A global proteomic study identifies distinct pathological features of IgG4-related and primary sclerosing cholangitis

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Aims: This combined proteomic and histopathological study was aimed to compare tissue characteristics of immunoglobulin (Ig)G4-related sclerosing cholangitis (ISC) and primary sclerosing cholangitis (PSC) in a global, non-biased manner.

Methods and results: Tissue proteomes and phosphorylomes of frozen large bile duct samples were analysed by a conventional liquid chromatography-tandem mass spectrometry (LC-MS/MS) protocol and additional phosphopeptide enrichment methods. The proteomic examination identified 23,373 peptides and 4,870 proteins, including 4,801 phosphopeptides and 1,121 phosphoproteins. The expression profiles of phosphopeptides discriminated ISC from PSC more clearly than those of non-phosphopeptides. In the pathway analysis, ISC was found to have 11 more activated signal cascades, including three immunological pathways, all B cell- or immunoglobulin-related. On immunostaining, two immunological markers (FYN-binding protein and allograft inflammatory factor-1) up-regulated in ISC were expressed mainly in M2 macrophages, consistent with increased phagocytic activity induced by the immunoglobulin (Ig)G-Fcγ receptor interaction. In contrast, PSC had two more activated signal pathways related to extracellular matrix (ECM) remodelling. Filamin-A involved in ECM remodelling was expressed aberrantly in injured bile ducts and associated cholangiocarcinomas in PSC, suggesting its possible roles in periductal fibrosis and carcinogenesis in PSC.

Conclusions: This study suggested crucial roles of B cells and macrophages in ISC, and more dynamic ECM remodelling in PSC.

Keywords: autoimmune pancreatitis, B cell, pathogenesis, phosphoproteome, proteome

Introduction

Immunoglobulin (Ig)G4-related sclerosing cholangitis (ISC) has been recognized increasingly as a hepatobiliary manifestation of systemic IgG4-related disease (IgG4-RD).1–4 This recently discovered form of cholangiopathy develops in association with IgG4-related autoimmune pancreatitis (type 1 AIP) in >90% of patients.3,5 Discriminating ISC from primary sclerosing cholangitis (PSC) is sometimes challenging, particularly when isolated ISC without other organ involvement diffusely affects the biliary tract. Another unique aspect is that hilar bile ducts involved in this condition show imaging features reminiscent of hilar cholangiocarcinoma, leading to unnecessary surgical resections in some patients.3–6 Unlike PSC, for which only liver transplantation is an effective treatment, ISC responds well to steroid therapy, which is the main reason why the two conditions have to be diagnosed correctly.3,4 ISC is characterized by elevated serum IgG4 concentrations and infiltration of IgG4+ plasma cells. However, serum and tissue IgG4
An interesting question is how ISC differs from PSC with respect to its pathogenesis, given the fact that their histological features (lymphoplasmacytic infiltration, tissue eosinophilia and periduct fibrosis) overlap.5,6 Our previous studies identified that T helper type 2 (Th2) and regulatory immune reactions are activated significantly in ISC.9 This observation has been validated by other studies, including those for IgG4-RD at other anatomical sites.10 CCL1–CCR8 interaction is another unique immune reaction seemingly involved in ISC.11 However, the pathogenetic processes of ISC are incompletely understood, as most of the basic studies on IgG4-RD involved molecular-targeted assays, where only selected immunological factors or cells were examined. Global studies have been awaited to obtain a clearer understanding of the underlying immunological features of this emerging cholangiopathy.12

Liquid chromatography-mass spectrometry (LC-MS)-based proteomics is a powerful approach for the comprehensive examination of proteins and post-translational modifications (PTMs) in a non-biased manner. LC-MS proteomics has been shown previously to enable simultaneous identification of thousands of proteins and PTMs that are significantly up- or down-regulated in tissue samples.1,3,14 Given that phosphorylation is a central modification process regulating the functional activity of proteins, global phosphoproteomic examination seems to be a promising approach.15,16 This novel approach has been used for in-vitro studies, and was rarely applied to tissue samples. We recently developed a workflow called SysQuant that enables examination of the phosphorylation status of proteins extracted from frozen human tissue samples, by means of several phosphopeptide enrichment steps with immobilized metal affinity columns (IMAC) or titanium dioxide (TiO2).17 Crucially, SysQuant also measures protein expression by means of a non-enrichment arm focusing on quantification of non-phosphorylated peptides. Our previous study on human pancreatic cancers revealed that 635 of 6543 phosphopeptides identified were modulated significantly between cancerous and background pancreatic tissue.17 The combined conventional proteomic and phosphoproteomic approach helped us to determine activated signalling pathways in pancreatic cancer tissue on a case-by-case basis.17

In this study, we applied the LC-MS proteomic technique to bile duct samples of ISC and PSC to examine both expression values and phosphorylation status of tissue proteins in a global, non-biased manner. This study was aimed to identify proteins and signal cascades that are up-regulated in either condition, and to learn how ISC differs from PSC in terms of the pathogenetic processes. To the best of our knowledge, this is the first attempt to examine the global tissue phosphoproteomes of inflammatory conditions.

Materials and methods

Study design and tissue samples

Ethical aspects and the research protocol were approved by the BioBank Committee of the Institute of Liver Studies, King’s College Hospital (Reference no. 08/H0704/117). All participants were diagnosed at our institutes and provided written informed consent for the use of their tissue samples for research.

This study consisted of ISC (n = 14) and PSC (n = 14). The proteomic discovery study was carried out using frozen tissue (four cases each of ISC or PSC), while the following validation study with immunohistochemistry was performed using formalin-fixed paraffin-embedded (FFPE) tissue (10 cases each). For the discovery study, bile duct tissues taken from surgically resected or explanted specimens were snap-frozen. The analysed samples were pure bile ducts with no pancreatic or liver tissue included. FFPE tissues of surgically resected specimens were used for single and double immunostaining.

Patient characteristics

Patients with ISC [median age: 65 years (range: 51–74); M/F = 12/2] underwent surgical resection for suspected pancreatic or biliary cancers. Eleven cases had both pancreatitis and cholangitis, while the remaining three presented with isolated mass-forming ISC involving the hilar bile ducts. Frozen tissues were obtained from the thick extrahepatic bile ducts (n = 4) and hilar mass (n = 1). The diagnosis of ISC was established histologically based on the International Consensus Diagnostic Criteria for AIP and HISOrt (histology, imaging, serology, other organ involvement and response to therapy) criteria for ISC18,19. Although serum IgG4 concentrations were not examined prior to surgery, they were found to be elevated in 12 patients tested after surgery (155–1840 mg/dl, normal range: < 135 mg/dl).

Of 14 patients with PSC [median age: 41 years (range: 25–53); M/F = 9/5], 12 underwent liver transplantation, while the remaining two had liver resection for hilar cholangiocarcinomas associated with PSC. The background livers in the latter two...
had only early bridging fibrosis with no cirrhosis. As liver transplantation is sometimes carried out for patients with non-cirrhotic PSC because of impaired quality of life (i.e. refractory itching), degrees of liver fibrosis varied in transplanted cases [established cirrhosis (n = 6), pre-cirrhosis (n = 3) and bridging fibrosis (n = 3)]. We selected four cases that still had active cholangitis and less advanced liver fibrosis to compare the proteomic profiles with inflammatory cholangitis in ISC cases. Hilar bile ducts sampled for the proteomic analysis were highly inflamed with ulceration, lymphoplasmacytic infiltration and xanthogranulomatous change. The diagnosis of PSC was based on diffuse bile duct abnormalities, including stricturing and dilatation on endoscopic retrograde cholangiopancreatograpy (ERCP) or magnetic resonance cholangiopancreatography (MRCP). Other potential causes of sclerosing cholangitis, such as ischaemia, sickle cell disease and immunodeficiency, were ruled out. Histological features of explanted livers of these patients were consistent with PSC without features suggestive of ISC. IgG4+ plasma cells were scarce or only focally aggregated with PSC without features suggestive of ISC.

**Histopathology**

Methods of conventional proteomic and phosphoproteomic analyses are described in detail elsewhere.17 Seven steps of the workflow are summarized below.

**Protein extraction**

Frozen tissue samples (91.6–115.5 mg) were pulverized and then ground into a fine powder using MultiSample BioPulveriser (MSBP; Biospec Products, Bartlesville, OK, USA) in the presence of liquid nitrogen. The powder was transferred to Eppendorf tubes containing 0.85 ml of ice-cold lysis buffer. Samples were then sonicated on ice (4°C). Following centrifugation at 12 500 g for 10 min at 4°C, the protein concentration of each sample was determined using the Bradford protein assay and a microplate luminometer. Samples were normalized to a concentration of 3.5 µg/µl and equal amounts of protein (3.0 mg) were used for each case.

**In-solution trypsin digestion**

Reduction, alkylation of cysteines and digestion were performed on lysates, according to a previous report.20 The digested samples were spun for 10 min at 2500 g and de-salted on 200 mg SepPak tC18 cartridges (Waters, Milford, MA, USA). Peptides were eluted with 50% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA) and lyophilized.

**Tandem mass tag (TMT) labelling**

Digested peptides from all samples were resuspended separately in 200 µl TEAB/10% ACN, mixed with their respective TMT8plex reagent (15 µl final concentration; Thermo Scientific, Waltham, MA, USA), and left to stand for 1 h at room temperature. The TMT reactions were then terminated with 0.25% hydroxylamine for 15 min. Samples were pooled into one TMT8plex and left to stand for another 15 min. The TMT8plex sample was acidified, the ACN concentration was reduced, and the sample was then divided into three aliquots, each of which was desalted on a 500 mg SepPak tC18 cartridge, eluted, and then lyophilized; 3.2 mg of total protein were calculated to be in the non-enriched aliquot while 10.4 mg of total protein were calculated to be in each of the enriched aliquots.

**Strong cation exchange-high-performance liquid chromatography (SCX-HPLC) and phosphopeptide enrichment**

The three independent aliquots of the TMT8plex sample were split further into 12 fractions by SCX chromatography, and then to reduce LC-MS/MS time fractions were pooled resulting in seven fractions for the non-enriched arm and eight fractions for each of the enriched arms. The seven non-enriched fractions were then analysed directly by LC-MS/MS, while the remaining two sets were first enriched for phosphopeptides using either IMAC (Thermo Scientific) or TiO2 (Thermo Scientific). The eight IMAC fractions were then merged with the eight complementary TiO2 fractions to further reduce LC-MS/MS time. Following phosphopeptide enrichment and pooling, peptides were purified using Pierce Graphite Spin Columns (Thermo Scientific).

**LC-MS/MS**

In total, 45 separate LC-MS/MS runs were performed [(seven fractions in the non-enriched arm + eight fractions in enriched arms) × three analytical repeats]. Peptides from all 15 fractions were resuspended in 35 µl of 2% ACN and 0.1% formic acid, and then 8 µl of each sample was injected onto a 0.1 × 20 mm column packed with ReproSil C18, 5 µm, using the Proxeon EASY-nLC II system (Thermo Scientific). Peptides were then resolved using an increasing gradient of 0.1% formic acid in ACN (10–25%) through a 0.075 × 150 mm self-packed column with ReproSil C18 at 3 µm, at a flow rate of 300 nl/min. Mass spectra were acquired on an LTQ Orbitrap Velos (Thermo Scientific) throughout the chromatographic run (115 min), using
10 higher-collision-induced dissociation (HCD) FTMS scans at 15 000 resolving power at 400 m/z, following each FTMS scan (2 × μScans at 30 000 resolving power at 400 m/z). HCD was carried out on 10 of the most intense ions from each FTMS scan and then put on a dynamic exclusion list for 30 s [10 parts per million (ppm) m/z window]. The AGC ion injection target for each FTMS1 scan was 1 000 000 (500 ms maximum injection time). The AGC ion injection target for each HCD FTMS2 scan was 50 000 (500 ms maximum ion injection time).

Peptide identification and quantification
All 45 raw data files from the TMT8plex sample were combined and searched as a MuDPIT file against the human UniProtKB/Swiss-Prot database using Mascot and Sequest [via Proteome Discoverer 1.3 (Thermo Scientific)]. Peptide spectrum matches (PSMs) were rejected if identified with only low confidence (≥5% FDR), showed ≤75% phospho-RS probability score or had missing quantification channels (e.g. not all peaks for isobaric tags were visible in spectra).

Bioinformatics
For normalization, sum-scaling was performed to remove potential experimental bias. For each peptide (phosphorylated and non-phosphorylated), a median normalized intensity was calculated from the four cases in each sample group. The median normalized intensity from the four ISC cases was then divided by the median normalized intensity from the four PSC cases to provide a ratio which was then transformed to the binary logarithm (log2). For protein ratios, all unique peptides that were not phosphorylated were used and combined with the median. For the phosphopeptide ratios, all peptides with a pRS probability of ≥75% for any phosphorylation position were used. A one-sided t-test (or one-sample location test) was used for the data analysis, as it can detect significant regulation. The software used for statistical analyses comprised EZR (http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmedEN.html), Multi Experimental Viewer (http://www.tm4.org/mev.html) and online DAVID Bioinformatics Resources version 6.7 (http://david.abcc.ncifcrf.gov/).

Immunohistochemistry
The primary antibodies used were monoclonal antibodies against FYN-binding protein (clone EPR2547Y; Abgent, San Diego, CA, USA), allograft inflammatory factor-1 (AIF-1) (clone 1022-5; LSBio, Seattle, WA, USA), filamin-A (clone PM6/317; Santa Cruz, Biotechnology, Santa Cruz, CA, USA), filamin-C (Atlas Antibodies, Stockholm, Sweden), CD3 (clone F7.2.38; Dako Cytomation, Glostrup, Denmark), CD68 (clone PG-M1; Dako Cytomation), CD163 (clone 10D6; Leica Microsystems, Wetzlar, Germany) and alpha-smooth muscle antigen (αSMA) (clone 1A4; Dako Cytomation). Sections were pretreated with a heated plate or micro-waved for 10 or 20 min. Single immunostaining on FFPE sections was performed using an autostainer based on the preinstalled protocol (Bond Max; Leica Microsystems). Dual fluorescent staining was performed manually. The reaction product was visualized with Alexa Fluor® fluorescent goat anti-mouse or anti-rabbit IgG antibodies (1:500; Abcam, Cambridge, UK), and was observed under a fluorescent microscope (AX80; Olympus, Tokyo, Japan).

Results
Clustering analyses based on peptide expression profiles
The proteomic examination enabled the identification and quantification of 23 373 peptides, including 4801 phosphopeptides. Peptide expression values were applied to the Multi Experimental Viewer to examine the differences between the peptide profiles of ISC and PSC. When both non-phosphopeptides and phosphopeptides were applied the two conditions were not well separated, with two dominant clusters consisting of both ISC and PSC cases (Figure 1). Another analysis using only non-phosphopeptides resulted in the same clustering pattern. Interestingly, when only phosphopeptides were considered for analysis, the separation appeared to be better, with two dominant clusters that were highly specific for either condition (Figure 1).

Protein identification and quantification
Peptide sequence information led to the identification of 4870 proteins. Of these, 3003 were non-shared, non-phosphorylated peptides used for protein quantification. The expression of 784 proteins (26%) was significantly higher in ISC than in PSC, whereas 164 (5%) were more abundant in PSC. ISC/PSC log2 expression ratios and P-values of those proteins are shown in Tables S1 and S2. The top 30 proteins up-regulated in ISC are described in Table 1.

Phosphorylation at each phosphorylation site was analysed quantitatively based on the expression values of phosphopeptides. Of 1121 proteins with quantifiable
phosphorylation site(s). 262 (23%) had at least one site that was more phosphorylated in ISC than in PSC. Similarly, 83 (7%) proteins were phosphorylated significantly in PSC \( (P < 0.05) \). Fourteen proteins selected by both comparisons had one phosphorylation site phosphorylated highly in ISC and another site phosphorylated significantly in PSC.

The proteomic analysis identified 32 peptide sequences (12 shared, 20 unique peptides) belonging to IgG4. Twenty of them were unique to IgG4, while 12 peptide sequences were shared with other IgG subclasses (there is >90% homology between IgG4 and other IgG subclasses). The expression of one shared and one unique peptide \( \text{gPSVFPLAPcSR, akGQPREPQVYTLPPSQEmTk} \) was significantly higher in ISC than in PSC \( (P = 0.013 \) and \( 0.041 \), respectively). Although a majority of the identified peptides were up-regulated in ISC (Figure 2), IgG4 protein expression values estimated based on all non-shared peptides did not reach statistical significance \( \text{ISC/PSC log}_2 \text{ ratio} = 0.78, \ P = 0.085 \). Two shared phosphopeptides belonging to IgG4 were identified, and they did not differ in expression between the two conditions.

**PATHWAY ANALYSIS**

Pathway analysis was performed by applying proteins that are significantly abundant or phosphorylated in individual conditions \( (P < 0.05) \). On the basis of protein–protein interactions of 784 proteins and 262 phosphorylated proteins up-regulated in ISC, 11 signalling pathways were estimated to be more activated in ISC than in PSC (Table 2). Interestingly, three immunological pathways were included, and they were all B cell- or immunoglobulin-related (Fcγ receptor-mediated phagocytosis, B cell receptor signalling pathway and Fce receptor I signalling pathway). Fcγ
receptor-mediated phagocytosis occurs mainly in macrophages, and is initiated by IgG molecules binding to Fcγ receptors (Figure 3). The B cell receptor signalling pathway is activated by the interaction of antigens and B cell receptors on the cell membrane.

The Fcε receptor I signalling pathway is a signalling cascade in mast cells, leading to Th2 response and eosinophilic activation (Figure 3).

A similar analysis based on 164 proteins and 83 phosphorylated proteins up-regulated significantly in

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PSC led to the identification of 17 signalling pathways probably activated in PSC (Table 2). Most of them were metabolism-associated, and no immunological pathways were identified. Interestingly, two of the selected pathways involved extracellular matrix (ECM) remodelling (focal adhesion and ECM-receptor interaction) (Figure 3).

Figure 2. Expressions of peptides belonging to immunoglobulin (Ig)G4. All but one peptide identified were up-regulated in IgG4-related sclerosing cholangitis (ISC). Twenty peptides unique to IgG4 are shown in red, while 12 peptide sequences shared with other IgG subclasses are in blue.

**IMMUNOHISTOCHEMISTRY FOR UP-REGULATED PROTEINS**

Single and dual immunostaining was carried out to examine the expressions of proteins that are potentially important in either condition. For ISC, FYN-binding protein and AIF-1 were selected for the validation...
study, as they are immune mediators ranked in the top 30 proteins up-regulated in ISC (FYN-binding protein: ISC/PSC log2 ratio = 1.28, \( P = 0.0007 \); AIF-1: ISC/PSC log2 ratio = 1.35, \( P = 0.0008 \)) (Table 1, Figure 4). FYN-binding protein was expressed in only lymphocytes in ISC, while this immunological factor appeared to be expressed diffusely in stromal cells in addition to lymphocytes in ISC (Figure 4). Dual fluorescent immunostaining revealed that the majority of the cells expressing FYN-binding protein in ISC were also positive for CD68 and CD163, consistent with macrophages (Figure 4). αSMA-positive myofibroblasts were negative for FYN-binding protein. AIF-1 was expressed in stromal cells in both conditions, but its expression was clearly up-regulated in ISC (Figure 4). Interestingly, AIF-1 and FYN-binding proteins were coexpressed in macrophages on dual fluorescent immunohistochemistry.

For PSC, filamins were selected for further analysis, as they are involved in the focal adhesion signalling pathway and are up-regulated in PSC (Figure 3). Stromal cells such as fibroblasts and macrophages require greater levels of filamin, as it is required for the focal adhesion process which is essential for cell crawling (a specific mechanism of cell motility). Two types of filamin (filamin-A and filamin-C) were identified by the proteomic study. Although the expression level of filamin-A did not differ between the two conditions (\( P = 0.068 \)), three phosphorylation sites were phosphorylated more significantly in PSC (Figure 5). The expression of filamin-C was higher in PSC than in ISC (ISC/PSC log2 ratio = 0.53, \( P = 0.028 \)). On immunostaining, macrophages and fibroblasts particularly forming xanthogranulomatous inflammation were positive for filamin-A in PSC, while its expression was weaker in ISC (Figure 5). Interestingly, filamin-A was positive in injured biliary epithelial cells in PSC, and cholangiocarcinomas associated with PSC seen in two cases. Filamin-C, which was expressed constantly in vascular smooth muscle cells, was expressed aberrantly in occasional fibroblasts and macrophages in PSC, while its expression was confined to blood vessels in ISC (Figure 5). Findings on immunostaining did not differ between early- and advanced-stage PSC.

**Discussion**

Pathogenetic studies on IgG4-RD including ISC have, until recently, focused on T cell responses such as cytokine expression and the activation of T cell subsets.9,10,21,22 However, a clinical observation that B cell depletion therapy with anti-CD20 antibodies is effective for IgG4-RD prompted us to suspect the critical involvement of B cells in the pathogenetic

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KEGG, Kyoto Encyclopedia of Genes and Genomes.
Activated signaling pathways in ISC

**FcyR-mediated phagocytosis**

**B-cell receptor signaling pathway**

**FcεRI signaling pathway**

Activated signaling pathways in PSC

**Focal adhesion**

**ECM receptor interaction**

Figure 3. Activated signalling pathways in immunoglobulin (IgG4-related sclerosing cholangitis (ISC) or primary sclerosing cholangitis (PSC). (A) Three immunological signalling pathways were determined to be more activated in ISC than in PSC by the pathway analysis. Many proteins involved in these cascades were more abundant (marked with red stars) or more phosphorylated (marked with ‘P’) in ISC. (B) Two extracellular matrix (ECM)-related signalling pathways were determined to be more activated in PSC than in ISC by the pathway analysis. Red stars indicate significantly more abundant proteins, while ‘P’ marks represent more phosphorylated proteins.

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Figure 4. Over-expressions of FYN-binding protein and allograft inflammatory factor-1 (AIF-1) in immunoglobulin (Ig)G4-related sclerosing cholangitis (ISC). (A, B) In the proteomic study, expressions of FYN-binding protein and AIF-1 were significantly higher in ISC than in primary sclerosing cholangitis (PSC). In addition, three phosphorylation sites in these proteins were significantly more phosphorylated in ISC. (C) On immunostaining, FYN-binding protein was diffusely expressed in stromal cells in addition to lymphocytes in ISC, while its expression was restricted to lymphocytes in PSC. The expression of AIF-1 was also more diffuse in ISC. (D) Dual fluorescent immunostaining showed that some of the cells expressing FYN-binding protein (FYN-BP) were positive for CD3 (T lymphocytes), while other cells expressed CD68 and CD163, in keeping with macrophages. No expression of αSMA was noted in FYN-BP⁺ cells. Some macrophages were double-positive for FYN-BP and AIF-1.
Two recent studies using a next-generation sequencing protocol have identified oligoclonal expansion of IgG4-switched B cells and plasmablasts in IgG4-RD. Circulating IgG4+ plasmablasts decrease rapidly with the B cell depletion treatment, but distinct clones reappear at the time of relapse. Another B cell subset of interest is regulatory B cells (Bregs). As with regulatory T cells (Tregs), circulating CD19+CD24highCD38high Bregs are known to increase in number in IgG4-RD patients. In line with this recent trend, the current global proteomic study also suggested the crucial roles of B cells in the pathogenesis of ISC. A remarkable finding in this study is that three immunological pathways determined to be more activated in ISC than in PSC were all B cell- or immunoglobulin-related.

Figure 5. Over-expressions of filamins in primary sclerosing cholangitis (PSC). (A, B) Filamin-A was significantly more phosphorylated at three phosphorylation sites in PSC than in immunoglobulin (Ig)G4-related sclerosing cholangitis (ISC), while filamin C was significantly more abundant in PSC with no difference in phosphorylation. (C) The expression of filamin-A was more diffuse in PSC than in ISC. Its expression was seen mainly in macrophages involved in xanthogranulomatous inflammation in the large bile ducts, and also in macrophages and stromal cells around intrahepatic small bile ducts in PSC. It was also expressed in the biliary epithelial cells. (D) In addition to smooth muscle cells, which constantly expressed filamin-C, macrophages and fibroblasts focally express the actin-binding protein in PSC.
The most significantly activated immunological pathway in ISC was Fcγ receptor-mediated phagocytosis (Figure 3). This signal cascade is initiated when IgG molecules bind to Fcγ receptors. It is worth mentioning here that IgG4 is considered as an anti-inflammatory antibody because of its poor capacity to bind to Fc receptors. Thus, one possible mechanism activating this pathway is that other IgG subclasses (i.e., IgG1) bind to the receptors. A similar discussion has proceeded to explain why hypocomplementaemia occurs frequently in IgG4-RD patients despite a relative inability of IgG4 to fix complement. Another potential explanation is that patients with IgG4-RD may have genetic polymorphisms that enable IgG4 to bind to Fcγ receptors more efficiently. A single-nucleotide polymorphism (SNP) in the CH3 exon of IgG4 (AGG → AAG at codon 409) was investigated recently in 25 patients with IgG4-RD, but the CH2 region, which is responsible for poor binding to Fcγ receptors, has not been examined. It is also interesting that an Fcγ receptor-related signalling pathway was activated in ISC (Figure 2). This finding seems understandable, given that serum IgG is elevated frequently in ISC patients, and this signal cascade leads to activation of Th2 lymphocytes and eosinophils, which is a known feature of ISC. This pathway may represent an aspect of B2T cell interaction in this condition.

The expression profiles of phosphopeptides discriminated ISC from PSC more effectively than those of non-phosphopeptides. It is not surprising that there are considerable overlaps in non-phosphopeptide expressions between the two conditions, as they have similar cellular populations in tissue, including lymphocytes, plasma cells, eosinophils, fibroblasts and macrophages. IgG4 plasma-cell infiltration is the best histological discriminator of ISC from PSC, but even expression values of IgG4 did not differ significantly between the two conditions in the current proteomic analysis. Frozen tissue of PSC might have had focally aggregated IgG4+ plasma cells, which are seen occasionally in PSC. The fact that the LC-MS/MS analysis is less sensitive than antibody-based assays (i.e., immunohistochemistry) might have caused this seemingly discrepant result. Unlike histological findings, clinical behaviours (i.e., steroid responsiveness) differ considerably between the two types of cholangiopathy. Given that phosphorylation regulates the functions of proteins, phosphopeptides should be linked closely to the biology of diseases, which is probably the reason why phosphopeptide profiles distinguished ISC from PSC very efficiently.

The current study also highlighted the potential involvement of macrophages in the pathogenesis of ISC, as the cells strongly expressed FYN-binding protein and AIF-1. FYN-binding protein is usually expressed in T cells and myeloid cells, and is possibly involved in the positive regulation of T cell activation as well as interleukin (IL)-2 production. The role of FYN-binding protein in macrophages is largely unknown, but its involvement in their phagocytic process has been suggested. AIF-1, cloned originally from a rat heart allograft under chronic rejection, is known to be up-regulated during the activation of macrophages. The expression of AIF-1 enhances the production of cytokines such as IL-6, IL-10 and IL-12, and augments the phagocytic activity of macrophages. These findings are in keeping with the result of pathway analysis that Fcγ receptor-mediated phagocytosis in macrophages was determined to be an activated signalling cascade in ISC. Another interesting aspect is that macrophages expressing FYN-binding protein and AIF-1 were positive for CD163, suggesting the M2-type phenotype. A recent study reported a possible role of M2 macrophages in the fibrosing process in IgG4-RD. To summarize, macrophages, particularly M2 type, are probably activated with enhanced phagocytic function due to the IgG-Fcγ receptor interaction, and may be involved in an orchestrated immune reaction by regulating cytokine production in ISC.

Two pathways related to ECM appeared to be up-regulated in PSC, while no significant activation was seen in immunological pathways, suggesting that PSC is a less inflammatory and more fibrogenic process than ISC. ECM remodelling seems to be highly dynamic in PSC. It is also interesting that filamin-A was expressed not only in stromal cells, but also the damaged biliary epithelium and associated cholangiocarcinomas in PSC. Although the roles of filamin-A in hepatobiliary diseases are poorly understood, a previous study revealed that filamin-A is overexpressed significantly in peripheral cholangiocarcinomas. This actin-binding protein has been studied in many neoplasms. Given that its expression in many cancers is involved in cell proliferation, migration, invasion and epithelial–mesenchymal transition (EMT), this multifunctional protein may contribute to periductal fibrosis in PSC by promoting EMT and may play a role in the development and progression of associated cholangiocarcinomas. A limitation of this study is that only transplanted cases of PSC were used for the proteomic analysis. Rare occasions are available to obtain frozen tissue from hilar bile ducts of early PSC. To overcome this...
limitation, we selected the four cases that still had active cholangitis and less advanced fibrosis for the proteomic study. PSC patients sometimes have liver transplantation at a non-cirrhotic stage for reasons other than chronic liver failure (i.e. refractory itching). Two cases of early-stage PSC were also examined in the validation study, which showed no significant difference in immunostaining for selected molecules between early and more advanced PSC. In addition, although inflammation usually becomes less conspicuous around intrahepatic small bile ducts along with bile duct loss in PSC, severe cholangitis remains in large bile ducts, which were used for the proteomic analysis.

In conclusion, the global tissue proteomic study identified distinct phosphopeptide expression profiles of ISC and PSC. Three immunological pathways more activated in ISC were all B cell- or immunoglobulin-related, suggesting the crucial roles of B cells in the pathogenesis. Enhanced phagocytotic function of activated macrophages in ISC was indicated by both the pathway analysis and the immunohistochemistry. PSC seems to have more dynamic ECM remodelling, which is mediated partly by filamins.

Acknowledgements

This study was supported in part by a King’s Liver Fund (the Institute of Liver Studies, King’s College Hospital) and a Grant-in-Aid for Scientific Research (the Ministry of Education, Culture, Sports, Science and Technology in Japan), and was also funded by Proteome Sciences plc. We thank Drs Gitte Bøhm, Petra Prefot, Christopher Lößner, Claudia Hoehle and Antje Berfeld (Proteome Sciences plc) for their technical support.

Conflicts of interest

All employees of Proteome Sciences plc (David Britton, Vikram Mitra and Ian Pike) hold stock or stock options in Proteome Sciences plc. Proteome Sciences produce the TMT reagents which are licensed for distribution by Thermo Fisher Scientific. There are no further patents, products in development or marketed products to declare.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:
Table S1. A list of proteins significantly more abundant in ISC than in PSC.
Table S2. A list of proteins significantly more abundant in PSC than in ISC.