Serum Proteomics Uncovers Biomarkers of Clinical Portal Hypertension in Children With Biliary Atresia

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Children with biliary atresia (BA) often develop portal hypertension (PHT) and its complications, which are associated with high morbidity and mortality. The goal of this study was to identify serum biomarkers of PHT by using large-scale proteomics. We applied the slow off-rate modified aptamer scan (SOMAscan) to measure 1,305 proteins in serum samples of children with BA with and without clinical evidence of PHT in validation and discovery cohorts enrolled in the Biliary Atresia Study of Infants and Children. Serum proteomics data was analyzed using logistic regression to identify protein(s) with an area under the receiver operating characteristic curve (AUROC) ≥ 0.90. Immunostaining was used to characterize the cellular localization of the new biomarker proteins in liver tissues. We identified nine proteins in the discovery cohort (n = 40 subjects) and five proteins in the validation cohort (n = 80 subjects) that individually or in combination predicted clinical PHT with AUROCs ≥ 0.90. Merging the two cohorts, we found that semaphorin 6B (SEMA6B) alone and three other protein combinations (SEMA6B+secreted frizzle protein 3 [SFRP3], SEMA6B+COMM domain containing 7 [COMMD7], and vascular cell adhesion molecule 1 [VCAM1]+BMX nonreceptor tyrosine kinase [BMX]) had AUROCs ≥ 0.90 in both cohorts, with high positive- and negative-predictive values. Immunostaining of the new protein biomarkers showed increased expression in hepatic endothelial cells, cholangiocytes, and immune cells within portal triads in BA livers with clinical PHT compared to healthy livers. *Conclusion:* Large-scale proteomics identified SEMA6B, SFRP3, COMMD7, BMX, and VCAM1 as biomarkers highly associated with clinical PHT in BA. The expression of the biomarkers in hepatic epithelial, endothelial, and immune cells support their potential role in the pathophysiology of PHT. (*Hepatology Communications* 2022;6:995-1004).

Biliary atresia (BA) is the most common cause of end-stage liver disease in children[(1)] and is often associated with portal hypertension (PHT) and its complications, resulting in significant morbidity and mortality.[(2)] PHT is defined as an increase in intraportal venous pressure (>10 mm Hg) as measured by hepatic venous pressure gradient (HVPG), with normal values ranging between 5 and 10 mm Hg. However, portal pressures are not commonly measured in children due to the invasive nature of the procedure, and therefore clinical and biochemical markers are used to phenotype and stage children with chronic liver disease.

The clinical diagnosis of PHT in children is currently made once they develop splenomegaly or

Abbreviations: ApoA1, apolipoprotein A1; AUROC, area under the receiver operating characteristic curve; BA, biliary atresia; BASIC, Biliary Atresia Study of Infants and Children; BMX, BMX nonreceptor tyrosine kinase; ChiLDReN, Childhood Liver Disease Research and Education Network; CI, confidence interval; COMMD7, COMM domain containing 7; cPHT, clinical portal hypertension; HVPG, hepatic venous pressure gradient; IQR, interquartile range; MMP7, matrix metalloproteinase 7; NIDDK, National Institute of Diabetes and Digestive and Kidney Diseases; PHT, portal hypertension; SEMA6B, semaphorin 6B; SFRP3, secreted frizzle protein 3; SOMAscan, slow off-rate modified aptamer scan; TGF-β1, transforming growth factor beta 1; TIMP1, tissue inhibitor of metalloproteinase 1; VCAM1, vascular cell adhesion molecule 1.

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complications of PHT. To uniformly define clinical PHT (cPHT), the Childhood Liver Disease Research and Education Network (ChiLDReN) investigators reported an operational definition where cPHT was "definite" if a patient had either 1) a history of a complication of PHT (esophageal or gastric variceal hemorrhage, ascites, or hepatopulmonary syndrome) or 2) clinical findings consistent with PHT (both splenomegaly [spleen palpable >2 cm below the costal margin] and thrombocytopenia [platelet count <150,000/mL]).[2] cPHT was defined as "absent" if none of the criteria were met. This method relies on the nonspecific findings of splenomegaly and thrombocytopenia or known complications. Sutton et al.[3] recently reviewed the available studies of noninvasive biomarkers of PHT in pediatrics; among the few published studies, platelet count was used as a biomarker alone or in models employing platelet count, spleen length z-score, and albumin.[3] Despite the strengths of these studies, there is a need for a minimally invasive biomarker(s) that closely correlates with the cPHT as an initial critical step to guide future validation with secondary markers (such as increased liver stiffness). To this end, we tested the hypothesis that serum proteomics identifies specific biomarkers of cPHT in children with BA by performing large-scale quantification of proteins in subjects with and without cPHT and immunolocalization of selected proteins in liver tissue from children with advanced stages of biliary cirrhosis secondary to BA.

Materials and Methods

STUDY DESIGN

We performed large-scale serum proteomics in a discovery cohort of subjects with BA with definite or absent cPHT. This was followed by the selection of biomarkers that had an area under the receiver operating characteristic curve (AUROC) ≥ 0.9; a similar approach was applied to a validation cohort. Biomarkers with AUROCs ≥ 0.9 in both cohorts were identified, and their discriminatory performance was compared against known biomarkers of PHT. Those with higher performance were selected for immunostaining analysis to identify cellular sources of expression.

HUMAN SAMPLES

Serum samples were obtained from children with BA enrolled in the Biliary Atresia Study of Infants and Children (BASIC) conducted by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)-funded ChiLDReN (www.childrennetwork.org; Clinicaltrials.gov identifier: NCT00061828).

Data on protein expression are submitted as Supporting Material. Further information and requests for resources and reagents should be directed to and will be fulfilled by the corresponding author Jorge A. Bezerra (Jorge.bezerra@cchmc.org).

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Tissue sections for immunostaining (protocol available in the Supporting Material) were obtained from de-identified paraffin-embedded liver tissues archived in the Biobank Repository of Cincinnati Children's Hospital Medical Center. The study protocols were approved by the human research review boards of all participating institutions.

PROTEOMICS ASSAY

Serum samples were subjected to the slow off-rate modified aptamer scan (SOMAscan) assay that measures 1,305 proteins (Somalogic Inc., Boulder, CO). Information on assay protocols has been described elsewhere[4] and is summarized in the Supporting Material.

STATISTICAL ANALYSIS

Clinical and biochemical characteristics of subjects were analyzed using the median and interquartile range (IQR). Statistically significance was defined as $P < 0.05$, and biochemical and sex differences were compared by the Mann-Whitney and Fisher's exact tests, respectively.

All protein results were log transformed to accommodate the wide range of assayed proteins and skewness of their measurement values. For the discovery cohort of 40 subjects with BA in the cPHT and non-PHT groups, a two-sample *t* test and a false discovery rate <0.05 were applied for all proteins, followed by a stepwise selection of those that segregated with cPHT by using multivariate logistic regression. Youden's index was used to select optimal cut-off points for sensitivity and specificity. Proteins showing the best discriminative potential for cPHT were used to build the best performing models for the discovery cohort. For the validation cohort of 80 subjects (40 each in cPHT and without cPHT), we had about 72% power to detect a departure of 0.10 from 0.90 in the discovery cohort. Data from this cohort were analyzed by the same methods. For calculations, we used an estimated prevalence of cPHT of 49% based on the entire cohort of patients enrolled in the BASIC study, as reported by Shneider et al.[2] Statistical analysis was performed with Prism (GraphPad Software, San Diego, CA) and SAS (SAS Institute, Cary, NC).

All authors had access to the study data and reviewed and approved the final manuscript.

Results

FIRST-PASS BIOMARKER IDENTIFICATION IN A DISCOVERY COHORT

To identify potential biomarkers of cPHT, we used the SOMAscan assay to quantify the relative abundance of 1,305 serum proteins (Supporting Table S1) from a discovery cohort of 40 children with BA enrolled in the BASIC study of the NIDDK-funded ChiLDReN consortium. This discovery cohort consisted of 21 subjects with definite cPHT and 19 without cPHT, according to the operational clinical definition discussed above (Fig. 1). All subjects had undergone hepatoportoenterostomy at the time of diagnosis and were predominantly female participants (n = 12 in the cPHT group and n = 16 without cPHT). Subjects had a median age of 8.5 years (IQR, 5.4-12.6) for the cPHT group and 11.7 years (IQR, 5.1-15.5) for the group without cPHT. The subjects with cPHT had higher serum aspartate aminotransferase (AST) and total bilirubin and a lower platelet count compared to the subjects without cPHT (Supporting Table S2).

We used multivariable logistic regression analyses of the relative abundance of proteins (expressed as pixel values) with the goal set *a priori* to select only proteins with an AUROC ≥ 0.90. This approach identified eight different models that best predicted subjects with cPHT (Table 1). Two proteins alone met this goal, with secreted frizzle protein 3 (SFRP3) having an AUROC of 0.93 (95% confidence interval [CI], 0.85-1.00) and a sensitivity of 90% and specificity of 84%, while semaphorin 6B (SEMA6B) had an AUROC of 0.90 (95% CI, 0.80-1.00) and a sensitivity of 90% and specificity of 84%. The other models consisted of a combination of two proteins (β2-microglobulin+insulin-like growth factor binding protein 2, SEMA6B+SFRP3, SFRP3+COMM domain containing 7 [COMMD7], SEMA6B+COMMD7, regenerating family member 4+natural killer P44, and vascular cell adhesion molecule 1 [VCAM1]+BMX nonreceptor tyrosine kinase [BMX]), all with an AUROC ≥ 0.90 and sensitivity ranging from 71% to 100% and specificity ranging from 84% to 95% (Table 1).

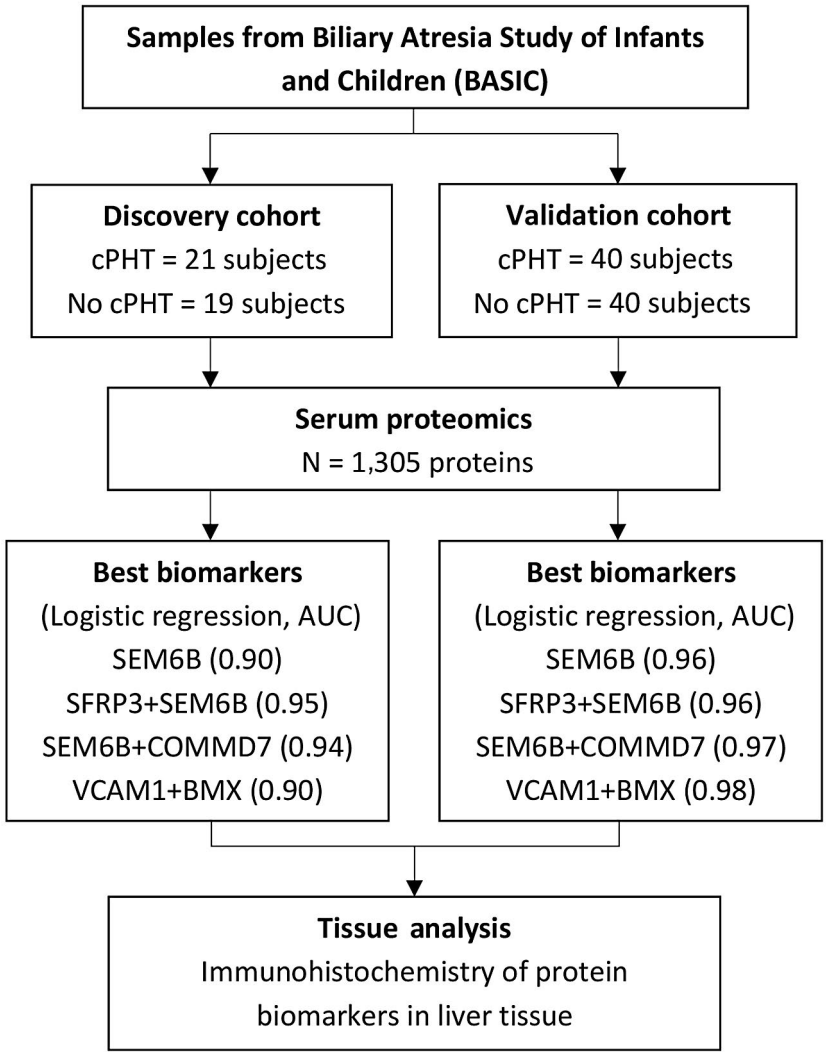

FIG. 1. Study design. Serum was obtained from subjects with BA who were clinically identified to have PHT or not to have PHT. Discovery and validation cohorts were formed. Serum proteins were measured by high-throughput proteomic assay (SOMAscan) separately for each cohort and analyzed to identify biomarkers that performed with an AUROC ≥ 0.90. Proteins identified as best biomarkers were then analyzed in liver tissue by immunohistochemistry staining. Abbreviation: AUC, area under the curve.

REPRODUCIBILITY OF SERUM BIOMARKERS IN A VALIDATION COHORT

To investigate the reproducibility of these findings, we performed a new SOMAscan assay in a validation cohort of 40 subjects each in definite cPHT and no cPHT groups for a total of 80 subjects (Fig. 1). The median age of the subjects was 2.5 years (IQR, 0.75-7.9) in the cPHT group and 5.3 years (IQR, 2.0-12.0) in the group without cPHT; both groups were predominantly girls (26/40 and 22/40, respectively). The cPHT group had higher AST, alanine aminotransferase, and total bilirubin and lower platelet count when compared to the subjects without cPHT (Supporting Table S3).

Applying the same statistical methods as the discovery cohort, we found five prediction models that met the highly stringent AUROC ≥ 0.90. One model was SEMA6B alone, which had an AUROC of 0.96 (95% CI, 0.92-1.00) with a sensitivity of 95% and specificity of 93%. Three models consisted of two proteins in combination (SEMA6B+SFRP3, SEMA6B+COMMD7, VCAM1+BMX), and the final model consisted of a combination of three different proteins with

TABLE 1. PREDICTION MODELS OF PROTEINS DIFFERENTIATING SUBJECTS WITH CPHT (N = 21) FROM THOSE WITH NO CPHT (N = 19) IN THE DISCOVERY COHORT

Biomarker Models	AUROC (95% CI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
SFRP3	0.93 (0.85-1.00)	90	84	84	90
SEMA6B	0.90 (0.80-1.00)	90	84	84	90
β2-microglobulin+IGFBP2	0.93 (0.86-1.00)	86	84	84	86
SEMA6B+SFRP3	0.95 (0.87-1.00)	90	95	95	91
SFRP3+COMMD7	0.97 (0.93-1.00)	90	95	95	91
SEMA6B+COMMD7	0.94 (0.87-1.00)	90	95	95	91
REG4+NKP44	0.97 (0.93-1.00)	100	89	90	100
VCAM1+BMX	0.90 (0.81-0.99)	71	95	93	77

Abbreviations: IGFBP2, insulin-like growth factor binding protein 2; NKP44, natural killer P44; NPV, negative-predictive value; PPV, positive-predictive value; REG4, regenerating family member 4.

TABLE 2. PREDICTION MODELS OF PROTEINS DIFFERENTIATING SUBJECTS WITH CPHT (N = 40) FROM THOSE WITH NO CPHT (N = 40) IN A VALIDATION COHORT

Biomarker Models	AUROC (95% CI)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
SEMA6B	0.96 (0.92-1.00)	95	93	93	95
SEMA6B+SFRP3	0.96 (0.92-1.00)	93	93	93	93
SEMA6B+COMMD7	0.97 (0.94-1.00)	93	95	95	93
VCAM1+BMX	0.98 (0.96-1.00)	95	93	93	95
VCAM1+galectin-4+angiotensinogen	0.99 (0.99-1.00)	98	95	95	98

Abbreviations: NPV, negative-predictive value; PPV, positive-predictive value.

VCAM1+galectin-4+angiotensinogen. All models had an AUROC ≥ 0.96 and sensitivity ranging from 93% to 98% and specificity ranging from 93% to 95% (Table 2).

SELECTION OF SERUM BIOMARKERS WITH DISCRIMINATIVE PROPERTIES IN BOTH DISCOVERY AND VALIDATION COHORTS

With the goal to identify biomarkers that meet the *a priori* set criteria of AUROC ≥ 0.90 in both cohorts, we combined the protein models and selected SEMA6B and three combinations of two proteins as follows: SEMA6B+SFRP3, SEMA6B+COMMD7, and VCAM1+BMX (Supporting Table S4). SEMA6B alone had an AUROC of 0.90 and 0.96 in the discovery and validation cohorts, respectively. The combination of SEMA6B+SFRP3 had AUROCs of 0.95 and 0.96, SEMA6B+COMMD7 had AUROCs of 0.94 and 0.97, and VCAM1+BMX had AUROCs of 0.90 and 0.98, each in the discovery and validation cohorts, respectively (Fig. 2A). The graphic presentation of SEMA6B alone and of the combination of two proteins as a single value for individual subjects showed the distinguishing features of the biomarkers between the group with and without cPHT (Fig. 2B; for a graphic presentation of each protein separately, see Supporting Fig. S1).

We further ranked the biomarker models based on sensitivity and specificity ≥90%. Two of the models met the criteria. SEMA6B+SFRP3 had a sensitivity for discovery and validation cohorts of 90% and 93%, respectively, and a specificity of 95% and 93%, respectively; SEMA6B+COMMD7 had a sensitivity of 90% and 93%, respectively, and a specificity of 95% and 95%, respectively (Supporting Table S5).

LIMITED DISCRIMINATORY VALUES FOR KNOWN BIOMARKERS OF PHT

Next, we examined the proteomic data platform to determine the AUROC for the following serum

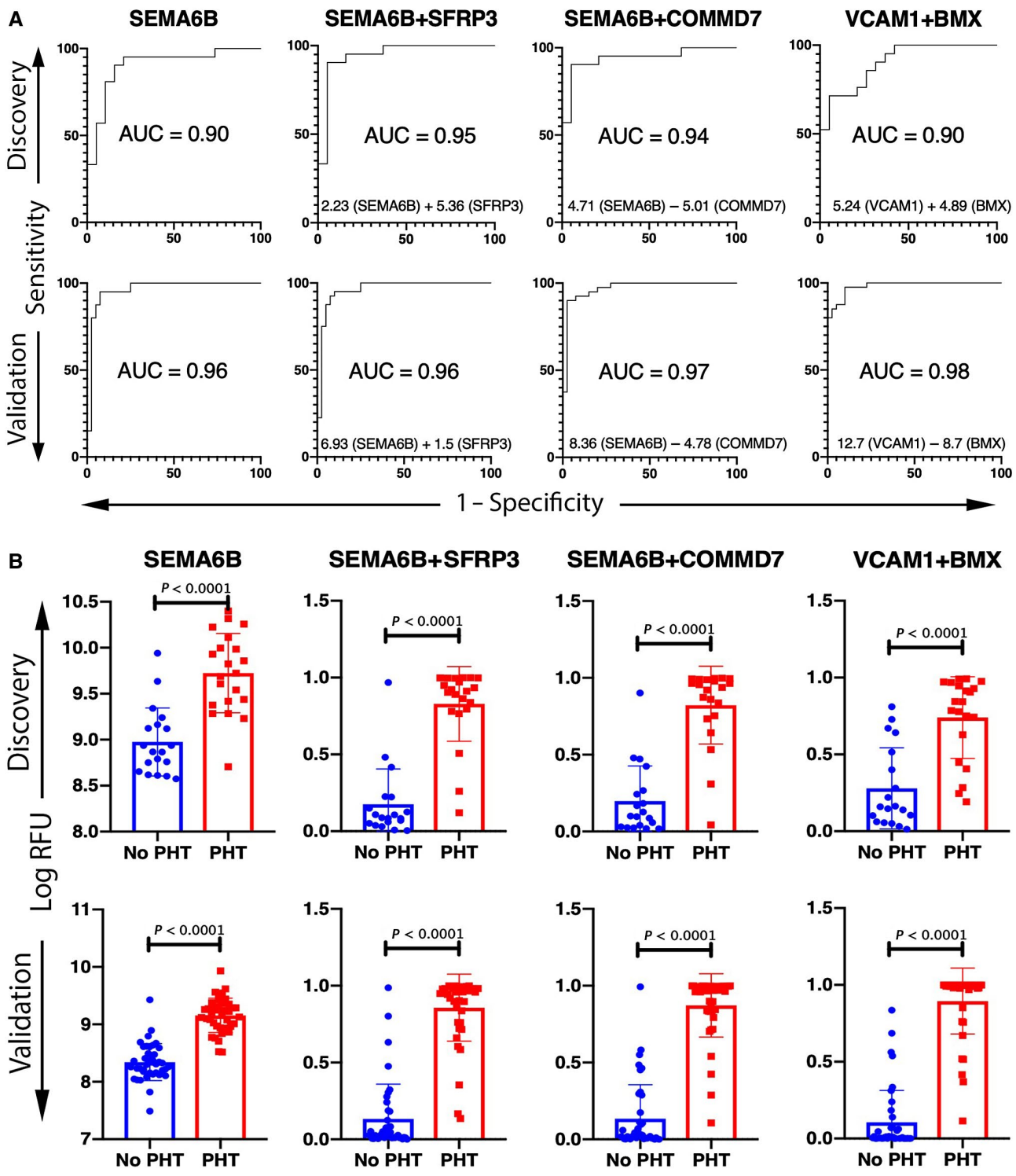

FIG. 2. Discriminatory features of serum biomarkers in children with cPHT and no cPHT. (A) AUROC values for serum biomarkers in a discovery cohort (top row) and validation cohort (lower row) distinguish cPHT from no cPHT in children with BA. (B) Dot plots of the same biomarkers and same subjects in the discovery and validation cohorts. Data are shown as the log of RFU; bars and whiskers represent the mean and SD. Abbreviation: RFU, relative fluorescent unit.

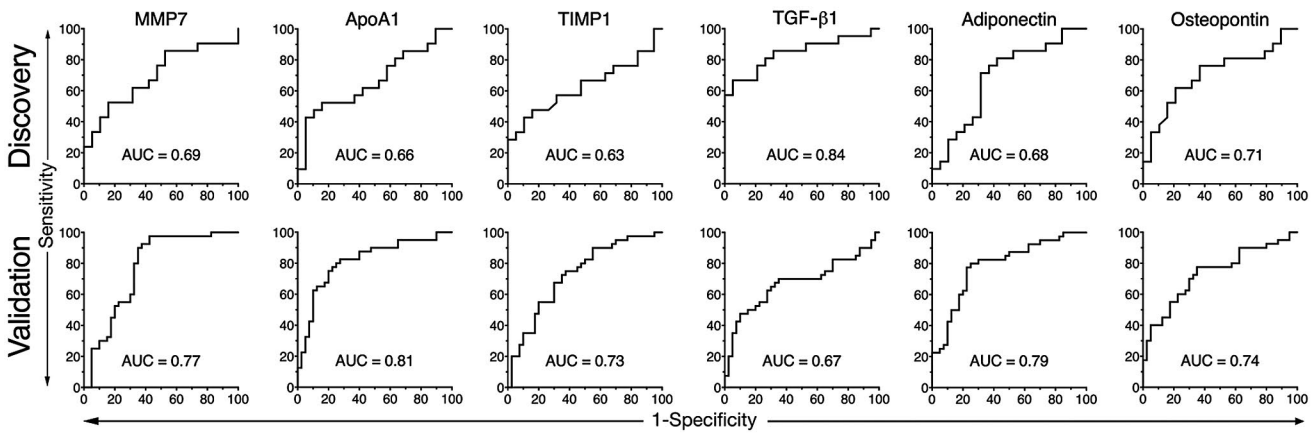

FIG. 3. AUROCs of previously reported fibrosis markers. AUROC values for proteins previously reported as fibrosis biomarkers in the discovery cohort (top panel) and validation cohort (lower panel) distinguishing subjects with and without cPHT in children with BA.

proteins previously reported as biomarkers of fibrosis or PHT in subjects with chronic liver disease: matrix metalloproteinase 7 (MMP7), tissue inhibitor of metalloproteinase 1 (TIMP1), transforming growth factor beta 1 (TGF-β1), apolipoprotein A1 (ApoA1), adiponectin, and osteopontin. Despite a satisfactory performance of these proteins in identifying cPHT, their AUROCs ranged from 0.63 to 0.84 in the discovery cohort and 0.67 to 0.81 in the validation cohort (Fig. 3; Supporting Fig. S2). Out of these previously described biomarkers, TGF-β1 had the best discriminatory performance in the discovery cohort with an AUROC of 0.84, while ApoA1 best distinguished cPHT in the validation cohort with an AUROC of 0.81. Osteopontin was the only protein that performed with an AUROC >0.70 in both the discovery and validation cohorts at 0.71 and 0.74, respectively.

LOCALIZATION OF PHT PROTEIN EXPRESSIONS IN LIVER TISSUE

As an initial step to understand the biological basis of these biomarkers in the serum and how individual proteins may relate to the pathogenesis of PHT, we performed immunostaining to define protein expression at the cellular level by using liver sections from an unused fragment of a donor normal liver transplant graft and explants from children with BA at the time of liver transplantation. In normal liver tissue, biliary epithelial cells had detectable expression of SEMA6B and BMX but minimal or no expression for SFRP3, COMMD3, and VCAM1. In BA sections, all five proteins had a strong expression in epithelial cells populating expanded bile duct profiles (Fig. 4). The increased signal of BMX seen in the portal tracts of BA sections was unexpected given the relative decreased serum concentration of BMX in patients with cPHT. In addition, BMX, SEMA6B, and SFRP3 were expressed in vascular endothelial cells in normal livers and in BA; BMX, SFRP3, COMMD7, and VCAM1 were also expressed in portal mononuclear cells in BA, and COMMD7 was also expressed in zone 1 hepatocytes in normal livers. This overall expression pattern was consistent with a substantial increase in the expression of SEMA6B, SRFP3, COMMD7, VCAM1, and BMX by biliary epithelial cells in expanded portal tracts of children with BA and cPHT at the time of liver transplantation.

Discussion

Using large-scale proteomics, we report the discovery and validation of protein biomarkers that accurately distinguish clinically evident PHT in children with BA with excellent performance based on an AUROC ≥ 0.90. From 1,305 proteins assayed simultaneously, the following five proteins had highly discriminative properties alone or in combination: SEMA6B performed well alone or in combination with SFRP3 or COMMD7, and VCAM1+BMX achieved AUROC ≥ 0.9 only in combination. When

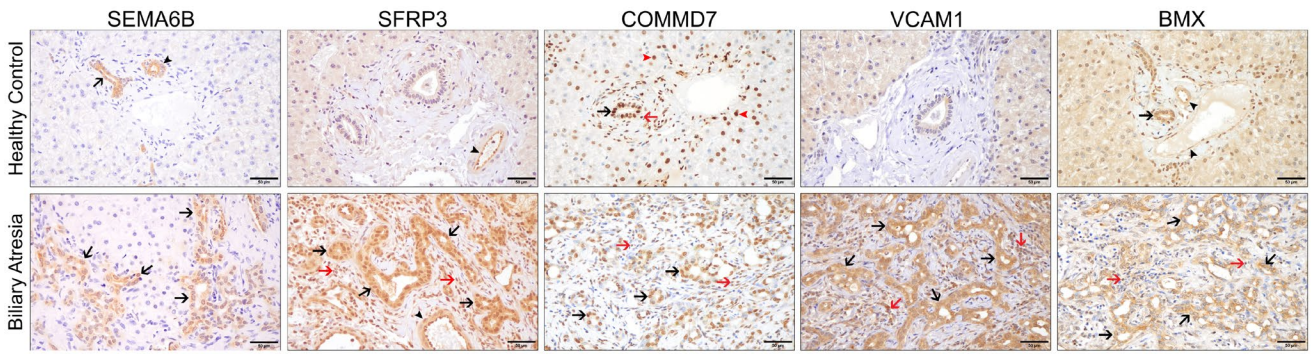

FIG. 4. Immunostaining of proteins that distinguished cPHT in children with BA. Representative immunostaining of proteins with discriminatory features of cPHT in children with BA. Top row represents liver sections from an unused fragment of a donor liver transplant graft; lower row sections are from explanted livers from children with BA and cPHT. Black arrows indicate biliary epithelial cells, black arrowheads designate endothelial cells, red arrows represent portal tract mononuclear cells, and red arrowheads highlight zone 1 hepatocytes. Magnification: ×400; scale bar: 50 μm.

the four models were further submitted to selection based on the additional requirement of having a sensitivity and specificity ≥90%, the combination of SEMA6B+SFRP3 and SEMA6B+COMMD7 performed best. Examining their expression in normal livers, the proteins had variable expression levels in different cell types, but all five proteins had higher expression in biliary epithelial cells in cirrhotic liver explants of subjects with cPHT secondary to BA.

The discriminative features of these protein biomarkers have superior performance when compared to described biomarkers of PHT. Serum MMP7 performs well in distinguishing BA from intrahepatic cholestasis, but its proposed correlation with liver fibrosis is not consistent among published reports[5-7]; our study showed that MMP7 has limited discriminative properties for cPHT with an AUROC of 0.69-0.77. Osteopontin, explored as a biomarker of fibrosis in a single cohort of patients with BA, was reported to predict esophageal varices with an AUROC of 0.93, a sensitivity of 73%, and a specificity of 96%,[8] but its performance to identify cPHT in our cohorts was limited with AUROCs of 0.71 and 0.74. Adiponectin also had low AUROCs of 0.68 and 0.79 in our cohorts, while a previous study reported its ability to predict the presence of esophageal varices with an AUROC of 0.65, 80% sensitivity, and 74% specificity in an adult population, with most patients having alcoholic or hepatitis C liver disease.[9] Other proteins, including laminin and hyaluronic acid, have been examined as PHT biomarkers but largely in adult populations and with inferior performance.[3] TIMP1 has been reported as a biomarker of liver fibrosis,[10] although with only a single pediatric study that measured TIMP1 in patients after liver transplant and distinguished only severe fibrosis.[11] Finally, TGF-β1 is well known to play a key role in hepatic fibrosis, but interestingly, TGF-β1 has been observed to be both increased[12] and decreased[13] in hepatic fibrosis, suggesting a complex physiologic interaction that has yet to be fully appreciated.

The pathophysiology of PHT is a result of a complex and dynamic process involving most hepatic cell types, chief among which sinusoidal endothelial cells and hepatic stellate cells contribute to endothelial dysfunction, hyperdynamic circulation, and the genesis of fibrosis.[14] Inflammation and immune activation have also been shown to be key mediators of this complex interaction.[15] The new protein biomarkers reported herein have not been directly linked to hepatic fibrosis but may indirectly influence molecular pathways controlling matrix production and/or removal. For example, SEMA6B belongs to the large family of semaphorin proteins, which have been reported to increase in liver fibrosis.[16,17] Similarly, SFRP3 is a member of the secreted frizzle protein family, which has been described to modulate the Wnt signaling pathway, which is indirectly linked to liver fibrosis.[18] The expression of SFRP3 also increases in aging endothelial cells.[19] VCAM1, a vascular cellular adhesion molecule and marker of endothelial activation,

angiogenesis,[20] and hyperdynamic circulation,[21] is overexpressed in chronic liver disease or cirrhosis.[22,23] Furthermore, VCAM1 has been demonstrated to correlate with increased HVPG in the adult population[23] and has been shown to be expressed by cholangiocytes and promote persistence of liver inflammation.[24] The two remaining proteins, COMMD7 and BMX, relate to inflammation through nuclear factor kappa B signaling (for COMMD7[25]) or the expression of tumor necrosis factor α, interleukin-1β, and toll-like receptor agonists (for BMX[26]).

While most of these novel proteins have not been directly linked to fibrosis, their expression by biliary epithelial cells, endothelial cells, and immune cells within the fibrotic tissue points to a potential role in the pathogenesis of PHT by matrix deposition or modulation of endothelial function. Their increased expression in biliary epithelial cells supports the theory that ductular reaction is not only associated with the severity of liver disease but also correlates with PHT, as recently suggested by Hamesch et al.[27] The increased expression of BMX was unexpected given the decrease in the serum concentration in cPHT. The precise mechanism of this divergence is yet to be determined and may relate to posttranslational processing that may influence its release to the blood. SEMA6B, SFRP3, and BMX all showed expression in hepatic endothelial cells, supporting their role in endothelial dysfunction. Additionally, the findings of SFRP3, COMMD7, VCAM1, and BMX expression in immune cells within the portal triads of the BA livers with cPHT may reflect an immune activation associated with PHT.

Our stringent strategy to only select proteins with AUROC ≥ 0.90 strengthens the evidence linking the proteins to cPHT. Despite this strength, future studies are needed to validate these discriminatory properties in children whose cPHT is corroborated by other direct or indirect methods of PHT or liver stiffness. In addition, studies are needed to investigate how SEMA6B, SFRP3, COMMD7, VCAM1, and BMX may directly contribute to the pathophysiology of PHT or if their release into the circulation reflects an unrelated mechanism, such as an increase in the splenic cellular mass that results from PHT. Although an optimal study design would classify PHT based on HVPG measurements, the lack of this measurement in clinical practice or in research cohorts (such as the ones included in our study) makes such a study not feasible. To overcome this experimental barrier, we used the well-described definition of cPHT as described above and the well-phenotyped cohort from the BASIC study.

In summary, the use of large-scale proteomics identified SEMA6B, SFRP3, COMMD7, VCAM1, and BMX as novel serum biomarkers of cPHT in children with BA. As biomarkers, they have excellent ability to distinguish cPHT with an AUROC ≥ 0.90 in discovery and validation cohorts. The expression of these proteins in biliary epithelial cells, hepatic endothelial cells, and immune cells within portal triads in BA livers with cPHT points to a potential role in the pathogenesis of PHT. Future studies to investigate their discriminatory performance at different phases of progression of hepatic fibrosis in BA and in cPHT secondary to other chronic liver diseases will provide insight into their value as noninvasive biomarkers of potential clinical value.

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