Morphometric assessment of liver fibrosis may enhance early diagnosis of biliary atresia

Ahmed F Abdalla, Abeer Fathy, Khaled R Zalata, Ahmed Megahed, Ahmed Abo-Alyazeed, Mohammed Ezz El regal

Mansoura, Egypt

Original article

Background: **Neonatal cholestasis syndrome is considered as a major challenge in pediatric practice. This study was undertaken to investigate the value of morphometric assessment of hepatic fibrosis in early diagnosis of biliary atresia.**

Methods: **We studied liver biopsy specimens from 53 patients with neonatal cholestasis. The patients were assigned to two groups: group 1 (25 patients with biliary atresia) and group 2 (28 patients with non-obstructive cholestasis). Morphometric assessment of fibrosis was performed for all biopsies; in addition, another twelve histological parameters were estimated and scored on a scale of 0 to 4. Biopsies of infants aged 60 days or younger were characterized and analyzed separately.**

Results: **Morphometric value of fibrosis was significantly higher in group 1 than in group 2 (16.8±8.4% *vs.* 5.9±2.3%, respectively; $P<0.001$). By multiple regression analysis, bile ductular plugs, morphometric assessment of fibrosis, rosetting, portal tract inflammation and pattern of cholestasis were found to be significant in discriminating the two groups. In infants aged 60 days or younger, a cutoff value for morphometric assessment of fibrosis of 7.5% was the discriminating point between the two groups with a sensitivity of 80% and a specificity of 84%.**

Conclusion: **Morphometric assessment of hepatic fibrosis could enhance the value of liver biopsy in early diagnosis of biliary atresia.**

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Key words: **biliary atresia;**
morphometry;
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Introduction

Neonatal cholestasis syndrome (NCS) is considered as a major challenge in pediatric practice.[1] The differential diagnosis of NCS can be dependent on extrahepatic and intrahepatic causes according to the anatomic location of its pathology.[2-5] Among the list of causes, biliary atresia (BA) and idiopathic neonatal hepatitis (INH) are considered the most important as they account for up to 50% to 70% of cases.[6,7] Moreover, the differentiation of BA from INH is crucial since the former needs urgent surgical intervention.[8]

Liver biopsy is the corner stone and the single most informative investigation for exact diagnosis of cholestatic syndromes of infancy and childhood.[3] Typical findings in BA include bile ductular proliferation, canalicular and cellular bile stasis, portal or periportal inflammation, and fibrosis with the presence of bile plugs in the portal tract bile ducts.[9] Hepatocyte giant cell transformation may be found in some early cases.[10,11] INH, classically, is characterized by lobular disarray, a variable inflammatory infiltrate with marked giant cell transformation of individual hepatocytes, hepatocyte necrosis, increased extramedullary hematopoiesis, and cellular bile stasis. Bile plugs are absent, and bile ductular proliferation is usually minimal or absent. Portal tract fibrosis is occasionally found but not extensive.[12,13] Paucity of intrahepatic bile duct is defined by Alagille as a ratio of intralobular bile ducts to the number of portal areas between 0 and 0.4, compared with 0.9 and 1.8 in normal children.[14] The bile duct loss, however, is progressive, and the diagnosis may not be established by liver biopsy at early stages.[15]

Author Affiliations: Pediatric Gastroenterology and Hepatology Unit, Mansoura University Children's Hospital, Faculty of Medicine, Mansoura University, Egypt (Abdalla AF, Fathy A, Megahed A, Ezz El regal M); Pathology Department, Faculty of Medicine, Mansoura University, Egypt (Zalata KR); Community and Public Health Medicine; Department of Public Health, Faculty of Medicine, Mansoura University, Mansoura, Egypt (Abo-Alyazeed A)

Corresponding Author: Abeer Fathy, Pediatric Gastroenterology and Hepatology Unit, Mansoura University Children's Hospital, Faculty of Medicine, Mansoura University, Egypt (Tel: 00201009832561; Fax: 0020502220679; Email: abeerfathy2000@yahoo.com)

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Moreover, bile duct loss also frequently occurs in infants suffering from neonatal hepatitis.[16]

Making a definitive diagnosis of BA versus INH on the basis of hepatic histology represents a great diagnostic dilemma particularly in early infancy with many overlapping features.[17-20] Some studies[21,22] based on controlled statistical grounds identified a set of histological parameters that may help in differentiating BA from INH. Morphometric assessment of fibrosis by image analysis is becoming more sensitive and accurate than semiquantitative methods for the assessment of hepatic fibrosis.[23,24] It is used mainly on chronic hepatitis C, chronic hepatitis B and alcoholic liver disease.[25,26] Mophometric assessment of hepatic fibrosis was found to be predictive of post kasi outcome in patients with BA.[27] To our knowledge, no previous studies evaluated the value of mophometric assessment of hepatic fibrosis in differentiation between BA and non-obstructive causes of NCS. The aim of this study was to evaluate whether morphometric assessment of hepatic fibrosis could add to the routine histopathology in differentiating BA and non-obstructive causes particularly in the early stage.

Methods

We retrospectively reviewed all patients presented with neonatal cholestasis to Pediatric GI and Hepatology Unit, Mansoura University Children's Hospital, Egypt between April 2005 and March 2007. Approach to diagnosis was done according to the North American Society for Pediatric Gastroenterology and Nutrition guideline recommendations.[28] Accordingly, fifty-three liver biopsies obtained from 53 infants had been re-examined.

The patients were grouped into two groups: group 1 (biliary atresia group) included 25 patients with extrahepatic BA as proved by intraoperative cholangiogram; group 2 (non-obstructive group) included 28 patients. In group 2, 21 patients had INH and 7 bile duct paucity. All the 28 patients had radiological documentation of patency of the biliary passages. Among the 21 INH patients, 14 became nonicteric; none had persistent clay stool. While 6 out of the 7 patients with bile duct paucity remained jaundiced and 3 showed intermittent clay stool (mean follow-up period: 18±6.3 months). Those with neonatal cholestasis due to other causes including α-1-antitrypsin deficiency, cystic fibrosis, congenital infections, metabolic disorders (n=14) were excluded from this study.

All biopsies were reassessed by a single pathologist who was blind about the final diagnosis of each case.

Histological examination

Sections were prepared from the paraffin blocks of the liver biopsy specimens that were stained by hematoxylin-eosin masson trichrom, Sirius red, periodic acid-schiff method, with or without diastase. All biopsy specimens were at least 1.0 cm length or presented 10 or more portal areas.

Each sample was described using Ludwig classification for fibrosis and inflammation.[29,30] Liver fibrosis was assessed as follows: grade 1, mild fibrosis with expansion of portal areas by fibrous tissue; grade 2, moderate fibrosis with portal to portal bridging fibrosis; grade 3, severe fibrosis with marked portal to portal bridging; grade 4, cirrhosis with a reconstruction of hepatic lobules. Inflammatory changes were classified as grade 1: minimal inflammation; grade 2: mild inflammation; grade 3: moderate inflammation; and grade 4: severe inflammation.

The following categories of lesions were investigated: bile ductular reaction, bile ductular plugs, giant cell transformation, severity of cholestasis, rosetting of parenchyma, cholangiolitis, eosinophils, steatosis, and sinusoidal extramedullary hematopoiesis. The histological criteria were as follows: grade 0: absent; grade 1: minimally present; grade 2: moderately present; and grade 3: severely present.[31,32] Pattern of cholestasis was assessed as cellular, canalicular or combined.

Morphometry

The morphometric assessment of liver fibrosis was performed by the fully automated Leica image processor with automated stage and Leica QWin software 2004, Wetzlar, D-35578, Germany.

The liver biopsy slides, stained with Sirius red, were placed on the x-y motorized stage of a Leica microscope. By magnification, automated sequential digitalized images were taken and stored, then a mosaic picture was created including all the images with minimal field overlapping. This enables fibrosis assessment of the entire core at the same time. After interactive thresholding, the image was converted into a binary image. Artifacts created during slide preparation were eliminated by both automatic and interactive procedures. The area of liver parenchyma was considered the reference area, and then the fractional surface occupied by fibrosis was measured within the above defined area as a percentage.[33]

In the BA group, we considered assessment of the degree of fibrosis irrespective of the associated congenital anomalies.

Statistical analysis

The statistical analysis of data was performed using Microsoft® Office Excel 2003 program and SPSS statistical package for social science version 10.

The description of the data was done in the form of mean±stand deviation (SD) for quantitative data and frequency & proportion for qualitative data.

The data were analyzed to test statistically significant differences between the groups. For quantitative data, Student's *t* test was used to compare between the two groups; the Chi-square test was used to compare qualitative data. All significant data in unvaried analysis were subjected to multivariate regression analysis for determining predictable variables of biliary atresia. $P \leq 0.05$ was considered significant at a confidence interval of 95%. ROC curve was drawn to predict cutoff point with highest sensitivity and specificity to diagnose biliary atresia by morphometric assessment of fibrosis.

Results

The clinical and laboratory parameters of the groups are shown in Table 1. The morphometric values of fibrosis for patients in groups 1 and 2 were 16.8±8.4 and 5.9±2.3, respectively (mean±SD) ($P<0.001$).

A significant difference was found in the stage of hepatic fibrosis, bile ductular reaction, bile ductular plugs, giant cell transformation, severity of cholestasis, rosetting of parenchyma, portal tract inflammation, and extramedullary hematopoiesis between the two groups. No significant difference exists in steatosis and eosinophil infiltration between the two groups. In the BA group, cholestasis was found to be canalicular in 23 patients (92%) and mixed in 2 patients (8%); none of these patients had cellular cholestasis. On the other hand, cholestasis was cellular in 16 patients of the non-obstructive group (57.1%), canalicular in 4 patients (14.3%), and mixed in 8 patients (28.6%) (Table 2). All data with statistically significant differences in univariate analysis between the two groups were reanalyzed using multiple regression (discriminate analysis) test; accordingly, the following variables were considered to be significant in discrimination of the BA and non-obstructive groups in a decreasing order: hepatic fibrosis stage, bile ductular plugs grade, morphometric assessment of fibrosis, and severity of cholestasis.

Fifteen (60%) patients aged less than two months at time of diagnosis in group 1 and 18 patients (64%) in group 2. Their mean age at liver biopsy was 51.2±8.3 days and 59±9 days for groups 1 and 2, respectively (mean±SD). In these patients, the mean morphometric value for fibrosis was 16.9±10.0% for group 1 and 5.9±2.3% for group 2 (mean±SD) ($P<0.001$). Hepatic fibrosis scale, bile ductular reaction, bile ductular plugs, giant cell transformation, pattern of cholestasis, rosetting, portal inflammation, and hematopoiesis

Table 1. Clinical and laboratory parameters of the two groups

Parameters	Group 1 (*n*=25)	Group 2		*P* value
		INH (*n*=21)	Paucity (*n*=7)	
Sex (Male:Female)	13:12	12:9	5:2	0.68
Age at biopsy (d)	70.8±30.5	75.8±30.4	77.1±33.0	0.99
AST(IU/L)	324.0±115.2	247.5±193.4	271.0±143.0	0.72
ALT(IU/L)	200.0±73.8	192.0±171.5	159.0±63.5	0.61
Alk. Phosphatase (IU/L)	916.8±228.3	855.6±536.2	824.5±233.3	0.65
GGT(IU/L)	580±180.5	198.3±137	157.5±323	0.001
Total bilirubin (mg/dL)	9.9±2.2	9.7±3.2	9.6±5.5	0.88

AST: aspartate aminotransferase; ALT: alanine aminotransferase; GGT: gamma-glutamyl transpeptidase; INH: idiopathic neonatal hepatitis.

Table 2. Semiquantitative histological parameters of the two groups

Parameters	Biliary atresia group *n*=25, *n* (%)	Non-obstructive group *n*=28, *n* (%)	*P*
Fibrosis score			
0	0 (0)	3 (10.7)	<0.001
1	0 (0)	21 (75)	
2	3 (12)	2 (7.1)	
3	22 (88)	2 (7.1)	
Bile ductular reaction			
0	0 (0)	3 (10.7)	<0.001
1	3 (12)	13 (46.4)	
2	11 (44)	10 (35.7)	
3	11 (44)	2 (7.1)	
Bile ductular plugs			
0	2 (8)	26 (92.9)	<0.001
1	13 (52)	2 (7.1)	
2	8 (32)	0 (0)	
3	2 (8)	0 (0)	
Giant cell transformation			
0	16 (64)	10 (35.7)	0.025
1	7 (28)	6 (21.4)	
2	2 (8)	6 (21.4)	
3	0 (0)	6 (21.4)	
Cholestasis severity			
1	6 (24)	18 (64.3)	0.013
2	16 (64)	8 (28.6)	
3	3 (12)	2 (7.1)	
Rosetting formation			<0.001
0	0 (0)	21 (75)	
1	2 (8)	2 (7.1)	
2	15 (60)	5 (17.9)	
3	8 (32)	0 (0)	
Cholangiolitis			
0	0 (0)	3 (10.7)	0.008
1	10 (40)	20 (71.4)	
2	13 (52)	5 (17.9)	
3	2 (8)	0 (0)	
Portal inflammation			
1	18 (72)	10 (35.7)	0.014
2	4 (16)	15 (53.6)	
3	3 (12)	3 (10.7)	
Sinusoidal hematopoiesis			
0	20 (80)	13 (46.4)	0.012
1	5 (20)	15 (53.6)	

0: absent; 1: minimal; 2: moderate; 3: severe.

Table 3. Semiquantitative histological parameters of infants less than 60 days of age

Parameters	Biliary atresia group *n*=15, *n* (%)	Non-obstructive group *n*=18, *n* (%)	*P*
Fibrosis score			
0	0 (0)	3 (16.7)	<0.001
1	0 (0)	15 (83.3)	
2	3 (20)	0 (0)	
3	12 (80)	0 (0)	
Bile ductular reaction			
0	0 (0)	3 (16.7)	<0.001
1	3 (20)	9 (50)	
2	3 (20)	6 (33.3)	
3	9 (60)	0 (0)	
Bile ductular plugs			
0	0 (0)	18(100)	<0.001
1	9 (60)	0 (0)	
2	6 (40)	0 (0)	
3	0 (0)	0 (0)	
Giant cell transformation			
0	12 (80)	0 (0)	<0.001
1	3 (20)	6 (33.3)	
2	0 (0)	6 (33.3)	
3	0 (0)	6 (33.3)	
Rosetting formation			
0	0 (0)	15 (83.3)	
1	0 (0)	0 (0)	<0.001
2	9 (60)	3 (16.7)	
3	6 (40)	0 (0)	
Cholangiolits			
0	0 (0)	3 (16.7)	0.02
1	6 (40)	12 (66.7)	
2	9 (60)	3 (16.7)	
3	0 (0)	0 (0)	
Portal tract inflammation			0.004
1	12 (80)	6 (33.3)	
2	0 (0)	9 (50)	
3	3 (20)	3 (16.7)	
Sinusoidal hematopoiesis			
0	12 (80)	3 (16.7)	<0.001
1	3 (20)	15 (83.3)	

0: absent; 1: minimal; 2: moderate; 3: severe.

were statistically significant between the patients of the two groups. Between the two groups, there was no significant difference in the severity of cholestasis, eosinophilic infilteration, and steatosis. Cholestasis was canalicular in all patients with BA, while it was cellular in 12 patients of the non-obstructive group (66.7%) and mixed in 6 patients (33.3%) (Table 3). Multivariate analysis showed that only bile ductular plugs, morphometric assessment of fibrosis, rosetting, portal inflammation and pattern of cholestasis were significant in discrimination of the BA and non-obstructive groups. The descriptive assessment of fibrosis could not predict the diagnosis of BA in those patients of less than two months at the time of diagnosis.

A value of morphometric assessment of fibrosis of 7.5% was found to be the discriminating point between the two groups with a sensitivity of 80% and a specificity of 84% (Fig).

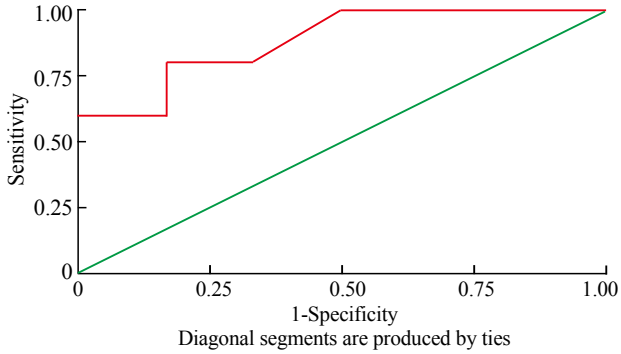

Fig. The receiver operating characteristic curve, a sensitivity and specificity of morphometric assessment of fibrosis in diagnosing biliary atresy in patients less than 60 days of age.

Discussion

The differentiation between BA and neonatal hepatitis remains difficult, particularly in patients before 8 weeks of age. Many histological parameters are powerful variables in differentiating BA from INH with variable degrees of sensitivity and specificity.[21,34] Moreover, these histopathological changes in neonatal cholestasis reflect a dynamic process, with different characteristics in distinct age groups.[35]

The current study demonstrated a significant portal fibrosis in patients with BA compared to the non-obstructive group. This finding is in agreement with the description of portal expansion and fibrosis as a feature of BA.[36,37] Desmet[38] described the presence of portal-portal bridges in infants with BA, which originates from fibrosing piecemeal necrosis. This leads to the formation of fibrous septa, which is still a potentially reversible lesion, since the intrahepatic vascular relationships are preserved. Ultimately the increase of fibrosis leads to a "jigsaw puzzle" pattern of cirrhosis characteristic of secondary biliary cirrhosis.[12] This is unusual in INH.[21,34] Bennett[39] differentiated the fibrosis pattern as being predominantly portal in extrahepatic cholestasis and lobular in intrahepatic cholestasis. Recently, Russo et al[40] developed a standardized system for histologic reporting of liver biopsies from infants with cholestasis. The histological features, which were found to predict BA on the basis of logistic regression, included bile duct proliferation, portal fibrosis, and absence of sinusoidal fibrosis. According to Cocjin's study, the fibrogenesis process seems to be due to the proliferation of Ito cells for the formation of the periductular stroma surrounding the neoductules at the periphery of the portal spaces. Although portal fibrosis is also detected in infants with

BA before 8 weeks of age, its extent is less marked.[41]

Descriptive assessment of fibrosis as well as the use of semiquantitative scores requires a fair amount of training and experience in liver histopathology. Also, because of the inherent subjectivity of these methods, inter-observer discrepancies of 10%-20% in assessing liver fibrosis were reported.[26] To overcome these problems, we have applied morphometry as a quantitative method to assess fibrosis. To our knowledge, this is the first time to use morphometry for differentiation between BA and non-obstructive causes of NCS. Application of morphometry on a mosaic picture of the entire core enables the assessment of the portal as well the parenchymal fibrosis at the same time.[33] Semi-quantitative methods describe the pathologic distribution and pattern of fibrosis, while morphometry can assess the actual amount of fibrosis, thus the two methods of assessing fibrosis should be looked as complementary.

An exciting issue in our study is that morphometric assessment of fibrosis in patients of less than 60 days could serve as a distinguishing criterion between atresia and non-atresia groups, while the descriptive assessment of fibrosis could not. In these patients, the pathologist judgment for BA diagnosis through routine assessment showed a sensitivity of 66.6% and a specificity of 83.3%. Thus, the use of morphometry at these young age groups could serve as a sensitive and specific tool (a sensitivity of 80% and a specificity of 84%) for early diagnosis of BA.

In our study, image analysis was made at a lower magnification (×10). The use of low (whole biopsy in one frame) magnification has many advantages; random loss of tissue caused by gaps between frames and double counting because overlap does not occur. Other dark staining tissues which are not collagen, such as nuclei, are below the level of resolution at a low magnification, and therefore do not falsely elevate the measured fibrosis area. Moreover, Wright et al[42] reported that image analysis at a lower magnification gives superior reproducibility, correlates well with its high magnification counterpart, and is 10-20 fold faster. However, low magnification has a potential disadvantage of loss of resolution, and it may lead to inclusion of areas containing capsular tissue, and at last the overestimation of fibrosis.[43]

Bile ductular plugs rather than bile ductular reaction could serve as a sensitive predictor for BA. This finding is in agreement with the reports by Desmet[38] and Shiraki et al.[44] In contrast, Brough and Bernstein[45] considered bile ductular plugs as a nonsignificant finding to differentiate between two groups. In this study extra medulary hematopoesis was more frequent in patients with INH. The same finding was reported by Alagille and Romero.[46,47] Subanalysis of the infants aged less than 60 days has shown that bile dductular plugs rather than extramedullary hematopoiesis is of value in differentiating BA from non-obstructive causes (INH and paucity of intrahepatic bile ducts); this is in agreement with the report of Lee et al.[16]

In conclusion, our study highlights the value of morphometric assessment of fibrosis for early diagnosis of BA. We propose that morphometric assessment of fibrosis could be used as a supplementary tool to the conventional liver biopsy for early diagnosis of BA.

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