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Bile duct matrix metalloproteinase-7 expression: a new modality for diagnosis of biliary atresia

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Abstract

Background Biliary atresia (BA) is an obliterative cholangiopathy of infancy that results in cholestasis and liver fibrosis. This fibrosis is due to an imbalance in extracellular matrix (ECM) breakdown and deposition. The mechanism by which the progressive injury occurs is not fully elucidated. Matrix metalloproteinases (MMPs) are involved in ECM turnover but also have non-ECM-related functions. Matrix metalloproteinase 7 (MMP7) has been suggested as a promising biomarker in diagnosing BA.

Objective The aim of this study was to assess the hepatic expression of MMP-7 in infants with BA.

Patients and methods The study was a retrospective-prospective case–control study that included 50 patients who were categorized into two groups, BA group (25 patients) and non-BA cholestatic patients as a control group (25 patients). Liver biochemistry, liver biopsy, histopathology, and immunohistochemical staining for primary antibody MMP-7 were performed for all studied patients.

Results Bile duct MMP7 expression was significantly higher in infants with BA than in non-BA cholestasis ($P=0.003$), While the hepatic MMP-7 intensity did not differ significantly between both groups ($P>0.05$). Bile duct expression of MMP-7 had a significant positive correlation with the BA Score ($P=0.017$), while hepatic MMP-7 intensity had a significant positive correlation with alanine transaminase levels ($P=0.007$) and a significant negative correlation with γ glutamyl transferase in the BA group ($P=0.038$). There was no statistically significant difference among different stages of fibrosis as regards the median of the hepatic MMP-7 intensity score and MMP-7 bile duct expression in infants with BA. There was no statistically significant difference between infants with successful and failed Kasai as regard the hepatic MMP-7 intensity and its bile duct expression.

Conclusion Bile duct expression of MMP-7 measured by immunohistochemistry is useful for the diagnosis of BA, but it is limited in predicting the stage of liver fibrosis and the outcome of Kasai portoenterostomy (KPE).

Keywords Biliary atresia, Immunohistochemistry, Kasai portoenterostomy, Matrix metalloproteinase-7, Neonatal cholestasis

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Introduction

Biliary atresia (BA) is a congenital biliary disorder, which is characterized by an absence or severe deficiency of the extrahepatic biliary tree [1]. It is one of the most common causes of neonatal cholestasis, often causing cirrhosis immediately and leading to death, and accounts for over half of children who undergo liver transplantation [2].

The cause of BA is not known. Auto-immune mechanisms may be partly responsible; BA could be triggered by a viral infection in susceptible infants. It is also thought that there are two kinds of BA: Embryonic (fetal) and perinatal. The perinatal type is often associated with a later onset of jaundice and may be caused by environmental factors [3].

Patients with BA are surgically treated with a Kasai portoenterostomy (KPE), which aims to restore bile flow from the liver to the intestines. After KPE, progressive liver fibrosis is often observed in BA patients, even despite surgical success and clearance of their jaundice [1].

Matrix metalloproteinases (MMPs) are a big family of proteases that use zinc to mediate their proteolytic activity. Their first function to be described was the turnover and modulation of the ECM by proteolytically degrading various proteins that are present in the ECM. The ECM is a mixture of cells and non-cellular components that function as a physical scaffold to many cells. The two classes of molecules that mainly constitute the ECM are proteoglycan, collagen, and elastin. Proteoglycan forms a hydrophilic gel that interacts with growth factors, cell receptors, and cytokines, while collagen gives the cell tensile strength. This allows the ECM to send signals to the cell that regulate different cell functions, such as differentiation and adhesion [4].

Besides ECM turnover, MMPs play a role in inflammation, vascularization, cell migration, and proliferation [5].

Matrix metalloproteinase-7 (MMP-7) (also referred to as matrilysin) is expressed by exocrine and mucosal epithelial cells in the skin, salivary glands, pancreas, liver, breast, intestine, urogenital tract, the lungs, and other tissues. Apart from epithelial cells and keratinocytes, MMP-7 secretion has been reported only in fibroblasts, macrophages, and neoplastic cells of epithelial origin, where it is often highly expressed and has important protumor effects [6].

Matrix metalloproteinase-7 may be a key mediator linking inflammation and fibrosis [7]. MMPs which were initially characterized as ECM cleaving proteolytic enzymes, can orchestrate the inflammatory functions at various levels. They can regulate the transmigration of inflammatory cells from vasculature to the site of inflammation in tissue and regulate the recruitment and influx of inflammatory cells to the site of inflammation [5].

Once Matrix metalloproteinase-7 can be blocked by targeted medication such as isofraxidin [8] and it could be a therapeutic target for the treatment of fibrosis, so we aimed to assess the expression of MMP-7 in the liver tissue of infants with BA and its relation to liver fibrosis (Fig. 1).

Patients and methods

The present study was conducted as a prospective-retrospective case–control study in the Pediatric Hepatology, Gastroenterology, and Nutrition Department of the National Liver Institute, Menoufia University. Informed

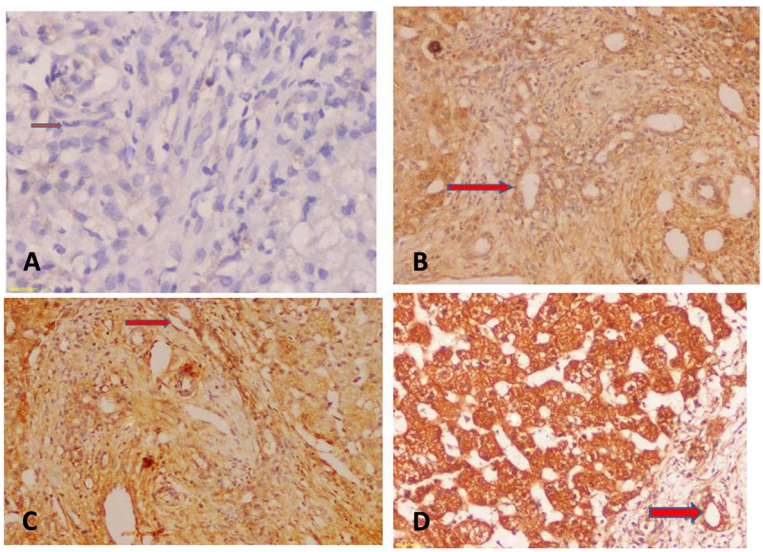

Fig. 1 Immunohistochemical hepatic expression of MMP-7. Immunohistochemical staining of MMP7 of cases with neonatal cholestasis (red arrow points to bile duct): **A** A case of PFIC showing negative expression of MMP7 in hepatocytes with focal apical staining in the bile duct (IHC × 200). **B** A case of bile inspissation showing mild expression of MMP7 in both parenchyma and bile ducts (IHC × 200. **C** A case of BA showing moderate expression of MMP7 in both parenchyma and bile ducts (IHC × 200). **D** A case of BA showing strong expression of MMP7 in both parenchyma and bile ducts (IHC × 200)

written consent was obtained from legal guardians. The study procedures were carried out and approved by the Ethical Committee of the National Liver Institute and in accordance with the declaration of Helsinki. The present study included 50 patients; 25 infants with BA whose liver tissues were compared with that of 25 infants with neonatal cholestasis other than BA in the same age group as a control group. The diagnosis of patients in the control group was 6 patients with progressive familial intrahepatic cholestasis (PFIC). They were diagnosed phenotypically based on a constellation of clinical, biochemical, and histopathological studies together with the exclusion of other causes of NC. Four cases were diagnosed as galactosemia based on reducing substances in urine and enzyme assay. Four cases had sepsis diagnosed by blood cultures and sepsis screen. Three cases had cytomegalovirus (CMV) infection, the diagnosis was established by isolation of the virus in blood within the first 3 weeks of life. Two cases with Alagille syndrome were diagnosed by neonatal cholestasis, specific facies, butterfly vertebrae, cardiac anomaly, and bile duct paucity in liver biopsy. Two cases were diagnosed as tyrosinemia. One patient with Niemann-Pick type C (NPC) was diagnosed by genetic analysis 1 case had glycogen storage disease diagnosed by clinical, biochemical, and histopathological studies. One patient had bile duct inspissation based on biochemical and histopathological studies. One case was diagnosed as idiopathic neonatal hepatitis (INH) after excluding other possible known etiologies. They were recruited from the inpatient and outpatient clinics of the Pediatric Hepatology, Gastroenterology, and Nutrition Department, National Liver Institute, Menoufia University. BA was diagnosed according to suggestive clinical, laboratory, radiological, and pathological criteria and BA score [9] and confirmed by intraoperative cholangiogram (IOC). Infants with BA receiving corticosteroid therapy were excluded from the study to avoid the drug effect on MMP7 orchestrating the inflammatory functions at various levels.

Each patient underwent: full history taking, thorough clinical examination, laboratory investigations, and abdominal ultrasonography (US) which was performed using Xario and Nemio US devices (Toshiba, Tokyo, Japan).

Liver biopsy and histopathological assessment

Ultrasonography-guided liver biopsy was done for all patients using a Tru-Cut needle size of 14 Gauge × 20 cm. A core of liver tissue containing at least 5 portal tracts was considered sufficient. Biopsy specimens were fixed in formalin, and embedded in paraffin and the prepared slides were stained by Hematoxylin and Eosin (H&E), Masson`s trichrome, Orcein, and Perl's stains and assessed blindly by a hepatopathologist according to Russo et al. [10].

Immunohistochemical (IHC) staining

Four micron-thick sections were cut by standard microtome and mounted on positively charged slides. Paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated. The sections were treated with 200 ml of tris–EDTA high PH retrieval solution (Dako, Ref K8000, Glostrup, Denmark) for 20 min. Endogenous peroxidase was blocked with peroxidase-blocking reagent. The following primary antibodies were used: MMP-7 [Concentrated form (100 microns) with a dilution of 1:100, Polyclonal rabbit anti-human antibody (Chongqing Biospes), Catalog # YPA2172].

A positive reaction was detected using DAB (3,3-Diaminobenzidine) as a substrate-chromogen solution. The sections were then counterstained with Mayer's hematoxylin. Each slide was dehydrated through graded alcohol and covered with a coverslip.

Histopathological assessment of liver biopsies

All cases were reassessed blindly by a hepatopathologist. MMP7 immunostaining was assessed by two different scores:

- The first score which describes the intensity of MMP-7 immunostaining of biliary epithelial cells only, bile duct expression (0 = no staining, 1 = apical staining, 2 = complete staining with weak, 3 = moderate staining, 4 = strong intensity) [11].
- The other one describes MMP-7 immunostaining in liver tissue, hepatic MMP7 intensity (0 = no staining, 1 = faint in hepatocytes, bile ductular epithelium, and some non-parenchymal cells morphologically identical to Kupffer cells, 2 = moderate immunoreactivity for MMP-7 is present in hepatocytes, bile ductular epithelial cells, Kupffer cells and the bile plugs in some bile ductules, 3 = hepatocytes, bile ductular epithelial cells, Kupffer cells and the interstitial fibrous tissue are all strongly stained with MMP-7 antibody) [12]. The positively stained cells were characterized by the presence of brownish nuclear staining for MMP-7.

Statistical analysis

Data were collected, tabulated, and statistically analyzed by an International Business Machines (IBM) compatible personal computer with Statistical Package for the Social Sciences (SPSS) version 22.

Types of statistics were used:

a) Descriptive statistics, e.g., number (N) and percent (%) for qualitative data, mean and standard deviation (SD) for quantitative data.
b) Analytic statistics, e.g., chi-squared test ($\chi 2$) was used to study the association between two qualitative variables. Pearson correlation coefficient test (r-test) is a test of significance used to study the correlation between parametric variables. Spearman correlation coefficient test (r-test) is a test of significance used to study the correlation between non-parametric variables.

Probability of error (p value): non-significant difference if $P > 0.05$. Significant difference if $P \leq 0.05$. Highly Significant difference if $P < 0.01$.

Results

The study included 25 infants with BA and 25 infants with non-BA cholestatic disorder who were diagnosed as follows: 6 patients with PFIC, 4 cases were diagnosed as galactosemia, 4 cases had sepsis, 3 cases had CMV infection, 2 cases with Alagille syndrome, 2 cases were diagnosed as tyrosinemia, 1 case was diagnosed as NPC, 1 case had glycogen storage disease, 1 case had bile duct inspissation, and 1 case was diagnosed as INH after excluding other possible known etiologies. Twenty-eight (56%) of the included patients were females, 22 (44%) were males and their median age was 59 days with a mean of 58.3 ± 21.46 days.

The histopathological characteristics of the studied cases

Infants with BA had significantly higher stages of fibrosis, mixed inflammatory cells, and Intraluminal bile plugs than those with non-BA cholestasis (P value = 0.012, P value = 0.041, P value < 0.0001 respectively) (Table 1).

The immunohistochemical expression of MMP-7 in the studied cases

Matrix metalloproteinase-7 expression in bile ducts was significantly higher in infants with BA than non-BA cholestasis ($P = 0.003$). While the hepatic MMP-7 intensity did not differ significantly between both groups ($P > 0.05$) (Table 2).

Bile duct expression of MMP-7 had a significant positive correlation with the BA Score ($P = 0.017$), while hepatic MMP-7 intensity had a significant positive correlation with the ALT ($P = 0.007$) and a significant negative correlation with the GGT in the BA group ($P = 0.038$).

There was no statistically significant difference among different stages of fibrosis as regards the median of the MMP-7 intensity score and MMP-7 bile duct expression in infants with biliary atresia ($P > 0.05$) (Table 3).

When hepatic MMP-7 intensity score and MMP-7 bile duct expression were compared in BA infants who had successful kassai operation (14 infants) with those who had failed operation (11 infants), no statistically significant difference was detected (P value > 0.05) (Table 4).

Discussion

Matrix metalloproteinases are the most important mediators of remodeling of the ECM [13]. In the liver, MMP-7 is thought to be produced by a variety of cells, such as glandular epithelial cells, and cholangiocytes but also by macrophages [12].

This study showed that MMP-7 expression in bile ducts was significantly higher in infants with BA. Our results are in agreement with Kerola and his colleagues [11] who

Table 1 Comparison of histopathological findings between infants with BA and infants with non-BA cholestasis

Parameter		Biliary atresia $N = 25$	Non-biliary atresia $N = 25$	$\chi 2$	P value
Fibrosis stage in biopsy	No fibrosis	0(0%)	2(8%)	11	0.012
	Mild fibrosis	9(36%)	18(72%)		
	Moderate fibrosis	15(60%)	5(20%)		
	Marked fibrosis	1(4%)	0(0%)		
Infiltrating cells	Chronic inflammatory cells	6(24%)	13(52%)	4.160	0.041
	Mixed inflammatory cells	19(76%)	12(48%)		
Eosinophils	Present	2(8%)	9(36%)	5.71	0.017
	Absent	23(92%)	16(64%)		
Intraluminal bile plug	Present	24(96%)	7(28%)	24.53	< 0.0001
	Absent	1(4%)	18(72%)		
Giant cell transformation	Present	13 (52%)	12(48%)	0.080	0.777
	Absent	12(48%)	13(52%)		

N number of patients, $\chi 2$ chi-squared test

Table 2 Comparison of MMP-7 intensity and bile duct expression between infants with BA and those with non-BA cholestasis

Parameter		Biliary atresia $N=25$	Non-biliary atresia $N=25$	$\chi 2$	*P* value
Hepatic MMP-7 intensity	0	3 (12%)	4 (16%)	2.164	0.539
	1	5 (20%)	8 (32%)		
	2	12 (48%)	11 (44%)		
	3	5 (20%)	2 (8%)		
Hepatic MMP-7 intensity	Median (25th–75th)	2 (1–2)	2 (1–2)	$Z=1.313$	0.189
Bile duct expression	0	2 (8%)	7 (28%)	10.12	0.038
	1	4 (16%)	6 (24%)		
	2	6 (24%)	9 (36%)		
	3	7 (28%)	2 (8%)		
	4	6 (24%)	1 (4%)		
Bile duct expression	Median (25th–75th)	3 (2–3)	1 (0–2)	$Z=2.935$	0.003

MMP-7 matrix metalloproteinase-7, *N* number of patients, $\chi 2$ chi-squared test

Table 3 Matrix metalloproteinase-7 intensity and bile duct expression among different stages of fibrosis in infants with biliary atresia

Biliary atresia	*N*	Hepatic MMP-7 intensity	Bile duct expression
		Median (25th–75th)	Median (25th–75th)
Mild fibrosis	9	2 (2–2)	3 (2–4)
Moderate fibrosis	15	2 (1–2)	2 (2–3)
Marked fibrosis	1	–	–
Z		2.77	3.96
P value		0.250	0.138

MMP-7 matrix metalloproteinase-7, *N* number of patients

reported a significant increase of MMP-7 expression in the biliary epithelium of bile ducts and proliferating ductules of children with BA when compared to controls. Also, Lertudomphonwanit and his colleagues [14] found that MMP-7 was primarily expressed by cholangiocytes at the time of diagnosis of BA and it was released upon epithelial injury and promoted the experimental disease phenotype.

As many studies approved the role of metalloproteinases in the turnover and degradation of extracellular matrix [13, 15] and focused on the role of MMP-7 in the degradation of specific ECM components such as gelatin, fibronectin, laminin, and elastin-elastin [16], we analyzed the relationship between MMP-7 hepatic expression and histological fibrosis stage. We did not detect a statistically significant difference among different stages of fibrosis as regards the median of the MMP-7 intensity score and bile duct expression (Table 4).

Table 4 Comparison of the MMP-7 intensity and bile duct expression between infants with successful and failed Kasai

		Successful Kasai $N=14$	Failed Kasai $N=11$	$\chi 2$	*P*
Hepatic MMP-7 Intensity	0	2 (14.3%)	1 (9.1%)	2.340	0.505
	1	3 (21.4%)	2 (18.2%)		
	2	5 (35.7%)	7 (63.6%)		
	3	4 (28.6%)	1 (9.1%)		
Hepatic MMP-7 intensity	Median (25th–75th)	2 (1–3)	2 (1–2)	$Z=0.264$	0.792
Bile duct expression	0	1 (7.1%)	1 (9.1%)	0.794	0.939
	1	3 (21.4%)	1 (9.1%)		
	2	3 (21.4%)	3 (27.3%)		
	3	4 (28.6%)	3 (27.3%)		
	4	3 (21.4%)	3 (27.3%)		
Bile duct expression	Median (25th–75th)	2.5 (1–3.25)	3 (2–4)	$Z=0.394$	0.694

MMP-7 matrix metalloproteinase-7, *N* number of patients, $\chi 2$ chi-squared test

Lertudomphonwanit and his colleagues [14] investigated whether the expression of MMP-7 correlated with fibrosis within the same liver tissue. They compared the surface area of hepatic fibrosis detected by trichrome staining and of the area immunostained by MMP-7 and reported that MMP-7 expression in the liver had no or very low correlation with hepatic fibrosis.

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 [12, 17, 18].

We detected that in BA patients MMP-7 bile duct expression and intensity were negatively correlated with GGT while MMP-7 intensity was positively correlated with ALT in the BA group.

Gamma-glutamyl transferase (GGT) is one of the factors measured in biochemical liver function tests and is widely used to differentiate BA from non-BA [19]. GGT is usually elevated during cholestasis. However, BA usually presents with a high GGT. While non-BA cholestasis patients sometimes present with normal or low GGT [20]. The GGT catalyzes the transfer of the glutamyl moiety from glutathione to restore intracellular glutathione levels [21], and the deficiency of GGT may aggravate oxidative stress damage in the liver [22]. Fried and his colleagues [23] demonstrated that decreases in glutathione initiated a pathway that resulted in cholangiocyte injury and bile duct obstruction.

A recent study by Luo et al. has demonstrated an upregulation of genes that encode proteins involved in the regulation of glutathione metabolism in BA infants who survived with their native livers up to 2 years of age [24]. Wang and his colleagues [25] reported that oxidative damage plays a crucial role in BA and it is associated with a higher grade of liver inflammation, and correlated with ALT and AST levels. MMP7 was experimentally implicated in the pathogenesis of bile duct epithelial injury [26].

Regarding histological findings, the results showed that infants with BA had significantly higher stages of fibrosis, mixed inflammatory cells, and intraluminal bile plugs than non-BA cholestasis. This was consistent with [27], who reported that hepatic fibrosis occurs earlier and faster in infants with BA than other causes of cholestasis.

Despite significant advances in the management of BA, establishing an early diagnosis for it and predicting post-KPE outcomes remain two major challenges. An early diagnosis will lead to a timely operation, which may promote surgical success rate [25]. So, we investigated the relationship between MMP-7 hepatic expression and outcome of KPE and we reported that MMP7 hepatic expression was comparable between infants with successful and failed KPE ($P > 0.05$) (Table 4).

Kasai portoenterostomy is deemed surgical successful when there is a potent connection between the liver and intestine, allowing drainage. The therapeutic success of KPE treatment is evaluated according to the levels of bilirubin at 6 months after KPE. If clearance of hyperbilirubinemia is achieved (< 20 μmol/L), KPE is deemed therapeutically successful. However, despite receiving a surgically and therapeutically successful KPE, liver fibrosis in BA patients often progresses to cirrhosis for which liver transplantation (LTx) is required [1] which may explain why we did not detect a significant relationship between MMP7 hepatic expression, which is involved in the pathophysiology of liver fibrosis, and outcome of KPE indicating that hepatic overexpression of MMP-7 can occur independent of cholestasis.

Kerola and his colleagues [11] reported marked gene and protein overexpression of MMP-7 also after effective surgical clearance of biochemical and histological cholestasis by successful KPE.

As downregulation of MMP-7 might be able to influence the course of tissue remodeling and to change the outcome of liver fibrosis [12]. Studies with a selective MMP7 inhibitor isofraxidin have been promising in counteracting the progression of fibrosis [8].

Lertudomphonwanit and his colleagues [14] reported that the use of Batimasat, an MMP7 inhibitor prevented epithelial injury and duct obstruction in 86% of neonatal mice infected with rhesus rotavirus (RRV) and decreased the expression of interleukin-8 (IL-8) orthologs and tumor necrosis factor-α (TNF-α).

The limitations of the current study are the relatively small study population and the different diagnoses of non-BA-cholestatic groups. The strength of the current study is that it is the first study on Egyptian infants with BA to evaluate MMP-7 bile duct expression as a diagnostic modality and to correlate with liver fibrosis and the outcome of kasai operation which may provide hope for further research of the pathophysiology of BA.

Conclusions

Bile duct expression of MMP-7 measured by immunohistochemistry is useful for the diagnosis of BA but is limited in predicting the stage of liver fibrosis and the outcome of KPE.

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Not applicable.

Authors' contributions

AA, MK, and OF were involved in the study concept and design. OF and TY were involved in the recruitment of patients, clinical management, and follow-up and contributed to data acquisition. SE, performed immunohistochemical staining, histopathological scoring evaluation and wrote the histopathological methodology. OF and TY performed the statistical analysis. OF and TY performed the interpretation of results and wrote the manuscript. All the authors reviewed and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The study procedures were carried out and approved by the Ethical Committee of the National Liver Institute and in accordance with the Declaration of Helsinki. Informed written consent was obtained from legal guardians.

Competing interests
The authors declare that they have no competing interests.

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