Bile proteome analysis by high-precision mass spectrometry to explore novel biomarkers of primary sclerosing cholangitis

（高精度質量分析を用いた胆汁プロテオーム解析による原発性硬化性胆管炎の新規バイオマーカー探索）

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Abstract:

Background: Primary sclerosing cholangitis (PSC) is a chronic inflammatory disease of unknown etiology that affects the intra- and extrahepatic bile ducts. The present study examined the utility of a bile proteome analysis using a high-sensitivity mass spectrometer to comprehensively screen for novel PSC biomarkers.

Methods: Bile endoscopically collected from patients with PSC, common bile duct stones, and biliary tract cancer were subjected to high-precision liquid chromatography/mass spectrometry. Some of the proteins specifically up-regulated in the bile of the PSC group were re-examined by an enzyme-linked immunosorbent assay.

Results: A total of 8,094 proteins were successfully identified and 332 were specifically up-regulated in the PSC group. The bioinformatics analysis showed that proteins involved in the proliferation and activation of diverse inflammatory cells were up-regulated in the PSC group. A receiver operating characteristic curve analysis showed good area under the curve values for interleukin-8 and annexin A1 (ANXA1) (0.836 and 0.914, respectively). Immunostaining for ANXA1 revealed its strong expression in inflammatory cells infiltrating the peripheral biliary tract in PSC livers.

Conclusion: A bile proteome analysis is a useful tool for elucidating the pathogenesis of PSC and developing new diagnostic approaches. Therefore, ANXA1 has potential as a

bile biomarker for PSC.

Abbreviations

ACN, acetonitrile; ANXA1, annexin A1; AUC, area under the curve; CBDs, common bile duct stones; CCa, biliary tract cancer; DIA, data independent acquisition; ELISA, enzyme-linked immunosorbent assay; ERCP, endoscopic retrograde cholangiopancreatography; FA, formic acid; FFPE, formalin-fixed paraffin-embedded; GO, gene ontology; IL8, interleukin-8; LC/MS, liquid chromatography-mass spectrometry; MRCP, magnetic resonance cholangiopancreatography; MS, mass spectrometry; PSC, primary sclerosing cholangitis; SDS, sodium dodecyl sulfate; SP3, single-pot solid phase-enhanced sample preparation.

Introduction

Primary sclerosing cholangitis (PSC) is a chronic inflammatory disease that is characterized by diffuse and multiple fibrotic stenoses of the intra- and extrahepatic bile ducts and leads to biliary stasis. Familial clustering and recurrence after liver transplantation from a blood donor indicate the involvement of genetic factors in the pathogenesis of PSC. The most extensive genome-wide association study analysis performed in 2017 successfully identified at least 23 disease susceptibility genes[1]. The trans-portal influx of pathogens into the liver (a leaky gut) and a reduction in the diversity (dysbiosis) of the intestinal flora may both be involved in the onset of PSC, which is strongly associated with inflammatory bowel disease (IBD).[2, 3]

PSC is typically diagnosed based on blood test results showing biliary stasis and characteristic cholangiograms, such as endoscopic retrograde cholangiopancreatography (ERCP) and magnetic resonance cholangiopancreatography (MRCP), after the exclusion of secondary sclerosing cholangitis. IBD and pathological findings (onion skin lesions) on liver biopsy are also helpful findings for diagnosing PSC. However, a wide range of diseases are responsible for bile stasis and bile duct stricture. Therefore, it is important to distinguish PSC from malignancy because PSC is a risk factor for biliary tract cancer (CCa). However, the sensitivity of a histological diagnosis of malignant bile duct stricture,

including CCa, is not necessarily sufficient.[4] Therefore, the development of biomarkers to differentiate between these conditions is an important and urgent issue.

Bile is a biological specimen that is present in the bile ducts, the seat of biliary disease. Cholangiography by ERCP and bile cytology are the standard tests performed to identify bile duct stricture, and bile may be simultaneously collected during these procedures. The utility of liquid biopsy using cell-free DNA in bile to diagnose CCa was recently reported.[5, 6] Collectively, these findings indicate that bile is a biological specimen that reflects the pathophysiology of biliary tract diseases. Liver biopsy is an invasive test with a risk of complications, such as bleeding. Therefore, the development of a novel diagnostic system using bile is warranted because limited histological information is obtained from liver biopsy samples.

Although recent proteome analyses of the bile of PSC patients identified a few hundred proteins, the analytical depth of bile proteins may not be sufficient. Furthermore, studies to validate bile as a biomarker for the diagnosis of PSC and bioinformatics analyses of its pathogenesis have been limited.[7] Therefore, we herein investigated whether a bile proteome analysis using a data independent acquisition (DIA) mass spectrometer (MS) enables a high analytical depth in comprehensive screening for novel PSC biomarkers.

Methods

Patients and bile samples

Subjects were patients with PSC, common bile duct stones (CBDs), and CCa who gave consent to obtain specimens at our hospital. Five bile samples from each group were used as the comprehensive analysis cohort, and thirteen samples from each group as the validation cohort. Bile samples were obtained at the time of ERCP. Bile was collected after catheter cannulation into the bile duct during ERCP, with 2.0–3.0 cc being used for cytology and the remainder (2.0–4.0 cc) being stored for later use. Bile was centrifuged for storage at 20,000×*g* for 10 min, separated into supernatant and precipitate, and stored in a freezer at −80°C until protein extraction.

Data acquired from medical records were analyzed. The Research Ethics Committees of the Graduate School of Medicine, Chiba University (approval number: 4,219) approved this study.

Diagnosis of PSC

PSC was diagnosed based on the following criteria: (i) PSC cholangiograms on ERCP or MRCP (band-like stricture, beaded appearance, pruned-tree appearance, and diverticulum-like outpouching); (ii) elevated biliary enzymes in blood tests indicating biliary stasis; and (iii) the exclusion of secondary sclerosing cholangitis, particularly

CCa.[8,9] The Mayo risk score was also used to assess the progression of PSC.[10]

Proteome analysis

Bioruptor II (CosmoBio) was used to sonicate 50 μL of bile dissolved in 200 μL of 100 mM Tris-HCl, pH 8.5, and 2% sodium dodecyl sulfate (SDS) for 5 min. Protein concentrations in dissolved bile were measured using a BCA protein assay kit (CAT# 23225, Thermo Fisher Scientific) and then adjusted to 1 μg/μL with 100 mM Tris-HCl (pH 8.5) containing 2% SDS.

(1) Protein digestion

Twenty micrograms of protein in bile was reduced and alkylated with 10 mM DTT at 50°C for 30 min and with 35 mM iodoacetamide at room temperature for 30 min while being protected from light. A single-pot solid phase-enhanced sample preparation (SP3) was used for cleaning up and digestion of alkylated protein samples.[11, 12] The SP3 method used two bead types (hydrophilic and hydrophobic Sera-Mag Speed-Beads; Cytiva, Marlborough, MA) combined at a 1:1 (v/v) ratio, rinsed with distilled water, and reconstituted in 500 mM Tris-HCl, pH 7.0 at 10 μg solids/μL. Twenty microliters of optimized beads was added to the treated sample followed by 99.5% ethyl alcohol to bring the final concentration to 75%. The supernatant was removed after stirring for 20 min,

and the pellet was washed with 80% ethyl alcohol and 100% acetonitrile (ACN). The bead pellet was resuspended in 100 μL of 100 mM Tris-HCl, pH 8.5 and 2% SDS, and a BCA protein assay was performed to measure protein concentrations. Regarding protein digestion, beads were resuspended in 50 μL of 50 mM Tris-HCl, pH 8.0 with 1 μg trypsin/Lys-C Mix (Promega, Madison, WI) and digested at 37℃ overnight. The digested sample was acidified with 20 μL of 5% trifluoroacetic acid and Bioruptor II (CosmoBio) was used to sonicate the sample at a high level for 5 min. GLTip SDB (GL Sciences Inc., Tokyo, Japan) was used for sample desalination according to the manufacturer's instructions, followed by drying with a centrifugal evaporator (miVac Duo concentrator, Genevac Ltd., Ipswich, UK). The desalted sample was redissolved in 3% ACN and 0.1% formic acid (FA) and then transferred to a hydrophilic-coated, low-adsorption vial (ProteoSave vial; AMR Inc., Tokyo, Japan). A Lunatic instrument (Unchained Labs, Pleasanton, CA, USA) was used to measure the concentration of peptides, which was adjusted to 250 ng/μL with 3% ACN and 0.1% FA.

(2) Proteome based on DIA-MS

Digested peptides were directly injected into a 75 μm-inner diameter × 25 cm capillary column (Aurora C18, particle size 1.6 μm, 120Å; IonOpticks, VIC, Australia) at 1.5 μL/min and 60℃ using the UltiMate 3000 RSLCnano system (Thermo Fisher Scientific),

and were then separated on a 120-min gradient at 200 nL/min. Quadrupole Orbitrap Exploris 480 hybrid MS (Thermo Fisher Scientific) was used to analyze the eluted peptides for DIA - MS. MS1 spectra were collected in the range of 495–745 m/z with 15,000 resolution to set an automatic gain control target of 3 × 106 ions and a maximum injection time of 55 ms. MS2 spectra were collected at >200 m/z with 45,000 resolution to set an automatic gain control target of 3 × 106 ions, a maximum injection time of "auto", and stepped normalized collision energies of 22, 26, and 30%. The MS2 isolation width was set to 4 m/z and mass range patterns of 500–740 m/z with overlapping 2-Da windows were used for window placement optimized by Skyline.[13]

(3) Protein identification and quantification

Scaffold DIA (Proteome Software, Inc., Portland, OR) with the human spectral library was used to search LC-MS/MS files. Spectral libraries were generated from the human protein sequence database (proteome ID UP000005640, reviewed, canonical, 20,381 entries) by Prosit.[14] Scaffold DIA search parameters were as follows: experimental data search enzyme, trypsin; maximum missed cleavage sites, 1; precursor mass tolerance, 10 ppm; fragment mass tolerance, 10 ppm; static modification; and cysteine carbamidomethylation. The protein identification threshold was set such that protein and peptide false discovery rates were both <1%. The EncyclopeDIA algorithm[15] in Scaffold

DIA was used to quantitate proteins and peptides.

(4) Bioinformatics analysis

The Enricher online tool (http://amp.pharm.mssm.edu/Enrichr/) was used to perform a functional gene ontology (GO) analysis.[16] Cluster proteins of interest were searched for from GO terms in the gene set library "GO_Biological_Process_2021" (terms, 6,036; gene coverage, 14,937) and those with $P < 0.05$ were selected. Functional network clusters based on biological processing terms were visualized using the ClueGO[17] Cytoscape v3.8.264 plug-in.[18]

Enzyme-linked immunosorbent assay (ELISA)

A sandwich ELISA was used to measure interleukin-8 (IL8) and annexin A1 (ANXA1) concentrations in the bile of the validation cohort according to the manufacturer's instructions (R&D systems, Minneapolis, US; ANXA1, Abcam, Cambridge, UK).

Immunohistochemistry

Liver tissue collected from PSC patients who underwent liver transplantation at our institution were subjected to a histological examination. Informed consent for the research use of specimens was obtained for all cases. Formalin-fixed paraffin-embedded (FFPE)

sections of the liver were examined by hematoxylin and eosin (H&E) staining and immunohistochemistry with an anti-ANXA1 antibody (Abcam, Cambridge, UK). Slides were evaluated by two pathologists (Y.K. and M.K.) blinded to patient information.

Statistical analysis

Results are shown as means ± SEM. Non-parametric tests (the Mann-Whitney U test or Kruskal-Wallis test) were used to perform statistical comparisons between three groups. The area under the curve (AUC) values for ANXA1 were measured in the ROC analysis. Perseus (version 1.6.15.0; available online: https://maxquant.net/perseus/) was used to perform statistical analyses in the proteome analysis.[19] Protein intensity values were log2 transformed and each protein was filtered by at least one group to include at least 70% valid values. The remaining missing protein values were imputed based on a normal distribution with the following parameters: width of 0.3 and downshift of 1.8. A one-way analysis of variance was performed on the imputed data set and p-values < 0.05 were selected as significant differences to identify differential proteins in the three groups (PSC, CBDs, and CCa). Significant protein intensity values were z-normalized followed by hierarchical clustering; PSC-specific cluster groups were extracted.

Results

Bile proteomes in patients with biliary diseases

The clinical backgrounds of the comprehensive analysis cohort consisting of PSC (n = 5), CBDs (n = 5), and CCa (n = 5) patients are shown in Table 1. Patients with PSC were significantly younger than those with CCa and CBD. C-reactive protein, hepatobiliary enzyme, jaundice, and tumor marker levels were significantly higher in CCa patients than in those with PSC and CBDs. These results were consistent with the pathophysiology of each disease. The mean Mayo risk score, a measure of PSC progression, was 4.4, indicating a relatively early stage of the disease. Bile samples in this cohort were subjected to a comprehensive proteomics analysis by LC-MS/MS. A total of 7,889 proteins were detected in at least one or more samples. The numbers of identified proteins in the PSC, CCa, and CBDs groups were 6,210, 7,299, and 6,858, respectively (Figure 1A). The three groups showed significantly different expression profiles for 905 proteins (Figure 1B). A subsequent clustering analysis successfully divided these proteins into the following four clusters: (i) stably up-regulated in the PSC group, (ii) stably up-regulated in the CCa group, (iii) stably up-regulated in the CBDs group, and (iv) stably down-regulated in the PSC group. We highlighted 332 proteins that were stably up-regulated in the PSC group. Among them, 147 up-regulated (≥2-fold increase) proteins

were subjected to a further bioinformatics analysis.

Functional analysis of proteins with up-regulated expression in the bile of PSC patients

To understand the functional network of a series of proteins that were significantly up-regulated in the PSC group, data were subjected to an enrichment analysis of GO biological processes by ClueGO and Enricher. Functional annotation based on ClueGO demonstrated the significant enrichment of genes involved in the regulation of various inflammatory cells, such as neutrophils (positive regulation of neutrophil degranulation [GO:0043315]), T cells (T cell proliferation [GO:0042098], regulation of T cell proliferation [GO:0042129]), B cells (positive regulation of B cell proliferation [GO:0030890]), and natural killer cells (natural killer cell-mediated cell injury [GO: 0042267]) (Figure 2A). Enricher showed the significant enrichment of genes involved in neutrophil function, such as neutrophil degranulation [GO:0043312], neutrophil activation involved in immune responses [GO:0002283], and neutrophil-mediated immunity [GO:0002446] (Figure 2B).

Differential expression of IL8 and ANXA1

We highlighted two proteins as diagnostic biomarkers for PSC among 147 proteins with up-regulated expression (≥2-fold increase) in the PSC group. One protein was IL8, the expression of which was previously reported to be up-regulated in the bile of PSC patients.[20] The other protein was ANXA1, which is common in GO terms such as neutrophil activation, lymphocyte proliferation, and inflammatory cytokine secretion. The expression of ANXA1 was shown to be up-regulated in the intestinal mucosa of ulcerative colitis, a common complication of PSC.[21] ELISA was subsequently performed on bile collected from patients with PSC (n=13), CCa (n=13), and CBD (n=13) in the comprehensive analysis cohort plus the validation cohort (8 patients per group) (Supplementary Figure 1A and Figure 3A). The clinical backgrounds of the clinical validation cohort are shown in Table 2. Similar to the comprehensive analysis cohort, significant differences were observed in age, C-reactive protein, hepatobiliary enzyme, jaundice, and tumor marker levels, and white cell blood counts between the CCa group and other groups. The validation cohort included patients with a slightly advanced stage of PSC because the mean Mayo risk score was higher in the validation cohort than in the comprehensive analysis cohort. ELISA using bile showed that IL8 concentrations were significantly higher in the PSC group than in the CBDs group ($p < 0.001$, Figure Supplementary Figure 1A), while ANXA1 concentrations were significantly higher in the

PSC group than in the CBDs and CCa groups (vs. CBDs; $p < 0.001$, vs. CCa; $p = 0.002$, Figure 3A). We then conducted ROC analyses to assess diagnostic performance for PSC. AUC values for IL8 and ANXA1 were 0.836 and 0.914, respectively (Supplementary Figure 1B and Figure 3B). IL8 had a sensitivity of 87.5% and specificity of 75.0% for the diagnosis of PSC when the optimal cut-off value was set at 3.9 ng/ml. Similarly, ANXA1 had a sensitivity of 87.5% and specificity of 87.5% when the optimal cut-off value was set at 7.7 ng/ml. Furthermore, a weak correlation was observed between IL8 and ANXA1 (Correlation coefficient 0.33, $p=0.04$, Supplementary Figure 1C).

Immunohistochemical analyses of ANXA1 in PSC

Immunostaining for ANXA1 was performed using FFPE specimens of PSC livers from two recipients to confirm the tissue expression of ANXA1 (Figure 4A-D). H&E staining showed the loss of lobular structures and fibrosis around the bile ducts (onion skin lesions), which is consistent with cirrhosis due to PSC. ANXA1 immunostaining showed its high expression in inflammatory cells infiltrating the peripheral bile ducts, in addition to its expression in bile duct cells, and vascular endothelial cells. Similar results were obtained for the two patients.

Discussion

In the present study, a comprehensive bile protein profile analysis of PSC, CCa, and CBDs using high-precision liquid chromatography-mass spectrometry (LC/MS) was performed to evaluate the utility of a bile proteome analysis in the search for new PSC biomarkers. We successfully identified 7,889 proteins in bile, which is a markedly higher number than previously reported. We also detected proteins that were specifically up-regulated in PSC by statistically analyzing their expression. The informatics analysis of the functions of these proteins revealed that proteins related to various inflammatory cell functions were enriched, particularly terms related to neutrophil function. This result suggests the presence of abnormal neutrophil function.

Proteome analyses of PSC bile have used different sample pretreatments and protein purification and analysis methods. Lankisch et al. compared the expression of peptides by CE-MS after the pretreatment of bile with a molecular weight cut-off filter and then identified peptide sequences using the data dependent acquisition (DDA) approach.[22] Navaneethan et al. and Rupp et al. performed protein digestion by in-gel digestion, followed by MS using the DDA approach.[23, 24] Since the DDA approach employed in these studies only analyzes proteins with high abundance, it is limited for the identification and reproducibility of proteins with low expression levels. On the other

hand, Holm et al. analyzed the proteome of serum and bile from patients with PSC using the DIA approach, similar to the present study.[25] However, only 301 proteins were identified. This finding may be attributed to samples being pretreated by the removal of proteins with high abundance, followed by acetone precipitation and urea dissolution. These processes may result in negative selection in the column, protein loss due to re-dissolution after acetone precipitation, and a decrease in digestion efficiency due to trypsin denaturation with urea. In the present study, a bioinformatics analysis of proteins up-regulated in the bile of PSC patients was performed to examine their functional relationship. However, this study listed terms such as Cell Morphology, Embryonic Development, and Hair and Skin Development and Function, but not those related to inflammation. In contrast to the method described above, the SP3 method does not result in protein loss or a decrease in digestion efficiency during sample pretreatment. Moreover, the DIA method used herein accurately quantifies proteins with low expression levels at high depth. This enables the identification of a large number of proteins. These approaches may reveal the relationship between the pathogenesis of PSC and various inflammatory cell functions, particularly neutrophil function.

Previous studies examined bile proteins as biomarkers in PSC using a proteome analysis with two-dimensional electrophoresis[26] and protein arrays[27] in addition to IL8, which was

also investigated in the present study. The findings obtained revealed elevated bile protein concentrations with the progression of PSC and their ratio to bile acid also increased. The S100A9 concentration ratio in serum to bile correlated with endoscopic activity and the Mayo risk score. A gene expression analysis of cytology specimens also showed that S100A9 expression was up-regulated in patients with more active disease. The latter study identified IL8 and S100A9 as well as S100A12, MMP-7, and tryptophan hydroxylase 2 as useful proteins for the diagnosis and severity stratification of PSC. S100A9, S100A12, and MMP-7 were also up-regulated in bile from PSC patients. These findings confirm the reproducibility and validity of the present analysis.

Based on these findings, we focused on ANAX1, one of the 147 proteins specifically up-regulated in PSCs. ANXA1 is a 37-kDa anti-inflammatory protein that is expressed in many cells, mainly neutrophils, but also in mature cells, such as monocytes and macrophages, lung epithelial cells, fibroblasts, and kidney mesangial cells.[28] Its anti-inflammatory effects are exerted by inhibiting the release of inflammatory mediators, such as prostaglandin E2 and leukotrienes, promoting tissue repair, and enhancing leukocyte migration.[29] ANXA1 also regulates neutrophil recruitment, apoptosis, and clearance. It is attracting increasing attention as a therapeutic agent for inflammatory diseases.[30] ANXA1 expression levels were elevated in the bile of PSC patients in the

present study. This result was attributed to the expression of ANXA1 being observed in inflammatory cells infiltrating the peripheral bile ducts by immunostaining and the secretion of ANXA1 by these cells being reflected in bile. ANXA1 functions as an anti-inflammatory protein, a countermeasure against inflammation around bile ducts induced by inflammatory proteins and cytokines that up-regulates the expression of ANXA1 in PSC, as shown in NASH model mice fed a methionine choline-deficient diet.[31] Although the role of ANXA1 in PSC remains unclear, its involvement in the maintenance and repair of intestinal mucosal homeostasis in IBD has been demonstrated. Da Paula-Silva et al. reported that ANXA1 contributed to responses to anti-TNF antibody therapy and may serve as a biomarker for treatment.[32] Babbin et al. showed the protective effects of ANXA1 in the intestinal mucosa of a mouse colitis model.[33, 34] Since PSC and IBD are consistently observed in some patients, ANXA1 may play an important role in the repair of the bile duct epithelium in patients with PSC.

Therefore, the potential of IL8 and ANXA1 as diagnostic PSC biomarkers was examined by ELISA using bile samples. IL8 and ANXA1 concentrations were elevated in patients with PSC. Furthermore, the AUC value for ANXA1 was higher than that for IL8 in the ROC analysis, suggesting the utility of ANXA1 as a new diagnostic biomarker for PSC. However, ANXA1 concentrations are not necessarily high in some patients with PSC.

Variations in ANXA1 concentrations may be attributed to heterogeneity in the pathogenesis of PSC. Another possibility is that inflammatory proteins other than ANXA1 contribute to the pathogenesis of PSC in some patients. Further studies using a larger number of patients are needed to clarify this issue.

There are several limitations that need to be addressed. This was a single-center retrospective analysis, and, thus, the results obtained need to be interpreted with caution due to the limited number of patients examined. Multicenter validation and larger cohorts are needed in the future. Furthermore, case selection may have affected the results obtained. Our preliminary LC/MS analyses demonstrated that the number of proteins identified in bile was lower in patients with than in those without biliary stenting (data not shown). This may be due to the reflux of intestinal fluid and bacterial biliary infection. In consideration of its potentially negative effects on protein identification by LC/MS, we excluded patients with biliary stenting from the present analysis. To assess the effects of active infection on protein identification, further analyses on bile collected from a larger number of patients with PSC and acute cholangitis are required. Another limitation is that liver biopsy was not performed because of its invasive nature; therefore, tissue activity and the degree of liver fibrosis were not evaluated. However, since disease activity may be assessed by the Mayo risk score and liver biopsy is not always required for a diagnosis,

a liver biopsy evaluation is not always necessary. Despite these limitations, this is the first study to perform a comprehensive bile PSC proteome analysis using high-sensitivity MS. In conclusion, a bile proteome analysis is a useful tool for elucidating the pathogenesis of PSC and developing new diagnostic and therapeutic approaches.

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Figure legends

Figure 1. Identification of proteins expressed in bile by a proteome analysis. (A) Distribution and flow chart of biliary proteins detected in patients with PSC, CBDs, and CCa. (B) Protein expression clustering analysis of the bile of patients with PSC, CBDs, and CCa.

Figure 2. Bioinformatics analysis of the bile proteome. An enrichment analysis of GO biological processes (GO-BP) by ClueGO (A) and Enricher (B). In Figure 2A, each circle is called a node and represents a GO term. Proteins involved in the proliferation and activity of various inflammatory cells were enriched. The highlighted node is the node containing ANXA1.

Figure 3. ELISA for ANXA1 in bile. (A) ANXA1 concentrations in bile were significantly higher in the PSC group than in the CBDs and CCa groups. ** $p < 0.01$, **** $p < 0.001$. (B) The ROC curve analysis confirmed the utility of ANXA1 expression in bile to discriminate between PSC, CBDs, and CCa. The AUC value was 0.94, sensitivity was 92.3%, and specificity was 88.5% at a cut-off value of 8.5 ng/ml.

Figure 4. Immunohistochemical analyses of ANXA1 in PSC livers. (A, B) H&E staining showed fibrosis around the bile ducts (onion skin lesions), which is consistent with cirrhosis due to PSC. (C) ANXA1 is expressed on bile duct cells, hepatocytes, vascular endothelial cells, and inflammatory cells infiltrating the bile ducts.

Supplementary Figure 1. ELISA for ANXA1 in bile. (A) IL8 concentrations in bile were significantly higher in the PSC group than in the CBDs groups. **** $p < 0.0001$. (B) The ROC curve analysis confirmed the utility of IL8 expression in bile to discriminate between PSC, CBDs, and CCa. The AUC value was 0.83, sensitivity was 91.7%, and specificity was 64.5% at a cut-off value of 3.9 ng/ml. (C) A weak correlation was observed between IL8 and ANXA1. The correlation coefficient was 0.33. $p=0.04$.

Figure 1

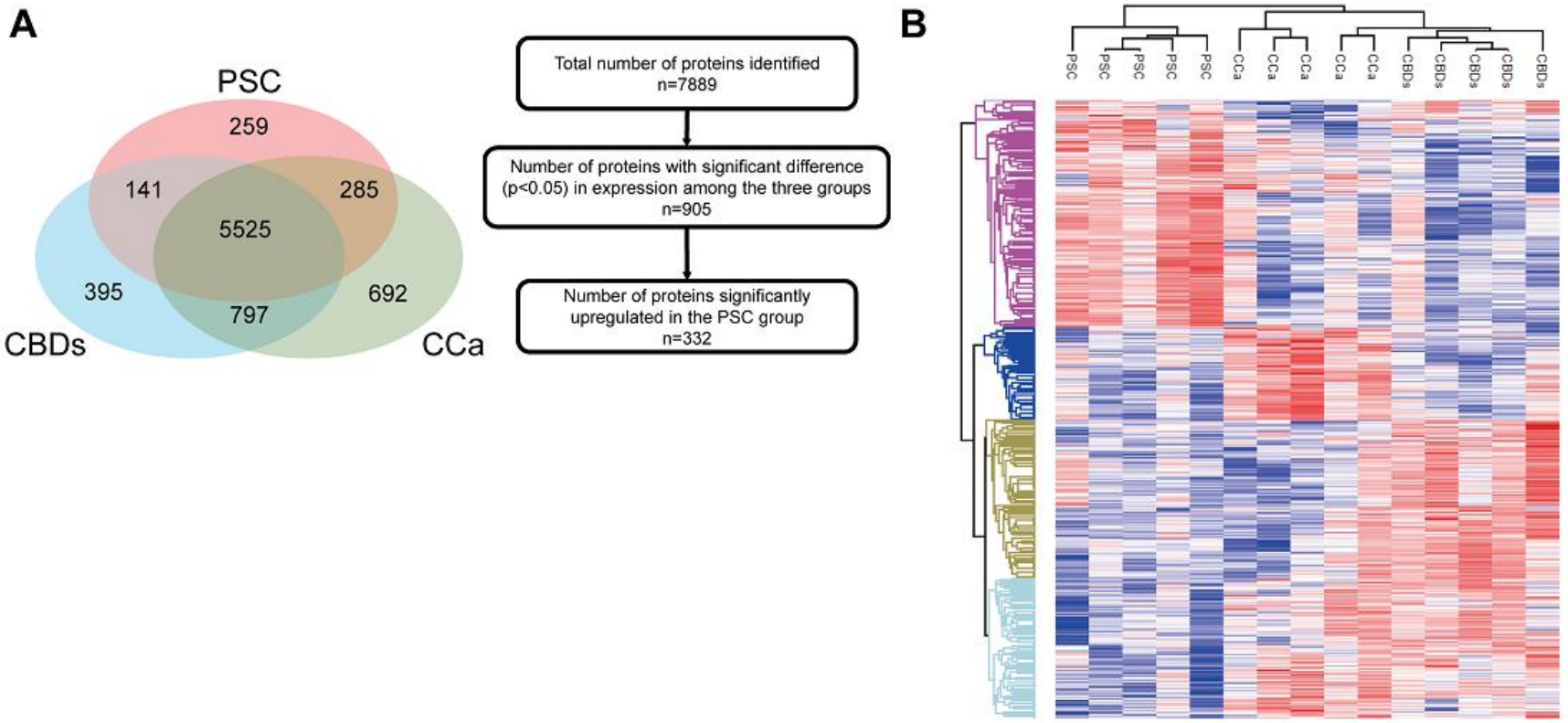

A
PSC
259
141
285
5525
395
797
692
CBDs
CCa
Total number of proteins identified
n=7889
Number of proteins with significant difference
(p<0.05) in expression among the three groups
n=905
Number of proteins significantly
upregulated in the PSC group
n=332
B
PSC
PSC
PSC
PSC
PSC
CCa
CCa
CCa
CCa
CCa
CBDs
CBDs
CBDs
CBDs
CBDs

Figure 2

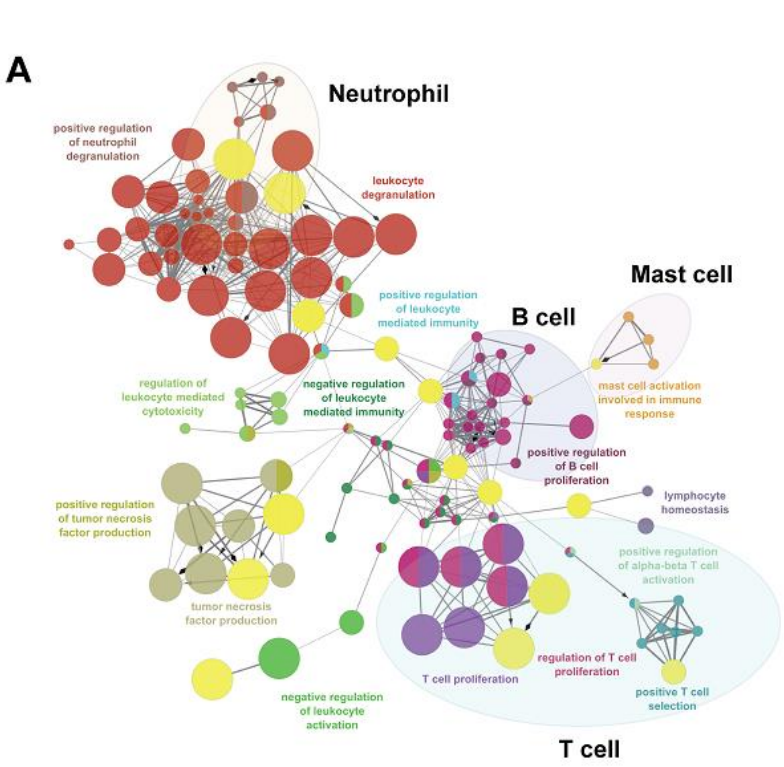

A
Neutrophil
positive regulation of neutrophil degranulation
leukocyte degranulation
Mast cell
positive regulation of leukocyte mediated immunity
B cell
regulation of leukocyte mediated cytotoxicity
negative regulation of leukocyte mediated immunity
mast cell activation involved in immune response
positive regulation of B cell proliferation
lymphocyte homeostasis
positive regulation of tumor necrosis factor production
positive regulation of alpha-beta T cell activation
tumor necrosis factor production
regulation of T cell proliferation
T cell proliferation
positive T cell selection
negative regulation of leukocyte activation
T cell
B
neutrophil degranulation (GO:0043312)
neutrophil activation involved in immune response (GO:0002283)
neutrophil mediated immunity (GO:0002446)
superoxide anion generation (GO:0042554)
vascular endothelial growth factor receptor signaling pathway (GO:0048010)
superoxide metabolic process (GO:0006801)
phagocytosis, engulfment (GO:0006911)
transmembrane receptor protein tyrosine kinase signaling pathway (GO:0007169)
cellular defense response (GO:0006968)
plasma membrane invagination (GO:0099024)

Figure 3

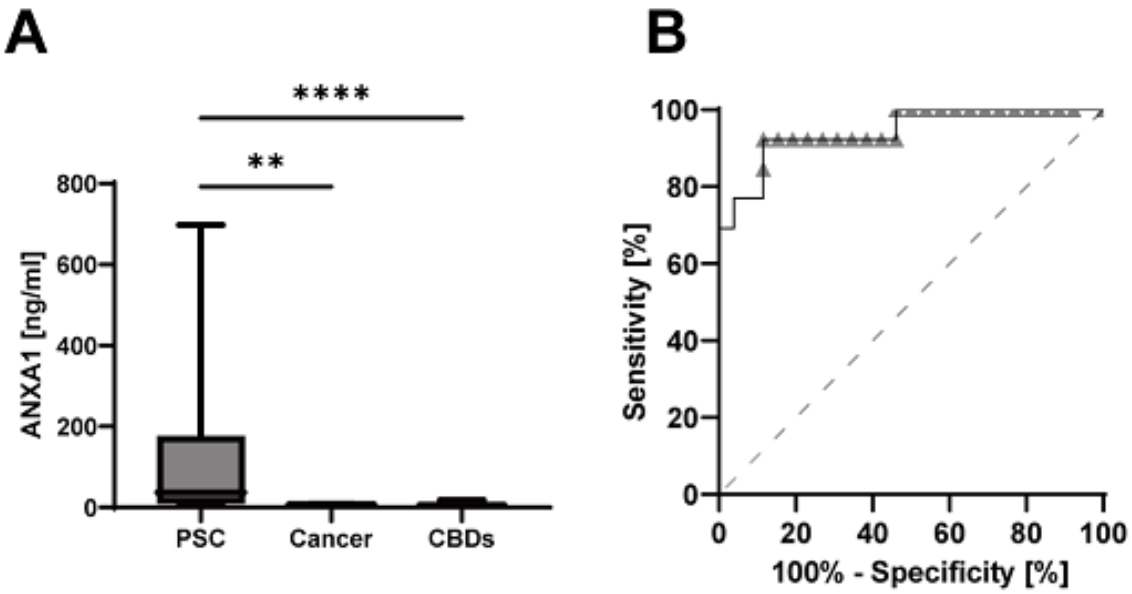
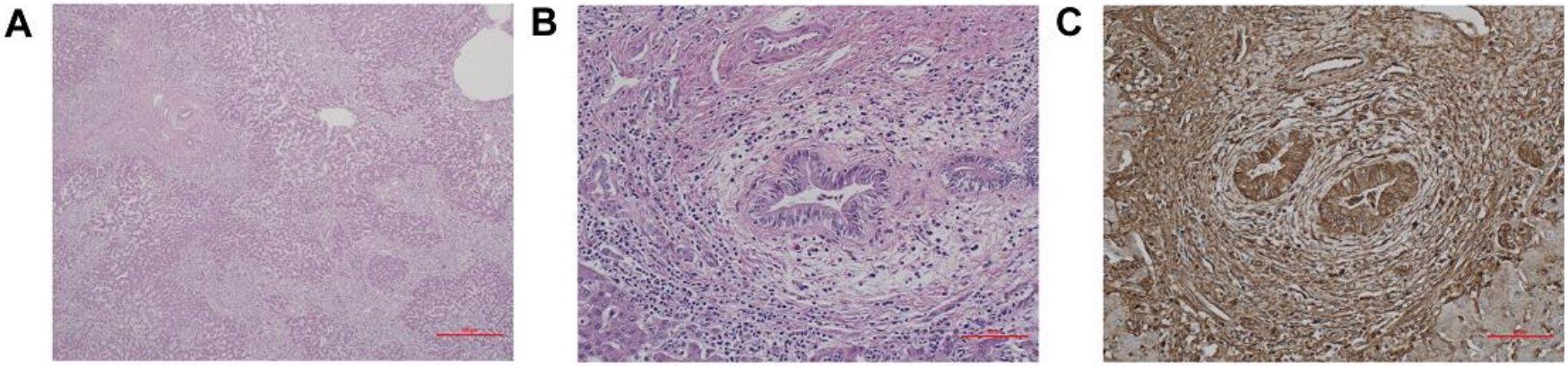

A
****
**
800
600
400
200
0
ANXA1 [ng/ml]
PSC
Cancer
CBDs
B
100
80
60
40
20
0
Sensitivity [%]
0
20
40
60
80
100
100% - Specificity [%]

Figure 4



Supplementary figure 1

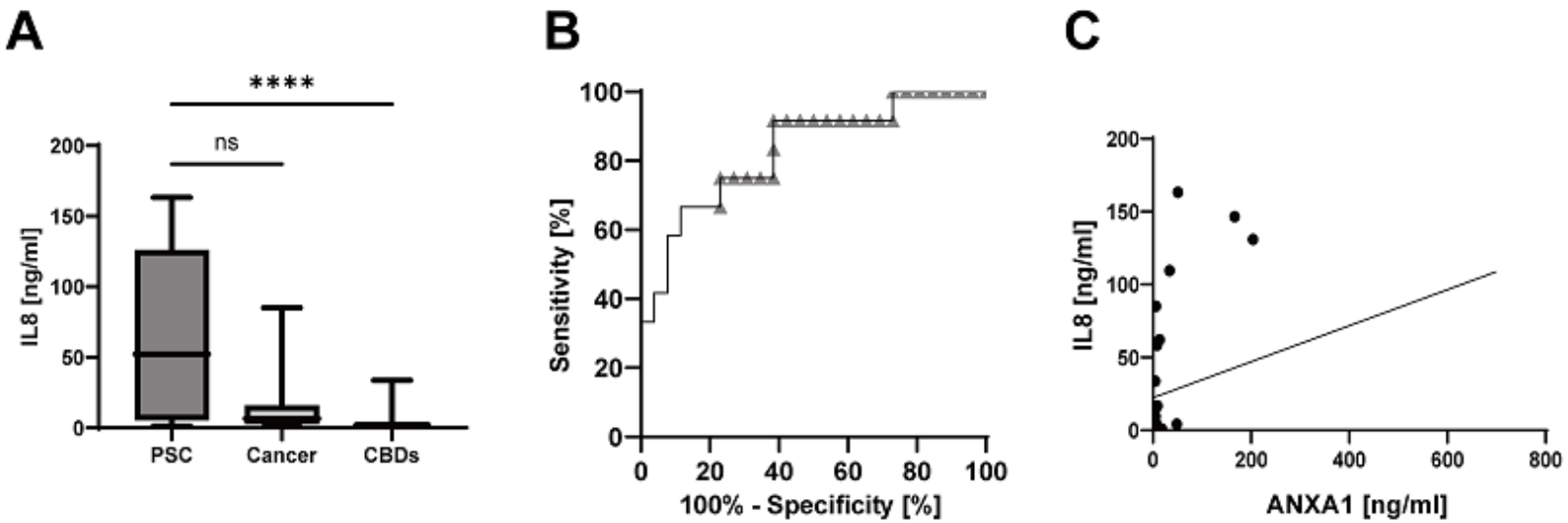


Table 1. Patient characteristics in the comprehensive analysis cohort

	PSC (n = 5)	CCa (n = 5)	CBD stones (n = 5)	P value
Age (years)				
Mean ± SD	33.6 ± 6.2	70.2 ± 14.9	77.0 ± 5.1	<0.05
Sex				
Male (%)	3 (60.0)	2 (40.0)	3 (60.0)	n.s.
WBC (/μl)				
Mean ± SD	5000 ± 1300	7900 ± 1900	6900 ± 2500	n.s.
CRP (mg/dl)				
Mean ± SD	0.34 ± 0.32	2.5 ± 2.6	0.06 ± 0.03	<0.05
AST (U/l)				
Mean ± SD	67 ± 37	170 ± 80	21 ± 6	<0.05
ALT (U/l)				
Mean ± SD	87 ± 41	200 ± 120	19 ± 7	<0.05
ALP (U/l)				
Mean ± SD	740 ± 700	1300 ± 1000	87 ± 16	<0.05
γ-GTP (U/l)				
Mean ± SD	290 ± 180	860 ± 560	22 ± 10	<0.05
T-Bil (mg/dl)				
Mean ± SD	2.6 ± 2.1	14.7 ± 13.1	2.4 ± 1.8	<0.05
CEA (ng/ml)				
Mean ± SD	1.9 ± 1.0	110 ± 240	6.3 ± 7.8	n.s.
CA19-9 (U/ml)				
Mean ± SD	31 ± 14	4700 ± 5700	31 ± 34	<0.05
Mayo risk score				
Mean ± SD	4.4 ± 1.7	N/A	N/A	N/A
UDCA				
Yes (%)	5 (100)	N/A	N/A	N/A

WBC, white blood cell; CRP, C-reactive protein; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; γ-GTP, γ-glutamyl transpeptidase; CEA, carcinoembryonic antigen; UDCA, ursodeoxycholic acid.

Table 2. Patient characteristics in the validation cohort

	PSC (n = 13)	CCa (n = 13)	CBD stone (n = 13)	P value
Age (years)				
Mean ± SD	48.7 ± 18.6	74.2 ± 11.0	72.7 ± 18.5	<0.05
Sex				
Male (%)	5 (38.5)	8 (61.5)	7 (53.8)	n.s.
WBC (/μl)				
Mean± SD	5400 ± 1100	7900 ± 2400	6800 ± 2400	<0.05
CRP (mg/dl)				
Mean ±SD	0.6 ± 0.8	1.7 ± 2.0	0.6 ± 1.6	<0.05
AST (U/l)				
Mean ± SD	72 ± 54	110 ± 81	44 ± 48	<0.05
ALT (U/l)				
Mean ± SD	82 ± 56	133 ± 104	76 ± 129	<0.05
ALP (U/l)				
Mean ± SD	720 ± 810	700 ± 810	140 ± 100	<0.05
γ-GTP (U/l)				
Mean ± SD	350 ± 390	670 ± 480	130 ± 150	<0.05
T-Bil (mg/dl)				
Mean ± SD	1.9 ± 1.7	11.4 ± 9.7	2.7 ± 3.4	<0.05
CEA (ng/ml)				
Mean ± SD	2.1 ± 1.3	46.3 ± 148.9	5.7 ± 4.9	n.s.
CA19-9 (U/ml)				
Mean ± SD	44± 36	2950 ± 4390	240 ± 610	<0.05
Mayo risk score				
Mean ± SD	5.1 ± 1.6	N/A	N/A	N/A
UDCA				
Yes (%)	11 (84.6)	N/A	N/A	N/A

WBC, white blood cell; CRP, C-reactive protein; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; γ-GTP, γ-glutamyl transpeptidase; CEA, carcinoembryonic antigen; UDCA, ursodeoxycholic acid.

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