generate hypotheses, but not to draw conclusions about disease etiology or pathogenesis based on our data. Nonetheless, these new data provide a rationale for additional studies to validate and extend our observations with the goal of developing reliable non-invasive diagnostic testing for BA. This work will require quantitative analysis of serum protein abundance for selected biomarkers (e.g., by ELISA) and the analysis of serum from many additional children with BA and non-BA liver disease. Definitive diagnosis may require a combination of serum biomarkers, clinical criteria, and imaging results to provide an unambiguous diagnosis without liver biopsy or cholangiogram. These results also support further investigations into the role of FXR and inflammation in BA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We greatly appreciate the Biliary Atresia Research Consortium investigators, coordinators, and families who participated since they made this work possible. We thank Dr. Richard LeDuc for bioinformatics assistance and preparation of Supplemental Table 1. This manuscript was not prepared in collaboration with other BARC investigators and does not necessarily reflect the opinions or views of BARC or the NIDDK. We thank Michael R. Narkewicz for providing serum samples to generate preliminary data as we began this study and David Rudnick, Ross Shepherd, Frances White and Rosemary Nagy for helpful comments.

This study was supported by the NIDDK (R21 DK68371, U01 DK062456) and the Proteomics Core of the Washington University Digestive Diseases Research Core Center Grant (DDRCC, #P30 DK52574), P41RR000954 and UL1 RR024992 from the National Center for Research Resources, a component of the National Institutes of Health (NIH) and NIH Roadmap for Medical Research.

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Figure 1.
Workflow for biomarker discovery.

Figure 2.

Representative 2-D and 3-D DIGE gels. (A,B) Identical images are shown (A) Highlighted spots are more abundant in BA than non-BA liver disease. (B) Highlighted spots are more abundant in non-BA than BA liver disease. Numbers beside spots correspond to numbers in Table 2. (C, D) Representative 2-D and 3-D images of single spots demonstrating quantitative differences between individuals. (C) Spot 4, Figure (A). (D) Spot 11, Figure (B).

A Classified results of 10 test samples

Index	Diagnosis	Test results	Accuracy
1	Non BA	Non BA	Correct
2	Non BA	Non BA	Correct
3	Non BA	Non BA	Correct
4	Non BA	Non BA	Correct
5	Non BA	Non BA	Correct
6	BA	Non BA	Wrong
7	BA	BA	Correct
8	BA	BA	Correct
9	BA	BA	Correct
10	BA	BA	Correct

B Receiver Operating Characteristic curve **Figure 3.**

Classifier Validation. (A) The relative abundance of 11 proteins in Table 2 correctly classified 9/10 of the children in the “test set”. (B) RDA algorithms are influenced by lambda and gamma parameters. Receiver operating characteristic (ROC) curve was generated by varying lambda and gamma, but maintaining the same 11 peptide spots as biomarkers. Sensitivity indicates the likelihood that the classifier generates an accurate classification. 1-specificity indicates the likelihood that the classifier generates an inaccurate classification.

Figure 4. Heat map. Relative abundance of potential biomarker proteins in infants with BA or non-BA cholestasis. Each column represents an individual child’s serum. Each row represents an individual protein. Colors show the protein abundance relative to that protein’s abundance in pooled samples. A scale bar is at the bottom. The abundance of single proteins cannot be used to classify serum samples, but trends in peptide abundance are evident. The top 7 proteins tend to be more abundant in serum of non-BA cholestatic infants than in children with BA. The bottom 4 proteins tend to be more abundant in children with BA than with non-BA liver disease.
* Alpha-1 antitrypsin deficiency, # CMV IgM positive.

Table 1

Demographic and clinical data for children at the time of diagnosis.

	Biliary atresia (BA)	Non-biliary atresia (non-BA)	P value	Test methods
Number of patients	19	19		
male/female ratio	10:9	15:4	0.300	Fisher Exact Test
Age *	55±3.7	47±5.6	0.235	t-test
Total bilirubin(TB)	8.8±1.1	7.8±0.9	0.456	t-test
Direct bilirubin(DB)	6.2±0.9	4.9±0.6	0.242	t-test
DB/TB ratio	0.80±0.03	0.68±0.05	0.057	Mann-Whitney Rank Sum Test
AST	177±25	146±23	0.330	Mann-Whitney Rank Sum Test
ALT	131±17	122±24	0.484	t-test
ALP	598±67	759±104	0.185	t-test
Albumin	3.5±0.1	3.4±0.1	0.621	t-test
GGT	564±78	307±99	0.051	t-test
PT	18.1±5.2	12.7±0.3	0.553	Mann-Whitney Rank Sum Test

Data presented as Mean±SEM (Standard Error of Mean)

* at serum sample day; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transpeptidase; PT, prothrombin time.

Table 2

Proteins used to differentiate BA from non-BA neonatal cholestasis

Serum proteins increased in BA patients					
Accession number	NCBI# Abbreviations	NCBI Official full name	Number in Fig 2A	BA/non-BA Relative protein abundance	P value
AAA51743	APOC2	Apolipoprotein C-II	4	3.2	0.0008
AAB59518	APOE	Apolipoprotein E	3	1.9	0.008
AAA16820	CFB	Complement factor B	1	1.7	0.021
AAA85332	C3	Complement component 3	2	1.4	0.030
Serum proteins decreased in BA patients:					
Accession number	NCBI Abbreviations	NCBI Official full name	Number in Fig 2B	Non-BA/BA Relative protein abundance	P value
NP_000362	TTR	Transthyretin	11	1.9	0.027
AAA60302	APCS	Amyloid P component, serum	9	1.7	0.017
P02749	APOH	Apolipoprotein H (beta-2-glycoprotein I)	7	1.7	0.0002
AAA52173	GC	Vitamin D-binding protein (group-specific component)	6	1.7	0.015
BAA22651	AHSG	alpha2-HS glycoprotein	5	1.6	0.039
CAA34079	MBL2	Mannose-binding lectin (protein C) 2, soluble (opsonic defect)	8	1.6	0.0004
AAA52700	HSPG2	Heparan sulfate proteoglycan 2	10	1.4	0.017

NCBI, National Center for Biotechnology Information.