Increased MMP-7 expression in biliary epithelium and serum underpins native liver fibrosis after successful portoenterostomy in biliary atresia

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Abstract

The molecular mechanisms underlying progressive liver fibrosis following surgical treatment of biliary atresia (BA) remain unclear. Our aim was to address hepatic gene and protein expression and serum levels of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) after successful portoenterostomy (PE), and relate them to histological signs of liver injury, clinical follow-up data and biochemical markers of hepatic function. Liver biopsies and serum samples were obtained from 25 children after successful PE at median age of 3.3 years. Serum MMP concentrations were determined by enzyme-linked immune sorbent assay. Hepatic gene expression of MMPs and TIMPs was analyzed using real-time reverse-transcription PCR. Liver expression of MMP-7 and cytokeratin-7 was studied using immunohistochemistry. Despite effective clearance of biochemical and histological cholestasis following PE, BA patients showed increased hepatic gene expression of MMP-7 (29-fold, \( p < 0.001 \)), MMP-2 (3.1-fold, \( p < 0.001 \)), MMP-14 (1.7-fold, \( p = 0.007 \)), and TIMP-1 (1.8-fold, \( p < 0.001 \)), when compared to controls. Similar to a biliary epithelial marker cytokeratin-7, expression of MMP-7 localized in biliary epithelium of bile ducts and ductal proliferations and periportal hepatocytes and was increased (\( p < 0.001 \)) in relation to controls. BA patients showed increased serum levels of MMP-7 (\( p < 0.001 \)), which correlated positively with hepatic MMP-7 gene (\( r = 0.548, p = 0.007 \)) and protein (\( r = 0.532, p = 0.007 \)) expression. BA patients showed a positive correlation between biliary MMP-7 expression and Metavir fibrosis stage (\( r = 0.605, p = 0.001 \)) and portal fibrosis grade (\( r = 0.606, p = 0.001 \)). Neither similarly increased MMP-7 expression nor correlation with liver fibrosis was observed in patients with intestinal failure-associated liver disease and comparable Metavir stage. In conclusion, our findings support an unique role of altered hepatic expression of MMP-7 in the progression of liver fibrosis after successful PE and introduce a potential therapeutic target to pharmacologically extend native liver survival by inhibiting MMP-7 hyperactivity. Serum MMP-7 may be a valuable postoperative prognostic tool in BA.

Keywords: cytokeratin-7; ductal proliferation; hepatocyte-to-cholangiocyte metaplasia; portal fibrosis; tissue inhibitor of matrix metalloproteinases; serum marker of fibrosis

Received 19 November 2015; Accepted 24 April 2016

Conflict of interest: None.

Abbreviations: BA, biliary atresia; LTx, liver transplantation; PE, portoenterostomy; CK, cytokeratin; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; IQR, interquartile range; APRI, aspartate aminotransferase to platelet ratio index.
Introduction

Biliary atresia (BA) is a progressive obliterative fibroinflammatory cholangiopathy of infancy and the leading indication for paediatric liver transplantation (LTx) worldwide [1]. The aetiology of BA is believed to be multifactorial. Viral infections, environmental factors or developmental defects may lead to bile duct injury and rapidly progressing liver fibrosis [2]. The first line surgical treatment is portoenterostomy (PE), which is considered successful when patients achieve normal serum bilirubin concentration below 20 \( \mu \text{mol/L} \) [1]. Despite successful PE, around 80% of the patients require LTx before adulthood due to progressive liver fibrosis [2–4]. The molecular mechanisms underlying this progressive liver injury after successful PE remain unclear.

In cholestatic liver fibrogenesis, activated hepatic stellate cells and portal myofibroblasts are thought to serve as the major producers of excessive extracellular matrix, while bile ductules proliferate in a deformed manner originating from pre-existing cholangiocytes, hepatocyte-to-cholangiocyte metaplasia, or liver progenitor cells [5–7]. The epithelial cells of bile ducts and proliferating ductules may function as key drivers of fibrogenesis in BA by epithelial-to-mesenchymal transition [6,8–11]. The extent of cytokeratin (CK)–7 positive biliary proliferation has been suggested to predict native liver survival more accurately than the extent of liver fibrosis at PE, while showing a close correlation with the liver fibrosis stage following successful PE [3,10].

Matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) are essentially involved with tissue remodeling during hepatic fibrogenesis and cancer invasion [12,13]. MMP-7 has been shown to be upregulated in liver and lung fibrosis, and studies with a selective MMP-7 inhibitor isofraxidin have been promising in counteracting progression of fibrosis [14–17]. Previous studies have shown significant alterations in hepatic expression and serum levels of MMPs and their inhibitors in BA patients at the time of PE and LTx [15,18–25]. Whether the altered liver expression occurs secondary to cholestasis remains unknown, while little is known about expression of MMPs and TIMPs after successful PE and clearance of jaundice.

The purpose in this study was to investigate whether MMPs and TIMPs are involved with progressive liver damage in BA patients after successful surgical clearance of jaundice, and whether extent of liver fibrosis could be assessed for follow-up purposes by measuring their serum concentrations. We hypothesized that expression of MMP-7 by proliferating biliary epithelium remains upregulated despite surgical clearance of biochemical and histological cholestasis and relates to liver fibrosis. To this end, expression of MMPs and TIMPs in liver and serum were evaluated in relation to immunohistochemical and biochemical markers of liver injury after successful PE.

Materials and methods

Study design

Of the 46 BA patients treated in Helsinki University Children’s Hospital between May 1991 and June 2011, 27 (59%) patients cleared their jaundice (serum bilirubin \(< 20 \mu \text{mol/L} \)). Of them, 25 patients were enrolled and they underwent liver biopsy and serum sampling as a part of their clinical follow-up as described previously [3].

Controls

Control blood samples were obtained from 78 day-surgery patients (53% males) without evidence of hepatobiliary disease at median age of 8.5 (interquartile range (IQR) 4.5–14) years. Liver biopsies of 10 children [11 (7.8–15) years] operated for complicated cholelithiasis with \( n = 3 \) or without underlying liver disease were used as controls for RNA expression analyses. Fourteen donor [15 (8–16) years] liver biopsies were used as controls for immunohistochemical studies. Ten patients with intestinal failure associated liver disease [4.6 (3.0–8.4) years] with advanced liver fibrosis were used as disease controls.

Clinical data

Blood samples were analyzed for platelets, bilirubin, gamma-glutamyl transferase (GGT), bile acids, alanine aminotransferase, aspartate aminotransferase (AST) and prealbumin using routine hospital laboratory methods. AST to platelet ratio index (APRI) was calculated [26]. Portal hypertension was defined by endoscopic verification of oesophageal varices, or the presence of splenomegaly and thrombocytopenia (platelet count below 150 \( \times 10^9/\text{L} \)) at the time of control biopsy [27]. Splenomegaly was defined as spleen length over two standard deviations above reference value of age and gender matched healthy children [28].
Liver biopsies, histological analyses and cytokeratin-7 immunohistochemistry

An experienced paediatric radiologist performed liver biopsies percutaneously under ultrasound guidance, while the patients were anaesthetized for upper gastrointestinal endoscopy for variceal surveillance [3]. Core needle liver biopsies were fixed in formalin, embedded in paraffin, cut, and stained as previously described [3]. Immunostaining for CK7 was performed using SP52 monoclonal antibody and ultraView Universal DAB Detection Kit (Ventana, Tuscon, Arizona) for analysis of bile ductal proliferation (0–2) and staining of periportal hepatocytes (0–4) as described previously [3]. Two experienced paediatric liver pathologists, blinded to clinical data, reviewed the sections together until a consensus was reached. Histological features were assessed according to a semiquantitative scoring system validated for BA differential diagnostics [29]. Fibrosis was analyzed by Metavir staging (0–4) and portal fibrosis was graded separately (0 = absent or fibrous expansions of some portal areas, 1 = fibrous expansion of most portal areas, 2 = focal portal-to-portal bridging, 3 = marked bridging and 4 = cirrhosis). Cholestasis was graded as 0–3. Inflammatory cells in portal areas (grade 0–3) were analyzed semiquantitatively and, when present, the percentage of neutrophils, lymphocytes and macrophages was counted.

Analyses of serum MMPs and TIMP-1

The concentrations of MMP-7, MMP-9 and TIMP-1 were determined by commercially available enzyme-linked immune sorbent assay (ELISA) kits as described previously [30]. The Quantikine® kit for MMP-7 (R&D Systems, Inc., Minneapolis, MN) and Biotrak ELISA systems MMP-9 and TIMP-1 (American Biosciences UK, Ltd., Buckinghamshire, UK) were used according to the manufacturer’s protocol [30]. MMP-8 analyses were carried out by time-resolved immunofluorometric assay (Medix Biochemicalia, Kauniainen, Finland) with the inter assay coefficient of variations (CV%) 7.3% and detection limit of 0.8 μg/L [31]. The CV% for MMP-7, -9 and TIMP-1 were 4.4, 8.8 and 13.1%, and detection limits 0.016, 0.6 and 1.25 ng/mL, respectively [30].

RNA isolation and gene expression analysis

Liver tissue specimens were embedded in RNA later solution (Ambion, Life technologies, Thermo Fisher Scientific, Inc., Waltham, MA) and frozen until analyzed. RNA was extracted using the RNeasy Mini Kit (QIAGEN, Frederick, MD). RNA integrity was assessed spectrophotometrically. Gene expression was analyzed in triplicate by quantitative real-time polymerase chain reaction using the Human Fibrosis RT² Profiler™ PCR Array (QIAGEN SABiosciences, Frederick, MD) on an ABI 7700 Sequence Detection System (Perkin-Elmer Life Sciences, Boston, MA) according to the manufacturer’s instructions. The primer for MMP-7 was a product from QIAGEN (Frederick, MD). Quantification of target gene mRNA expression was performed using the ΔΔCt method and expressed after normalization to housekeeping genes (B2M, HPRT1, RPL13A, GAPDH, ACTB) and relative to control subjects.

Immunohistochemistry of MMP-7 and TIMP-1

For MMP-7 immunohistochemistry, formalin-fixed and paraffin-embedded liver samples were freshly cut into 4 μm thick sections, fixed on slides and dried for 12–24 h at 37°C. Subsequently, sections were deparaffinized in xylene and rehydrated through ethanol and distilled water with gradually decreasing concentrations. Then slides were treated in a Pretreatment Module (Lab Vision Corp., Fremont, CA) in Tris-EDTA buffer (pH 9.0) for 20 min at 98°C. Dako REAL EnVision Detection System, Peroxidase/DAB +, Rabbit/Mouse (Dako, Glostrup, Denmark) was used for staining in Autostainer 480 (Lab Vision Corp.). Then, slides were incubated with a monoclonal MMP-7 antibody, clone 141-7B2 (Merck Millipore, Merck KGaA, Darmstadt, Germany), diluted 1:1500 in Dako REAL Antibody Diluent (Dako), for 60 min, followed by a 30 min incubation with peroxidase-conjugated Dako REAL EnVision/HRP, Rabbit/Mouse (ENV) Reagent. Slides were finally visualized by Dao REAL DAB+ Chromogen for 10 min. Between each step in above-mentioned procedure, slides were washed with phosphate-buffered saline containing 0.04% Tween-20 (Dako). Counterstaining was performed with Meyer’s haematoxylin, washed in tap water for 10 min, and mounted in aqueous mounting medium (Aquamount, BDH, Poole, UK). For TIMP-1 immunostaining, same procedure was done with Tris–HCl buffer (pH 8.5) for antigen retrieval and incubating overnight with a monoclonal TIMP-1 antibody, clone 63515 (R&D Systems, Minneapolis, MN) with a 1:50 dilution.

Immunohistological analyses were performed by the primary author (A.K.), using a Leica DM RXA microscope (Leica Microsystems GmbH, Wetzlar, Germany) and blinded to other patient data. Immunostaining of MMP-7 was confined to bile duct epithelial and perportal hepatocytes. The localization and intensity of MMP-7 immunostaining of biliary epithelial cells was graded semiquantitatively: no staining = 0, apical staining = 1, or complete staining.
with weak = 2, moderate = 3 and strong = 4 intensity (Figure 1). TIMP-1 staining was observed as brown cytoplasmic particles and graded as follows: 0 = no staining, 1 = staining of individual spindle-shaped stromal cells in portal or parenchymal areas, 2 = staining of less than 30% of hepatocytes, 3 = over 30% of hepatocytes. Staining of periportal hepatocytes was analyzed dichotomously for both MMP-7 and TIMP-1.

Figure 1. Grading of MMP-7 immunohistochemical expression in biliary epithelial cells (×400). (A) Grade 1: No staining. (B) Grade 2: Staining only at the apical/luminal side. (C) Grade 3: Biliary epithelial cells completely stained with weak intensity, (D) Grade 4: moderate intensity with staining of periportal hepatocytes (asterisk) and (E) Grade 5: strong intensity.

Ethics
This study was approved by the Ethics Committee of the Hospital District of Helsinki and Uusimaa (protocol number 345/13/03/03/2008), and complied with the ethical guidelines of the 1975 Declaration of Helsinki. Informed consent was obtained from all participating childrens’ legal guardians and adults.
Matrix metalloproteinases in biliary atresia

Table 1. Patient characteristics

| Characteristic                      | Number of patients | Age at portoenterostomy (months) | Male gender, n (%) | Associated anomalies, n (%) | Age at follow-up (years) | Splanomegaly at follow-up, n (%) | Portal hypertension at follow-up, n (%) | Liver biochemistry at follow-up |
|-------------------------------------|--------------------|----------------------------------|--------------------|----------------------------|--------------------------|-----------------------------------|--------------------------------------|----------------------------------|
| Number of patients                 | 25                 | 2.0 (1.3–2.9)                    | 13 (52%)           | 9 (36%)                    | 3.3 (2.1–7.4)             | 9 (36%)                           | 13 (52%)                            | Bilirubin total (μmol/L) 10 (4–17) |
|                                    |                    |                                  |                    |                            |                          |                                   |                                      | Gamma-glutamyl transferase (GGT) (U/L) 51 (24–156) |
|                                    |                    |                                  |                    |                            |                          |                                   |                                      | Bile acids, total (μmol/L) 32 (18–87) |
|                                    |                    |                                  |                    |                            |                          |                                   |                                      | Prealbumin (mg/L) 146 (115–169) |
|                                    |                    |                                  |                    |                            |                          |                                   |                                      | Alanine aminotransferase (ALT) (U/L) 41 (24–88) |
|                                    |                    |                                  |                    |                            |                          |                                   |                                      | APRI* 1.17 (0.43–1.93) |

Data are median (interquartile range) or frequency (%).

*APRI, [(Aspartate aminotransferase (AST, U/L)/50)/Platelet count (E9/L)]

Statistical analyses

Unless otherwise stated, the data are expressed as median and IQR. Statistical analysis was performed using SPSS software version 21.0 for Windows (IBM Corp., Armonk, NY). Comparisons between groups were performed using Mann-Whitney U test for continuous variables. Fisher’s exact test was used when comparing dichotomous variables between the groups. Correlations were calculated using Spearman’s rank correlation. Predictive values were perceived as area under the receiver operating characteristics curves (AUROC). A p value of less than 0.05 was considered statistically significant.

Results

Patient characteristics and liver histology

Laboratory values and base line clinical patient data are shown in Table 1. Median age at PE was 62 (41–88) days and 36% had associated malformations, including polysplenia, vascular or cardiac anomalies, situs inversus or malrotation. After median follow-up time of 3.3 years, median plasma conjugated bilirubin concentration was 4 μmol/L and 52% of patients had developed clinically evident portal hypertension.

Evolution of liver histology is displayed in Table 2. Despite resolution of histological cholestasis and diminishing portal inflammation after PE, Metavir fibrosis stage, ductal proliferation and CK-7 positivity of periporal hepatocytes remained high at follow-up (Table 2, Figure 2). There was no correlation between age at follow-up and fibrosis (portal fibrosis: r = −0.112, p = 0.594; Metavir stage: r = −0.025, p = 0.906).

Hepatic gene expression of MMPs and TIMPs

As presented in Table 3, BA patients showed significantly increased hepatic RNA expression of MMP-7 (29-fold, p < 0.001), MMP-2 (3.1-fold, p < 0.001) and MMP-14 (1.7-fold, p = 0.007), when compared to controls. Of MMP tissue inhibitors only TIMP-1 (1.8-fold, p < 0.001) gene expression was increased. There was a positive correlation between the liver gene expression of MMP-7 and TIMP-1 (r = 0.531, p = 0.008) among patients, but not among controls (r = 0.006, p = 0.987).

Upregulated liver expression of MMP-7 localizes in biliary epithelium and peripoatal hepatocytes, similar to CK-7

As shown in Figure 3, expression of MMP-7 was significantly increased in biliary epithelium of bile ducts and proliferating ductules when compared to controls. In most patients (56%) biliary epithelial cells completely stained with moderate or strong intensity. In control specimens, staining was restricted to the apical cytoplasm without any detectable staining in five

Table 2. Liver histology at portoenterostomy and after follow-up of median 3.3 years

| HISTOLOGY                                      | Scale | Portoenterostomy (n = 20) | Follow-up (n = 25) | p value |
|------------------------------------------------|-------|--------------------------|--------------------|---------|
| Portal fibrosis                                | 0–4   | 3 (2–3)                  | 3 (2–4)            | 0.480   |
| Metavir fibrosis                               | 0–4   | 2 (2–3)                  | 3 (2–4)            | 0.016   |
| Intracellular cholestasis                      | 0–3   | 2 (1–3)                  | 0 (0–0)            | <0.001  |
| Intracanalicular cholestasis                   | 0–3   | 2 (1–3)                  | 0 (0–0)            | <0.001  |
| Bile ductal cholestasis                        | 0–3   | 1 (0–2)                  | 0 (0–0)            | 0.002   |
| Ductal proliferation*                          | 0–2   | 1 (1–2)                  | 1 (1–1)            | 0.302   |
| Cytokeratin-7 expression of periporal hepatocytes | 0–4   | 1 (1–2)                  | 1 (0–4)            | 0.014   |
| Portal inflammatory cell infiltrate            | 0–3   | 2 (2–3)                  | 1 (0–1)            | <0.001  |
| Lymphocytes (%)                                | 50 (20–80) | 70 (60–90) (0–5) | 0.005   |
| Neutrophils (%)                                | 25 (5–60) | 5 (0–5) | 0.005   |
| Macrophages (%)                                | 10 (5–20) | 5 (0–5) | 0.005   |

Data are median (interquartile range). Significance was evaluated by Wilcoxon Signed Rank Test.

* Ductal proliferation was analyzed using cytokeratin-7-immunostaining.
Expression of TIMP-1 did not differ between BA patients and controls (2 vs 2, \( p = 0.189 \)). Among patients, biliary MMP-7 expression correlated positively with liver MMP-7 RNA expression (\( r = 0.453, p = 0.026 \)). Similar to MMP-7 immunostaining of CK-7 localized in biliary epithelium of bile ducts and ductal proliferations, and in perportal hepatocytes (Figure 4). Biliary epithelial MMP-7 expression correlated positively with ductal proliferation (\( r = 0.454, p = 0.023 \)), and periportal MMP-7 staining of hepatocytes associated with CK-7 positive perportal hepatocytes (Figure 4). MMP-7 expression of biliary epithelium was increased in patients with MMP-7 positive perportal hepatocytes (4 vs 2, \( p = 0.046 \)). Patients with CK-7 positive perportal hepatocytes had also significantly increased biliary epithelial staining of MMP-7 (3 vs 2, \( p = 0.002 \)).

Increased serum levels of MMP-7 and TIMP-1 reflected their enhanced liver expression

As presented in Table 4, in comparison to control subjects, BA patients had significantly higher serum levels of MMP-7 (sixfold, \( p < 0.001 \)) and TIMP-1 (\( p < 0.001 \)) and lower levels of MMP-9 (\( p < 0.001 \)). In patients, serum levels of MMP-7 correlated positively with MMP-7 RNA expression (\( r = 0.548, p = 0.007 \)) and immunohistochemical MMP-7 expression in biliary epithelium (\( r = 0.532, p = 0.007 \)). A respective positive correlation was also seen between

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**Figure 2.** After successful portoenterostomy (PE), fibrosis and ductal proliferation increased while cholestasis resolved. Liver histology from the same patient at PE and 2 years after successful PE (×200). (A) At PE, portal fibrosis grade 3 (0–4), Metavir stage 2 (0–4) and intracellular cholestasis grade 3 (0–3). (B) At PE, CK-7 positive biliary proliferation grade 1 (0–2, asterisk). (C) At follow-up fibrosis progressed (both portal fibrosis grade and Metavir stage 4) and intracellular cholestasis resolved. (D) At follow-up, CK-7 staining demonstrated increased ductal proliferation (grade 2, asterisk) and CK-7 staining of periportal hepatocytes (grade 4, (0–4), arrow) indicating hepatocyte-to-cholangiocyte metaplasia.
serum TIMP-1 and TIMP-1 RNA expression ($p = 0.027$). Among patients, there was a strong positive correlation between serum levels of MMP-7 and TIMP-1 ($r = 0.600$, $p = 0.002$), which was weaker among controls ($r = 0.225$, $p = 0.047$).

Increased expression of MMP-7 in biliary epithelium and serum was coupled with liver fibrosis

Patients with Metavir stage 1 fibrosis ($n = 6$) had significantly weaker MMP-7 staining of biliary epithelial cells than patients with more advanced fibrosis (1 vs 3, $p = 0.005$). As shown in Figure 5, there was a positive correlation between biliary epithelial MMP-7 expression and Metavir fibrosis stage ($r = 0.605$, $p = 0.001$), portal fibrosis grade ($r = 0.606$, $p = 0.001$) and portal inflammatory cell infiltrate ($r = 0.694$, $p < 0.001$). The patients without portal fibrosis ($n = 4$) had significantly lower serum MMP-7 levels when compared to patients with portal fibrosis (5.78 vs 17.6 ng/mL, $p = 0.008$). Serum MMP-7 showed significant predictive value for portal fibrosis with AUROC of 0.925 (CI 95%: 0.817–1.000, $p = 0.008$, Figure 5).

Patients with established histological cirrhosis (Metavir stage 4, $n = 9$) showed increased hepatic gene expression of TIMP-1 (1.7-fold, $p = 0.014$) and MMP-2 (1.7-fold, $p = 0.05$) and biliary epithelial expression of MMP-7 (3 vs 2, $p = 0.053$), when compared to those without cirrhosis. Upregulated TIMP-1 gene expression also correlated positively with Metavir fibrosis stage ($r = 0.449$, $p = 0.028$) and portal fibrosis grade ($r = 0.482$, $p = 0.017$), as did MMP-2 ($r = 0.483$, $p = 0.017$ and $r = 0.454$, $p = 0.026$, respectively). Liver gene expression (1.5-fold, $p = 0.019$) and serum level (194 vs 126 ng/mL, $p = 0.004$) of TIMP-1 was increased in patients with portal hypertension ($n = 13$) in relation to those without.

Correlations of MMP-7 and TIMP-1 with biochemical cholestasis and liver function

MMP-7 expression in biliary epithelium correlated positively with plasma conjugated bilirubin ($r = 0.514$, $p = 0.009$) and bile acids ($r = 0.639$, $p = 0.001$). Similarly, plasma bile acids ($r = 0.502$, $p = 0.012$) and conjugated bilirubin ($r = 0.449$, $p = 0.028$) correlated positively, and prealbumin negatively ($r = -0.526$, $p = 0.008$), with serum MMP-7 concentration.

Overexpression of TIMP-1 RNA correlated positively with plasma levels of conjugated bilirubin ($r = 0.779$, $p < 0.001$), bile acids ($r = 0.564$, $p = 0.005$) and APRI ($r = 0.556$, $p = 0.005$), and negatively with plasma prealbumin ($r = -0.692$, $p < 0.001$). Similarly, plasma bile acids ($r = 0.696$, $p < 0.001$) and conjugated bilirubin ($r = 0.569$, $p = 0.003$) were positively related to serum TIMP-1, and negatively to prealbumin ($r = -0.637$, $p = 0.001$). APRI correlated positively with serum TIMP-1 ($r = 0.486$, $p = 0.014$).

MMP-7 overexpression is unique for BA

Intestinal failure patients with comparable Metavir fibrosis stage [2 (2–2) vs 3 (2–4), $p = 0.247$] showed substantially lower hepatic RNA expression [2.7 (1.2–4.0)-fold vs 29 (6.1–70)-fold, $p < 0.001$, immunohistological staining [grade 1 (0–1) vs 3 (2–3), $p < 0.001$], and serum levels [3.09 (2.10–4.44) vs 14.4 (7.01–28.6) ng/mL, $p < 0.001$] of MMP-7 than BA patients. Metavir stage showed no correlation with hepatic gene expression ($r = -0.174$, $p = 0.631$), protein expression ($r = 0.272$, $p = 0.447$) or serum levels ($r = 0.290$, $p = 0.416$) of MMP-7 in intestinal failure patients.

Discussion

In this study, we have systematically assessed the expression of MMPs and their tissue inhibitors in BA following successful surgical clearance of jaundice by PE. Identification of the molecular mechanisms driving advancing liver fibrosis after successful PE would potentially offer a possibility to hinder progression of liver injury and extend native liver survival by postoperative pharmacological manipulation.

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**Table 3. Hepatic gene expression of MMPs and TIMPs in patients and controls**

|        | Controls (N = 10) | Patients (N = 24) | P value |
|--------|------------------|-------------------|---------|
| MMP-1  | 1.00 (0.92–1.55) | 0.90 (0.74–1.11)  | 0.597   |
| MMP-2  | 1.00 (0.81–1.10) | 3.12 (2.26–4.01)  | <0.001  |
| MMP-3  | 1.00 (0.85–1.37) | 0.77 (0.63–0.95)  | 0.273   |
| MMP-7  | 1.00 (0.80–1.90) | 29.2 (6.11–70.3)  | <0.001  |
| MMP-8  | 1.00 (0.92–1.55) | 0.90 (0.74–1.13)  | 0.650   |
| MMP-9  | 1.00 (0.57–1.52) | 1.25 (0.78–2.45)  | 0.186   |
| MMP-13 | 1.00 (0.92–1.55) | 0.90 (0.74–1.11)  | 0.623   |
| MMP-14 | 1.00 (0.76–1.18) | 1.70 (1.16–2.20)  | 0.007   |
| TIMP-1 | 1.00 (0.83–1.23) | 1.80 (1.52–2.93)  | <0.001  |
| TIMP-2 | 1.00 (0.79–1.02) | 1.05 (0.90–1.32)  | 0.623   |
| TIMP-3 | 1.00 (0.62–1.37) | 0.72 (0.54–1.06)  | 0.273   |
| TIMP-4 | 1.00 (0.92–1.62) | 0.87 (0.69–1.10)  | 0.650   |

Data are median (interquartile range). Transcript levels are expressed after normalisation to housekeeping genes and relative to control subjects using the ΔΔCT method. Significance between groups was evaluated by Mann-Whitney U test.
Our major findings show, first, that of the various MMPs and their inhibitors, liver expression of MMP-7 was the one mainly upregulated after successful PE and that this substantial overexpression was unique for BA. Second, the increased MMP-7 liver expression principally localized in the biliary epithelium of the bile ducts and ductular proliferations, but also in periportal hepatocytes with similar expression pattern to CK-7. Third, hepatic MMP-7 overexpression correlated with its markedly increased serum levels, and both associated with the liver fibrosis stage, which was not observed among intestinal failure patients with comparable liver fibrosis. Collectively, these findings suggest that MMP-7 may be uniquely involved in tissue remodeling during progression of liver injury after successful PE despite effective clearance of biochemical and histological jaundice, and that serum MMP-7 may be a valuable prognostic follow-up tool in BA.

Besides being expressed by developing fetal human liver, MMP-7 is involved with progression of liver fibrosis and overexpressed in various liver cancers, including cholangiocarcinoma and hepatocellular carcinoma [14,33–36]. In rhesus rotavirus infected rats, an animal model of BA, liver expression of MMP-7 is increased [37]. In several previous studies, hepatic gene expression of MMP-7 has been shown to be upregulated both at the time of PE and LTx, implicating its potential role in liver fibrogenesis in BA [15,18–21]. It should be emphasized that at these extreme stages of the disease the majority of patients are severely cholestatic. Intriguingly, we found marked gene and protein overexpression of MMP-7 also after effective surgical clearance of biochemical and histological cholestasis by successful PE. Only one previous study has explored protein expression of MMP-7 in BA, where the patients were studied at PE and LTx [15]. Similar to our findings, MMP-7 expression localized mainly in biliary epithelial cells and hepatocytes, but also in endothelial cells and local macrophages (Kupffer cells). As liver fibrosis progressed from PE to LTx, staining intensity of hepatocytes and especially biliary epithelium increased [15]. Here, a positive correlation between

**Figure 3.** Following successful portoenterostomy (PE) biliary epithelial protein expression of matrix metalloproteinase (MMP)-7 was significantly increased when compared to controls. (A) Immunostaining of a control specimen showing weak luminal staining (grade 1) of bile duct epithelial cells in the portal area (×400). (B) Enhanced epithelial MMP-7 immunostaining (grade 4) of biliary proliferation three years after successful PE (×400). (C) Biliary epithelial expression of MMP-7 was significantly increased in patients (n = 25) when compared to controls (n = 14). Box plots display median (bold transverse line), interquartile range (rectangle) and range. Significance evaluated by Mann-Whitney U test.
MMP-7 expression in biliary epithelial cells and Metavir fibrosis stage and portal fibrosis grade was found, while serum MMP-7 strongly predicted presence of portal fibrosis. Collectively, these data suggest that MMP-7 is essentially involved with progression of liver fibrosis also after successful PE, offering a potential therapeutic target to extend native liver survival by inhibition of MMP-7 hyperactivity [16,32]. Preliminary in vitro studies with a selective MMP-7 inhibitor isofraxidin have been promising, while inhibition of MMP hyperactivity in cystic cholangiocytes decreases cystic biliary deformation and fibrosis in polycystic liver disease [16,32].

The increased expression of MMP-7 localized in the biliary epithelial cells of the bile ducts and ductal proliferations, but also in periportal hepatocytes similar to CK-7 expression. Cytokeratin-7 is a cytoskeletal protein expressed by bile duct epithelial cells, intermediate hepatobiliary cells of ductular proliferations but not by normal hepatocytes. Ductular and biliary transformation of periportal hepatocytes, interpreted by CK-7 staining among specific hepatocyte...
markers, has been demonstrated in vivo [7,38,39]. Here, overexpression of MMP-7 and CK-7 shared similar patterns in ductal proliferation and in periporal hepatocytes, suggesting that MMP-7 is involved with hepatic tissue remodeling by ductal proliferation in BA. MMP-7 is able to cleave extracellular matrix and basement membrane, while ductal proliferation may drive fibrogenesis through epithelial-to-mesenchymal metaplasia [8,9,12,14,17,40].

Activated hepatic stellate cells produce TIMP-1, modulating liver fibrogenesis by inhibition of MMPs and by antiapoptotic and proliferating effects independent of MMP interactions [5,17,41]. In previous BA studies, gene expression and serum levels of TIMP-1 have been shown to be upregulated in patients with end-stage cirrhosis compared to healthy controls [20,22,23]. Controversially, others have found no correlation between gene or protein expression of TIMP-1 and different stages of fibrosis at the time of PE [24,25]. In our study, increased hepatic gene expression of TIMP-1 associated with advanced histological liver fibrosis, established cirrhosis, APRI and portal hypertension. These findings suggest that TIMP-1 contributes to hepatic tissue remodeling and fibrotic change along with MMP-7 following successful PE. MMP-14 is able to activate MMP-2 on cell membranes, having both anti-fibrogenic and profibrogenic effects [17,42]. Here, gene expression of MMP-2 and MMP-14 was modestly increased in BA compared to control subjects, while MMP-2 expression correlated positively with liver fibrosis.

Our findings also implicate that serum MMP-7 might be a useful biomarker for fibrosis and liver injury in BA. Serum MMP-7 concentration positively correlated with gene and protein expression in the liver. Both increased serum concentration and biliary epithelial expression of MMP-7 was related to liver fibrosis, while serum MMP-7 accurately predicted presence of portal fibrosis. Further prospective studies are required to confirm these findings.

This study had limitations. The age range of the patients was relatively wide and the number of patients studied limited. However, to our best knowledge, this is one of the first studies to systematically explore the molecular mechanisms underlying the progressive liver damage after successful PE.

In conclusion, our findings support an important role of altered gene and protein expression of MMP-7 in the progression of liver fibrosis and tissue remodeling after successful surgical clearance of

![Figure 5](image-url)

**Figure 5.** Liver expression of MMP-7 is coupled with fibrosis. (A) Biliary epithelial MMP-7 expression correlated positively with Metavir fibrosis stage after successful portoenterostomy. Box plots display median (bold transverse line), interquartile range (rectangle) and range. Spearman’s rank correlation (n = 25). (B) Area under the receiver operating characteristics curve (AUROC) showing predictive value of serum MMP-7 for histological portal fibrosis after successful portoenterostomy.
jaundice and may introduce a potential therapeutic target to pharmacologically extend native liver survival after PE. Further studies are needed to evaluate effects of MMP-7 inhibition on progression of liver injury after PE.

Acknowledgements

The authors thank Tuike Helmiö, Päivi Peltokangas and Kerttu Järvinen for the technical assistance. This study was supported with research grants by the Finnish Pediatric Research Foundation, Sigrid Juselius Foundation and the Research Foundation of Helsinki University Hospital. The work was independent of the funding.

Author contributions

Anna Kerola: study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript; statistical analysis. She has approved the final draft submitted. Hanna Lampela: acquisition of data; critical revision of the manuscript for important intellectual content. She has approved the final draft submitted. Jouko Lohi: acquisition of data; analysis and interpretation of data; technical, material and administrative support; critical revision of the manuscript for important intellectual content. She has approved the final draft submitted. Jaana Hagström: acquisition of data; technical, material and administrative support; critical revision of the manuscript for important intellectual content. She has approved the final draft submitted. Annika Mutanen: acquisition of data; analysis and interpretation of data; critical revision of the manuscript for important intellectual content. She has approved the final draft submitted. Taina Tervahartia: acquisition of data; analysis and interpretation of data; critical revision of the manuscript for important intellectual content. She has approved the final draft submitted. Timo Sorsa: acquisition of data; technical, material and administrative support; critical revision of the manuscript for important intellectual content. He has approved the final draft submitted. Caj Haglund: acquisition of data; technical, material and administrative support; critical revision of the manuscript for important intellectual content. He has approved the final draft submitted. Hannu Jalanko: acquisition of data; technical, material and administrative support; critical revision of the manuscript for important intellectual content. He has approved the final draft submitted. Mikko Pakarinen: study concept and design; acquisition of data; analysis and interpretation of the data; technical, material and administrative support; critical revision of the manuscript for important intellectual content; obtained funding; study supervision. He has approved the final draft submitted.

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