Journal of Pediatric Surgery 51 (2016) 1445–1449

Contents lists available at ScienceDirect

Journal of Pediatric Surgery

journal homepage: www.elsevier.com/locate/jpedsurg

Congenital choledochal malformation: search for a marker of epithelial instability

Enrico La Pergola [a], Yoh Zen [b], Mark Davenport [a,*]

[a] Department of Paediatric Surgery, Kings College Hospital, London, UK (now, Department of Diagnostic Pathology, Kobe University Graduate School of Medicine, Kobe, Japan)
[b] Institute of Liver Studies, Kings College Hospital, London, UK (now, Department of Diagnostic Pathology, Kobe University Graduate School of Medicine, Kobe, Japan)

ARTICLE INFO

Article history:
Received 16 December 2015
Received in revised form 9 February 2016
Accepted 12 March 2016

Key words:
Congenital choledochal malformation
Choledochal cyst
CA19-9
Amylase
Intrabiliary pressure
MIB-1

ABSTRACT

Purpose: There is a predisposition to the development of malignancy in congenital choledochal malformation (CCM) although the degree of risk is unknown. We investigated the role of CA19-9 in bile and the MIB-1 (Ki-67) epithelial proliferation index as markers of an *at risk* choledochal epithelium at the time of definitive surgery.
Methods: Bile collected at surgery was analyzed for levels of amylase (as a surrogate of pancreatic reflux) and CA19-9. Immunohistochemical staining for CA19-9 and MIB-1 index (expressed as %) was performed on resected specimens. Data are quoted as median (IQR) and differences assessed using non-parametric statistics. A P value of 0.05 was regarded as significant.
Results: Our study group consisted of 78 children with CCM (Type 1 fusiform, $n = 34$; Type 1 cystic, $n = 30$ and Type 4, $n = 14$). Median bile CA19-9 was 159,400 (6–1 × 10^6) kU/L. There was no correlation with bile amylase ($P = 0.49$) or biliary pressure ($P = 0.17$) but modest correlation with bilirubin ($r_s = 0.24$; $P = 0.02$). In contrast, bile amylase was correlated with plasma γ-glutamyl transpeptidase ($P = 0.02$), alkaline phosphatase ($P = 0.05$) and aspartate aminotransferase ($P = 0.02$); and inversely correlated with biliary pressure ($r_s = -0.38$; $P < 0.0008$).
Epithelial expression of CA19-9 and MIB-1 was assessed in 43 specimens. CA19-9 was diffusely expressed on all choledochal epithelium. MIB-1 expression was divided into: high expression (>40%)
n = 3; moderate (20–40%) $n = 5$, low (6–20%) $n = 7$ and very low (≤5%) $n = 28$. There was no correlation with choledochal pressure ($P = 0.87$), CA19-9 ($P = 0.51$) or bile amylase ($P = 0.55$).
Conclusion: Biliary CA19-9 levels were grossly (and unexpectedly) raised in choledochal malformation and appear to arise from biliary rather than pancreatic epithelium. MIB-1 confirms that a small proportion (19%) has marked epithelial proliferation but no clinical correlates could be identified.
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Congenital choledochal malformation (CCM) may be defined as biliary dilatation in the absence of acute obstruction, of which there are three principle types. Extrahepatic dilatation with a cystic morphology (Type 1c), extrahepatic dilatation with a fusiform or cylindrical morphology (Type 1f) and either of these with intrahepatic bile duct dilatation (Type 4) [1,2]. Common to all these variants is usually some degree of pancreato-biliary malunion allowing free intermixing of pancreatic juice and bile, and here termed a common channel.

The epithelial lining of the structural abnormality varies from normal biliary, through epithelial hyperplasia, ulceration and complete mucosal loss, to dysplasia and overt malignancy. Progression may take many years but seems inexorable and clinically, while most malignant transformation presents in adults in their 30s and 40s [3,4,5], there are case reports in children and adolescents [6,7]. Perhaps more worryingly are those cases who have had resection of their CCM but who still go on to develop malignancy in the residual bile duct within the liver, at the anastomosis or within head of pancreas [3,8].

The protein CA19-9 (variously carbohydrate or cancer antigen) was discovered in adults with colon cancer and pancreatic cancer in 1981 [9] and its serum levels have been used as a screening and diagnostic aid particularly for bile duct and pancreatic cancer [10]. Similarly Ki67 is a non-histone nuclear protein expressed in all cell cycle stages except G0. The antibody, MIB-1, binds to antigen Ki67 and has been used to quantitate epithelial nuclear proliferation, and has been used as a histological marker of dysplasia [11,12,13].

The aim of this study was to identify any markers of epithelial "instability" in this group known to be at risk of later malignant change using established histological techniques and molecular biomarkers in bile at the time of CCM resection. This then might define those most at risk and hence require more careful surveillance and follow-up.

1. Methods

Kings College Hospital is the largest referral institution in England & Wales for pediatric hepatobiliary conditions. Children with CCM were

* Corresponding author at: Kings College Hospital, Denmark Hill, London SE5 9RS. Tel.: +44 203 299 3350; fax: +44 203 299 4021.
E-mail address: Markdav2@ntlworld.com (M. Davenport).

http://dx.doi.org/10.1016/j.jpedsurg.2016.03.012
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identified from a prospectively-maintained database for the period January 2007–November 2015.

The classification of CCM used here is a simplification of the Todani classification used in previous studies from our institution [1,2,14,15]. The classic choledochal cyst is referred to as Type 1c, the more fusiform extrahepatic choledochal dilatation as Type 1f and the combination of dilated intrahepatic ducts and extrahepatic choledochal (be it c or f) as Type 4.

From our prospectively maintained database we identified the predominant clinical presentation (antenatal detection; obstructive jaundice or recurrent abdominal pain/pancreatitis), maximum measured common bile duct diameter (expressed in mm, from ultrasound scanning) and liver biochemistry.

It has been our practice to measure resting choledochal pressure during the laparotomy for CCM [2,15] and obtain and store bile for later analysis of amylase (since 1997) and CA 19-9 levels (since 2009). The resected bile duct was fixed in 10% neutral buffered formalin for 12–24 h, processed through alcohol and xylene overnight, and embedded and stored in paraffin wax. For this study, specimens were immunostained against MIB-1 antibody (Dako Cytomation Inc., Carpenteria, California, USA) processed using an auto-stainer (BondMax, Leica Microsystems). Quantification of positive nuclei was performed using a cell-counting graticule.

The MIB-1 index (positive cell ratio, %) was calculated by counting 300–500 cells in each case. For this study, the index was classified into four groups based on degree: high (>40%); moderate (20–40%), low (6–20%) and very low (≤5%).

Where possible and practical all children had annual follow-beginning 6 weeks after surgery. The follow-up studies in the first year included liver ultrasound, blood tests (liver biochemistry) and clinical evaluation. Thereafter, if asymptomatic, follow-up became annual or bi-annual based on clinical evaluation and liver ultrasound. Those who developed or had persistent symptoms (typically intermittent abdominal pain) would be investigated with liver biochemistry and serum amylase levels, upper gastrointestinal endoscopy and radionuclide biliary imaging. ERCP was also performed if necessary for those where a pancreatic etiology was possible.

We have reported the clinical results of some of these patients before with respect to their bile amylase, choledochal pressure and epithelial histology [2].

1.1. Statistical analysis

Data are quoted as median (IQR). Non-parametric statistical tests were used to compare groups (Kruskal–Wallis with Dunn's test post hoc, Mann–Whitney U test) and the Spearman statistic (r_S) to illustrate correlation. GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA was used throughout. A P value of ≤0.05 was regarded as significant.

2. Results

The study group consisted of 78 infants and children who had had a resection of a CCM (Type 1f, $n = 34$; Type 1c, $n = 30$ and Type 4, $n = 14$) during the period January 2007–November 2015. The median age at surgery was 4.4 years (range 13 days to 16 years). Table 1 illustrates the age at surgery, resting choledochal pressures and bile amylase and CA19-9 in the three types. As expected, these data showed that Type 1f was characterized as having lower choledochal pressure ($P = 0.05$) and higher levels of bile amylase ($P = 0.0002$), whereas Type 4 had the highest pressure and lowest bile amylase levels.

Median bile CA19-9 was 64,700 (range 6–1 × 10 (6)) IU/ml, with no significant difference between the three common types of CCM ($P = 0.85$) (Fig. 1A). There was modest correlation between CA 19-9 and bilirubin ($r_s = 0.24$; $P = 0.02$) (Fig. 2) but no relationship was found with other indices of liver biochemistry (i.e. AST, γ-GT, alkaline phosphatase). Although the highest levels of CA19-9 were found in those presenting with jaundice, no significant differences between the different groups ($P = 0.13$) were found (Fig. 3A). There was no correlation between CA19-9 and age at surgery ($P = 0.44$).

Median bile amylase was 5390 (0–97,600) IU/L, with a significant stepwise decrease from Type 1f to Type 4 ($P = 0.0002$) (Fig 1B). The highest levels of amylase were found in those presenting with pancreatitis with significant differences between the different groups ($P < 0.0001$) (Fig. 3B). Bile amylase levels correlated with γ-GT respectively ($r_S = -0.24$, $P = 0.02$), alkaline phosphatase ($r_S = -0.20$, $P = 0.05$) and AST ($r_S = -0.24$, $P = 0.02$) but not bilirubin ($P = 0.10$). There was also an inverse relationship of bile amylase with age at surgery ($r_S = -0.41$; $P = 0005$).

As expected there was a significant inverse relationship of choledochal pressure and levels of bile amylase ($r_s = -0.38$; $P < 0.0008$) but no such relationship was identified with CA19-9 levels ($r_S = 0.12$; $P = 0.16$).

2.1. Choledochal epithelial immunostaining

CA-19 immunostaining was present on the epithelium of all the specimens examined in this study ($n = 43$) (Fig. 4). The MIB-1 index (positive cell ratio, %) was calculated in this group and found to be highly variable among this cohort: high (>40%) $n = 3$; moderate (20–40%) $n = 5$, low (6–20%) $n = 7$ and very low (≤5%) $n = 28$ (Fig. 5). To investigate the relationship with other variables we combined those in the high expression groups ($n = 8$) with those in the low and very low ($n = 35$). The high expression group consisted of Type 1f ($n = 4$), Type 1c ($n = 3$), Type 4 ($n = 1$) with an age range of 13 days of life to 10 years. We could find no significant differences within this group with respect to common bile duct diameter ($P > 0.99$), choledochal pressure ($P = 0.87$), biliary amylase ($P = 0.3$) or CA19-9 ($P = 0.51$) (Fig. 6), bilirubin ($P = 0.9$), GGT ($P = 0.78$), AST ($P = 0.8$), alkaline phosphatase ($P = 0.99$) or age at operation ($P = 0.45$).

Median follow-up is currently 71 (54–105) months in the group ($n = 8$) with high and moderate (i.e. 20–65%) levels of expression of MIB-1. Two out of eight children do complain of recurrent abdominal pain and have been thoroughly investigated. Both have had normal upper gastrointestinal endoscopies, normal liver biochemistry (including amylase) and normal liver ultrasounds. The other children in this group are asymptomatic and have normal ultrasound scans.

3. Discussion

We have previously shown a fundamental inverse relationship between resting choledochal pressure and bile amylase in CCM [2,15] and inferred that it is the pressure which influences the range of

Table 1
Characteristics of Choledochal Malformations (n = 78).

	Fusiform ($n = 34$)	Cystic ($n = 30$)	Type 4 ($n = 14$)	P value*
Age (yr)	4.6 (2.4–7.6)	2.0 (0.3–6.8)	2.8 (0.9–4.1)	0.008 1F vs. 1C
Pressure (mmHg)	9.5 (7–13)	10.5 (7–17)	17 (9–23)	0.05 1F vs. 4
Amylase (IU/L)	18000 (4800–46,750)	328 (12–20,000)	640 (2–5292)	0.0002 1C vs F 1C vs 4
CA19-9 (IU/ml)	80,450 (5900–214,000)	39,000 (20,300–204,000)	67,430 (22,500–9,100)	0.85

All values expressed as median (IQR).
* Kruskal–Wallis test, post hoc Dunn's test.

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