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Bile proteomics for differentiation of malignant from benign biliary strictures: a pilot study

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Abstract

Background: Determining the etiology of biliary strictures is challenging, and the sensitivities of the current tests to diagnose them are low. Protein biomarkers in bile, in combination with other tests, may improve sensitivity in diagnosing biliary strictures.

Objective: To analyse the differential abundance of proteins in benign and malignant biliary strictures through proteomic analysis of bile.

Methods: In this prospective, cross-sectional study, bile was aspirated in 24 patients undergoing endoscopic retrograde cholangiopancreatography (ERCP) including six patients with primary sclerosing cholangitis (PSC), three with cholangiocarcinoma (CCA), ten with pancreatic cancer, and five with benign biliary conditions. Liquid chromatography/mass spectrometry was used to examine the bile for differential abundance of protein biomarkers. The relative abundance of various proteins was compared in the malignant vs. benign groups and in CCA vs. PSC.

Results: The majority of the proteins identified in bile were similar to those of the plasma (plasma proteins) and certain proteins were differentially expressed among the different groups (CCA, pancreatic cancer, PSC or benign). A total of 18 proteins were identified as being more abundant in the malignant group (CCA and pancreatic cancer) than in the benign strictures group, including myeloperoxidase, complement C3, inter-alpha-trypsin inhibitor heavy chain H4, apolipoprotein B-100, and kininogen-1 isoform 2. A total of 30 proteins were identified to be less abundant in the malignant group than in the benign group, including trefoil factor 2, superoxide dismutase [Cu-Zn], kallikrein-1, carboxypeptidase B and trefoil factor 1.

Conclusions: Protein biomarkers in bile may differentiate malignant from benign biliary strictures. Larger studies are warranted to validate these observations.

Key words: pancreatic cancer; proteomics; bile; cholangiocarcinoma

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Introduction

Biliary strictures can be due to benign or malignant causes. Cholangiocarcinoma (CCA) and pancreatic cancer account for the majority of malignant biliary strictures, and are often associated with a poor prognosis at the time of diagnosis [1, 2]. Detecting malignancies at an earlier stage is of paramount importance for effective management. Diagnosing malignant strictures at an early stage remains very challenging, especially when there is no evidence of a mass lesion (undetermined biliary stricture).

Endoscopic retrograde cholangiopancreatography (ERCP) with brush cytology and endoscopic ultrasound (EUS) with fine-needle aspiration (FNA) are the usual modalities for diagnosing biliary strictures. EUS-FNA is highly sensitive and superior to ERCP cytology in diagnosing malignant biliary strictures associated with pancreatic mass lesions, yet its sensitivity is similar to brush cytology for diagnosing undetermined strictures in the setting of CCA [3]. Also, the sensitivities of diagnosing malignant lesions through ERCP brushings in the published studies is low [4–6]. Although the addition of Fluorescence in situ hybridization (FISH) to cytology improves the sensitivity rates of ERCP brushings [7–9], the combined sensitivity is still low in detecting malignant strictures. Lower diagnostic sensitivities of the current techniques to detect malignant strictures warrant additional methodologies to improve the diagnosis.

Use of proteomics to detect biomarkers in bile may hold promise in aiding differentiation of malignant from benign biliary strictures [10]. Previous studies have attempted to study bile proteomics to identify peptides that will differentiate malignant from benign biliary strictures [11–15]. Most of the studies are limited because of lack of co-existing clinical information and a small sample size. The main aim of our study was to analyse the differential abundance of proteins in benign vs. malignant biliary strictures.

Methods

Patients

The Cleveland Clinic biliary fluid database is a prospectively maintained database of bile obtained by direct aspiration from the common bile duct in patients referred to our center for ERCP. We established this database in 2012 and included all patients in our center who had bile aspirated prior to contrast injection at the time of ERCP. The study was approved by the Cleveland Clinic Institutional Review Board and registered with the National Institute of Health (NIH) clinical trials registry. (NCT01565460) The patients included in our study were recruited between September 2012 and November 2012 and had a minimum of 1 year of clinical follow-up. Informed consent was obtained from each patient included in the study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008) as reflected in a priori approval by the institution’s human research committee.

Inclusion and exclusion criteria

The inclusion criteria were ability to give informed consent and age >18 years. Patients who had acute cholangitis were not included in our biliary fluid database. The diagnosis of pancreatic cancer and CCA was based on tissue diagnosis, either at surgery or on fine needle aspiration on subsequent endoscopic ultrasound on follow-up. Tissue diagnosis was established, based on histology in all patients with cancer in our cohort.

Biliary fluid sampling procedure

At the time of ERCP, once we had cannulated the common bile duct, approximately 1 to 5 mL of bile was aspirated through the sphincterotome. We transported these bile samples to the laboratory on ice; they were then frozen at −80°C until use.

Measurement of protein/peptides in bile

Bile samples were fractionated on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. Each sample was divided into three bands and analysed by liquid chromatograph mass spectrometer (LC-MS) or MS. To determine the protein content, dilutions were made to several samples in an attempt to try and equalize the overall amount of protein present in the SDS-PAGE gel. Several gels were run and the bands were cut from each gel. The protein bands were digested according to an in-gel digestion procedure. Briefly, the bands were cut from the gel and washed in 50% ethanol/5% acetic acid, alkylated with iodoacetaamide and reduced with Dithiothreitol (DTT). All bands were completely digested ‘in-gel’ using trypsin, by adding 5 μL trypsin (10 ng/μL) in 50 mmol/L ammonium bicarbonate and incubating overnight at room temperature. The peptides that were formed were extracted from the polyacrylamide in two aliquots of 30 μL 50% acetonitrile with 5% formic acid. These extracts were combined and evaporated to <10 μL in Speedvac (Thermo Fischer Scientific, San Jose, CA, USA) and then suspended in 1% acetic acid to make up a final volume of 30 μL for LC-MS analysis.

The LC-MS system was a Finnigan LTQ-Orbitrap Elite hybrid mass spectrometer system. The HPLC column was a Dionex 15 cm × 75 μm. Acclaim Pepmap C18, 2 μm, 100 Å reversed phase capillary chromatography column. The extract was injected in 5 μL volumes and the peptides, eluted from the column by an acetonitrile/0.1% formic acid gradient at a flow rate of 0.25 μL/min, were introduced into the source of the mass spectrometer on-line. The microelectrospray ion source was operated at 2.5 kV. The digest was analysed using the data-dependent multitask capability of the instrument, acquiring full scan mass spectra, to determine peptide molecular weights and product ion spectra to determine amino acid sequence in successive instrument scans.

Database searching

Tandem mass spectra were extracted by Proteome Discoverer software, version 1.4.1.288. Charge state de-convolution and de-isotoping were not performed. All LC-MS/MS samples were analysed using Mascot (Matrix Science, London, UK; version 2.3.02), Sequest (Thermo Fisher Scientific, San Jose, CA, USA; version 1.4.0.288) and X! Tandem (The CPM, thegpm.org; version CYCLONE (2010.12.01.1)). Mascot, Sequest, and X! Tandem were set up to search the Human Reference Sequence database (33292 entries) assuming the digestion enzyme trypsin, fragment ion mass tolerance of 1.2 Da and a parent ion tolerance of 20 PPM. Carbamidomethyl of cysteine was specified as a fixed modification and oxidation of methionine was specified as a variable modifications.

Criteria for protein identification

Scaffold software (version Scaffold_4.3.2; Proteome Software Inc., Portland, Oregon, USA) was used to validate LC-MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than...
95.0% probability by the Peptide Prophet algorithm [16]. Protein identifications were accepted if they could be established at greater than 99.0% probability to achieve a false discovery rate (FDR) of less than 1.0% and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [17]. Proteins that contained similar peptides and could not be differentiated based on LC-MS/MS analysis alone were grouped to satisfy the principles of parsimony.

**Comparison of malignant and benign biliary strictures**

The relative abundance of the proteins was compared by using the label-free quantitative method, spectral counting [18]. This method utilizes a data-dependent analysis, which involves an initial mass scan followed by 20 LC-MS/MS scans on the most abundant peptides. The selection of peptide ions for LC-MS/MS analysis was based on the abundance of the peptides and, therefore, the more abundant the protein in the sample the more often peptides from this protein were selected for LC-MS/MS analysis. The relative quantity of these proteins was determined by comparing the number of spectra (termed ‘spectral counts’), used to identify each protein. The numerical values used in the quantification correspond to the normalized spectral abundance factor (NSAF; SC/SC’protein length) [19]. The error observed for the SC measurements is greater for less-abundant proteins than for more-abundant proteins. Because of this, different filtering criteria were used to determine whether proteins are differentially present, based on the overall abundance, as an NSAF ratio.

The schematic representation of the proteomics approach to identifying proteins in each bile sample is shown in Figure 1. The error rating of the label-free method used in this analysis is greater for less-abundant proteins and we therefore utilized different filtering criteria for proteins of varying abundance levels (Table 1) [20]. The relative abundances of the proteins in these samples was determined by spectral counts and different cut-off values and P-values. Based on the criteria, the relative abundances of 304, 425, and 459 proteins were compared in the CCA vs. PSC, and malignant (pancreatic cancer and CCA) vs. benign (PSC and benign samples) respectively as set out below.

**Results**

A total of 24 bile samples were analysed, including six patients with PSC, three with CCA, ten with pancreatic cancer, and five with benign biliary conditions. The patients were recruited from...
September 2012 to November 2012. Among the five patients with benign biliary conditions, three had sphincter stenosis, one had chronic pancreatitis, and one had benign stricture secondary to biliary stones. All the six patients with PSC had both intrahepatic and extrahepatic PSC. None of the patients had any dominant strictures. Of the three patients with CCA, one had hilar strictures, while the remaining two had distal biliary stricture. All three presented with indeterminate biliary strictures. All patients had a minimum follow-up of at least 1 year up to November 2013. The basic demographic information is summarized in Table 2.

Each of the 24 bile samples was subjected to SDS-PAGE fractionation, tryptic digestion and LC-MS/MS analysis for both protein identification and relative protein quantification. Each gel lane was cut into three areas and analysed by a 120-minute LC gradient. The LC-MS/MS experiments identified a total of 459 proteins by at least two peptides. The overall FDR for this analysis was less than 1%.

### Malignant vs. benign strictures

A total of 459 proteins were quantified in the malignant and benign groups. The identification of proteins of different abundance was based on the criteria given in Table 1. Eighteen proteins (4%) were identified as more abundant in the cancer samples, including such proteins as MPO, complement C3, inter-alpha-trypsin inhibitor heavy chain H4, apolipoprotein B-100, and kininogen-1 isoform 2. Thirty proteins (7%) were identified as less abundant in cancer and included trefoil factor 2, superoxide dismutase [Cu-Zn], kallikrein-1, carboxypeptidase B and trefoil factor 1. The details were summarized in Tables 3 and 4.

### PSC vs. CCA

A total of 304 proteins were quantified in the CCA and PSC groups. Fourteen proteins (6%) were identified as more abundant in cholangiocarcinoma, including such proteins as intercellular adhesion molecule 1, ceruloplasmin, haptoglobin, complement c3, and -c4b. Nine proteins (3%) were identified as less abundant in cholangiocarcinoma, which included pro-activator polyopeptide isoform a pro-protein, carboxypeptidase B, chymotrypsin-like elastase family member 2A, chymotrypsin like elastase family member 3B, and chymotrypsin-C. The details are summarized in Tables 5 and 6.

### Discussion

Biliary tract and pancreatic malignancies are often diagnosed at advanced stages, with palliative measures being the major option for management of many cases. If diagnosed early, surgical resection or liver transplantation can offer increased survival. The sensitivities of various modalities for diagnosing malignancies in biliary strictures are currently low. We aimed to differentiate benign from malignant biliary strictures by analysing the bile for proteins using LC-MS/MS. We observed that the majority of the proteins identified in bile were similar to those of the plasma (plasma proteins) and certain proteins were differentially expressed among the different groups (CCA, pancreatic cancer, PSC, or benign). Some of those proteins, such as S100A8 and S100A9, which were significantly elevated, and TFF-2, which was significantly decreased in malignant strictures in our study, were found to be associated with tumorigenesis.

Serum CA 19-9 has been used as a routine diagnostic and prognostic tool for pancreatic cancer [21, 22]. Detecting malignancies at an earlier stage through lipidomics and proteomics seems promising, and these techniques are in the preliminary stages of being translated into clinical practice. We earlier reported the value of VEGF-1, oxidized phospholipids and volatile organic compounds in malignant biliary strictures and the results appear promising [23–25].

Tissue proteins are altered during the process of carcinogenesis, and this can occur during the earliest stages of malignancy [26]; these proteins can be detected in the extracellular fluid spaces. Detecting these changes in protein/peptide levels in the ECF can increase the diagnostic sensitivity, especially for early lesions. Since bile is in direct contact with biliary tract epithelial cells, malignant transformation of these cells is likely to affect expression of certain proteins in bile. Bile can be a representative of the products of molecular changes in biliary tract epithelia (novel proteins) as it is in direct contact with them. In an earlier study, proteomic analysis of bile fluid revealed that a larger percentage of proteins were cellular, from surrounding organs/tissues [10]. Many proteins associated with cancer, such as S100A9, MUC 1, NGAL, CEACAM6 and several other biomarkers [15, 27, 28] have been identified in bile in patients with malignant biliary strictures, further suggesting the importance of bile in proteomic

### Table 2. Demographic and clinical characteristics of study cohort

| Variables                  | Benign biliary conditions (n = 5) | Cholangiocarcinoma (n = 3) | Pancreatic cancer (n = 10) | Primary sclerosing cholangitis (n = 6) |
|----------------------------|----------------------------------|---------------------------|---------------------------|---------------------------------------|
| Age, mean (SD); years      | 44.0 (13.7)                      | 78.0 (7.1)                | 72.1 (8.2)                | 59.8 (15.0)                           |
| Female/male (n)            | 5/0                              | 1/2                       | 3/7                       | 2/4                                   |
| Albumin, mean (SD); mg/dL  | 3.3 (1.9)                        | 3.4 (0.7)                 | 3.3 (0.7)                 | 2.6 (2.0)                             |
| Bilirubin, mean (SD); mg/dL| 0.42 (0.41)                      | 1.3 (0.9)                 | 5.9 (6.4)                 | 1.1 (1.2)                             |
| AKP, mean (SD); U/L        | 66.6 (37.0)                      | 161 (69.1)                | 505.2 (296.7)             | 224.3 (214.4)                        |
| AST, mean (SD); U/L        | 26.8 (16.8)                      | 44.7 (23.1)               | 94.1 (63.4)               | 42.3 (39.7)                           |
| ALT, mean (SD); U/L        | 28.2 (16.8)                      | 33.0 (4.9)                | 136.7 (13.1)              | 49.2 (45.8)                           |
| CA 19-9, mean (SD)         | NA                               | 85.8 (61.7)               | 2591.7 (1191.2)           | 958 (817.3)                           |

AKP = alkaline phosphatase; AST = aspartate aminotransferase; ALT = alanine aminotransferase; NA = not available
### Table 4. Proteins identified as less abundant in cancer (malignant strictures) than in controls (primary sclerosing cholangitis and benign strictures)

| Proteins                                      | Accession  | MW (kDa) | Average SC Cancer | Average SC Control | NSAF ratio Cancer/Control | P-value (t-test) |
|-----------------------------------------------|------------|----------|-------------------|--------------------|---------------------------|------------------|
| Malate dehydrogenase, mitochondrial precursor | 21735621   | 36       | 0.0               | 3.5                | 0.0                       | 0.000897         |
| Prostate stem cell antigen pre-pro-protein    | 289547757  | 12       | 0.0               | 3.6                | 0.0                       | 0.00926          |
| Enteropeptidase precursor                     | 223942069  | 113      | 0.0               | 2.9                | 0.0                       | 0.00964          |
| Kallikrein-1 pre-pro-protein                  | 4504875    | 95       | 63.3              | 9.3                | 0.0                       | 0.00031          |
| Chymotrypsinogen B2 precursor                 | 118493350  | 29       | 17.6              | 1.1                | 0.0                       | 0.00133          |
| Ceruloplasmin precursor                       | 4557485    | 122      | 47.3              | 5.6                | 0.0                       | 0.00114          |
| Predicted: complement C4-A isoform 1          | 341916194  | 193      | 32.1              | 4.5                | 0.0                       | 0.00791          |
| Alpha-1B-glycoprotein precursor               | 21071030   | 54       | 10.9              | 1.2                | 0.0                       | 0.00458          |
| Inter-alpha-trypsin inhibitor heavy chain H4 isoform 1 precursor | 31542984  | 103      | 5.5               | 0.2                | 0.0                       | 0.00484          |
| Apolipoprotein B-100 precursor                | 105990532  | 516      | 49.3              | 1.0                | 0.0                       | 0.02595          |

MW = molecular weight; SC = spectral count; NSAF = normalized spectral abundance factor

### Table 3. Proteins identified as more abundant in cancer (malignant strictures) than in controls (primary sclerosing cholangitis and benign strictures)

| Proteins                                      | Accession  | MW (kDa) | Average SC Cancer | Average SC Control | NSAF ratio Cancer/Control | P-value (t-test) |
|-----------------------------------------------|------------|----------|-------------------|--------------------|---------------------------|------------------|
| Fibrinogen beta chain isoform 1 pre-pro-protein | 70906435   | 56       | 138.8             | 52.1               | 2.2                       | 0.01481         |
| C4b-binding protein alpha chain precursor      | 4502503    | 67       | 37.3              | 11.5               | 3.3                       | 0.04311         |
| Serum albumin pre-pro-protein                  | 4502027    | 69       | 986.0             | 332.8              | 2.4                       | 0.00006         |
| Vitamin D-binding protein isoform 1 precursor  | 32483410   | 53       | 51.9              | 19.2               | 2.4                       | 0.03919         |
| Kininogen-1 isoform 2 precursor               | 4504893    | 48       | 21.3              | 5.5                | 3.8                       | 0.01109         |
| Alpha-2-macroglobulin precursor                | 66932947   | 163      | 127.9             | 29.3               | 3.9                       | 0.00618         |
| Haptoglobin isoform 1-pre-protein              | 4826762    | 45       | 83.8              | 18.0               | 4.2                       | 0.00024         |
| Fibrinogen gamma chain isoform gamma-B precursor | 70906439  | 52       | 89.3              | 17.3               | 4.3                       | 0.00133         |
| Complement C3 precursor                        | 115298678  | 187      | 174.7             | 35.6               | 4.3                       | 0.00612         |
| Myeloperoxidase precursor                     | 4557759    | 84       | 70.2              | 11.0               | 5.3                       | 0.02501         |
| Apolipoprotein A-1 pre-pro-protein             | 4557321    | 31       | 29.8              | 5.9                | 5.7                       | 0.02170         |
| Fibrinogen alpha chain isoform alpha-E pre-pro-protein | 4503689 | 95       | 63.3              | 9.3                | 6.4                       | 0.00031         |
| Chymotrypsin gamma chain isoform gamma-B precursor | 4501647  | 16       | 13.9              | 3.7                | 3.9                       | 0.00511         |
| Ceruloplasmin precursor                        | 4557485    | 122      | 47.3              | 5.6                | 7.5                       | 0.00114         |
| Predicted: complement C4-A isoform 1           | 341916194  | 193      | 32.1              | 4.5                | 7.7                       | 0.00179         |
| Alpha-1B-glycoprotein precursor                | 21071030   | 54       | 10.9              | 1.2                | 8.3                       | 0.00468         |
| Inter-alpha-trypsin inhibitor heavy chain H4 isoform 1 precursor | 31542984  | 103      | 5.5               | 0.2                | 35.4                       | 0.00484         |
| Apolipoprotein B-100 precursor                 | 105990532  | 516      | 49.3              | 1.0                | 55.7                       | 0.02595         |

MW = molecular weight; SC = spectral count; NSAF = normalized spectral abundance factor
Bile proteomics in biliary strictures

Table 5. Proteins identified as more abundant in cholangiocarcinoma than in primary sclerosing cholangitis

| Proteins                                      | Accession   | MW (kDa) | Average SC | NSAF ratio | CCA  | PSC  | P-value (t-test) |
|-----------------------------------------------|-------------|----------|------------|------------|------|------|-----------------|
| Serum albumin                                 | 4502027     | 69       | 1338.0     | 334.5      | 2.16 | 0.03297        |
| Alpha-2-macroglobulin precursor               | 66932947    | 163      | 173.0      | 29.5       | 3.28 | 0.03422        |
| Haptoglobin isoform 1 pre-pro-protein         | 4826762     | 45       | 108.7      | 14.7       | 3.79 | 0.02149        |
| Complement C3 precursor                       | 115298678   | 187      | 269.7      | 31.7       | 4.52 | 0.04634        |
| Ceruloplasmin precursor                       | 4557485     | 122      | 55.7       | 6.5        | 5.34 | 0.01608        |
| Apolipoprotein A-1 pre-pro-protein            | 4557321     | 31       | 40.3       | 1.7        | 13.81| 0.02004        |
| Carbonic anhydrase 1                          | 192447430   | 29       | 29.7       | 0.7        | 15.31| 0.00834        |
| Peroxiredoxin-2 isoform a                     | 32189392    | 22       | 29.7       | 0.8        | 18.27| 0.00005        |
| Hemoglobin subunit alpha                      | 4504345     | 15       | 271.7      | 3.7        | 26.52| 0.01789        |
| Apolipoprotein B-100 precursor                | 105990532   | 516      | 127.7      | 0.0        | PSC  | only 0.01591    |
| Hemoglobin subunit delta                      | 4504351     | 16       | 26.7       | 0.0        | PSC  | only 0.00001    |
| Complement C4-B pre-pro-protein               | 17855739    | 193      | 52.3       | 0.0        | PSC  | only 0.02317    |
| Intercellular adhesion molecule 1 precursor   | 167466198   | 58       | 4.7        | 0.0        | PSC  | only 0.00097    |
| Peptidyl-prolyl cis-trans isomerase A         | 10863927    | 18       | 5.7        | 0.0        | PSC  | only 0.00309    |

MW – molecular weight; SC – spectral count; NSAF – normalized spectral abundance factor.

Table 6. Proteins identified as less abundant in cholangiocarcinoma than in primary sclerosing cholangitis

| Proteins                                      | Accession   | MW (kDa) | Average SC | NSAF Fratio | CCA  | PSC  | P-value (t-test) |
|-----------------------------------------------|-------------|----------|------------|------------|------|------|-----------------|
| Pro-activator polypeptide isofrom a pre-pro-protein | 11386147    | 58       | 0.0        | 8.0        | 0.00 | 0.00496        |
| Trypsin-1 pre-pro-protein                     | 4506145     | 27       | 0.0        | 38.7       | 0.00 | 0.01173        |
| Carboxypeptidase B pre-pro-protein            | 54607080    | 47       | 0.0        | 70.2       | 0.00 | 0.02961        |
| Chymotrypsin-like elastase family member 2A pre-pro-protein | 15559207    | 29       | 0.0        | 35.5       | 0.00 | 0.03517        |
| Chymotrypsin-like elastase family member 3B pre-pro-protein | 6679625    | 29       | 0.0        | 43.0       | 0.00 | 0.03623        |
| Chymotrypsin-C pre-pro-protein                | 62526043    | 29       | 0.0        | 21.2       | 0.00 | 0.04669        |
| Trefoil factor 2 precursor                    | 4885629     | 14       | 1.3        | 21.0       | 0.05 | 0.00235        |
| Antithrombin-III precursor                    | 4502261     | 53       | 10.7       | 43.3       | 0.18 | 0.03221        |
| IgG Fc-binding protein precursor              | 154146622   | 572      | 73.7       | 180.2      | 0.33 | 0.00129        |

analysis. Patients with PSC are at an increased risk of developing biliary tract malignancies [29–31], and multiple ERCPs are performed in those patients for relieving the obstruction in the ducts, as well as to rule out malignancies. Also, obtaining bile during routine ERCPs does not impart any major risk to the patients, beyond the baseline risks of the procedure.

We identified several proteins showing differential abundance. Comparing the overall benign (PSC and benign) and malignant lesions (pancreatic cancer and CCA), several proteins including myeloperoxidase, inter-alpha-trypsin inhibitor heavy chain H4, complement C3, ceruloplasmin, alpha-2-macroglobulin, apolipoprotein B-100 and kinogen-1 isoform 2 were found to be significantly elevated in patients with malignant biliary strictures. An earlier study created a model for identification of CCA using proteomic analysis based on 22 peptides, in which they found various proteins—including inter-alpha-trypsin inhibitor heavy chain H4, serum albumin, hemoglobin sub-units alpha and beta, and others—to be significantly elevated in malignant strictures caused by CCA [14]. We observed that similar proteins were also identified in malignant strictures in our study. These proteins are normally present in bile; however their levels are elevated significantly in the setting of CCA. As in our study, the fact that the above-mentioned proteins were significantly elevated in malignant strictures could be indicative of the changes in protein catabolism/apoptosis taking place in the malignant cells. In another study involving analysis of bile in patients from pancreatic adenocarcinoma, several proteins were identified [10]; of these, proteins such as serum albumin, ceruloplasmin, alpha-2-macroglobulin, vitamin D-binding protein, apolipoprotein A-1 were also found to be abundantly expressed in malignant strictures in our study. Our study further confirms the elevation of these above-mentioned proteins in malignant biliary strictures.

Trefoil factor 2 plays an important role in maintaining the integrity of the gut, providing mucosal protection; it has also been found to activate CXCR4 chemokine receptors in epithelial and lymphohytic cancer cell lines, affecting their proliferation [32]. In our study, we found that trefoil factor 2 was significantly lower in the malignant biliary strictures, when compared to the benign ones. Down-regulation of TFF-2, suggestive of tumor invasiveness and metastasis, has been recently described in gastric cancers [33]. We also found that proteins including intercellular adhesion molecule 1, ceruloplasmin, haptoglobin, complement c3, and -c4b were found to be significantly elevated in CCA when compared to patients with PSC. However, considering that only three patients in our study had CCA, the clinical significance of this observation warrants further validation from future studies. The S100 group of proteins, which are especially the markers of schwannomas, have been linked with many malignant conditions [34–36]. A significant elevation of S100 A9 in bile in patients has been demonstrated in CCA [27]. In our study, S100 A8, S100 A9 and a few other biomarkers were
significantly elevated in pancreatic cancer, when compared with benign lesions and PSC.

Although the complex constitution of bile can limit proteomic analysis, recent improvements in technology have facilitated major advances in proteomic studies. Summarizing, proteomic analysis of bile reflects the cellular changes in the surrounding tissues/organs, which can be identified through differential expression of various peptides/protein markers. Our preliminary study, involving quantitative proteomic analysis of 24 patients with biliary strictures, shows that the protein biomarkers may differentiate malignant- from benign biliary strictures and may at an earlier stage detect malignancies (CCA) in PSC patients with indeterminate biliary strictures, and hence improve their prognoses. Future large-scale studies on proteomic analysis of bile, comparing benign and malignant strictures, will further enhance the results and its application in clinical practice.

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