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Titolo di Tesi di Dottorato:

L'ESPRESSIONE DELLA CITOCHERATINA 20 (CK20)

NELL'ATRESIA DELLE VIE BILIARI: UN NUOVO POSSIBILE MARKER DI PROGNOSI

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ABSTRACT

Background

The aetiology and the prognostic factors of biliary atresia (BA), a progressive obstructive cholangiopathy of infants, are still not well known. Four different subtypes of BA have been described in relation to the age of the developmental failure during gestational age (i.e. Biliary Atresia Splenic Malformation (BASM), Cystic BA, CMV IgM +ve BA and Isolated BA).

We aimed to investigate the relation between the bile duct damage in the porta hepatis and liver and cells with an intestinal phenotype (expressing Cytokeratin 20 (CK-20)) in order to establish if this could be considered a prognostic marker of BA.

Methods

Samples were orientated intra-operatively then immunostained with anti-cytokeratin 20 (CK20). Sections were then digitised and analysed. Clinical and immunohistochemical data were compared. Data are quoted as median (range). Non-parametric statistical comparisons were made as appropriate. $P < 0.05$ was regarded as significant.

Results

48 consecutive infants were treated with Kasai Portoenterostomy (KPE) or primary liver transplantation in a single-centre between 1999 and 2017. CK20 expression in the liver was not associated with a successful KPE ($P = 0.69$) or native liver survival ($P = 0.91$). By contrast, remnant porta hepatis CK20 expression was associated with a successful KPE ($P=0.04$, HR: 0.49). Diffuse expression (distribution > 50% of slide) was associated with a longer period of native live survival ($P=0.01$; HR: 0.29).

Conclusions

CK20 diffuse expression in the porta hepatis is associated with a successful KPE and it is a predictor of native liver survival.

Biliary atresia (BA) is a progressive, usually inflammatory, obliterative cholangiopathy of infants that affects both the intra- and extra-hepatic biliary ducts. There is no analogous pathological process in older children or adults. The aetiology is probably multifactorial[1].

Historical Observations

The earliest reference to what was probably an infant with BA was reported in 1817 by Dr John Burns as an "incurable state of the biliary apparatus"[2]. Towards the end of the 19th century in 1892, the Scot John Thompson made the first accurate description of the clinical features and post mortem findings in an infant who appeared to have no common hepatic duct[3].

The first surgical success was probably described by the Boston surgeon Dr WE Ladd in 1935 in a series of patients with congenital biliary obstruction. Typically he anastomosed dilated proximal parts of the obstructed biliary tree with the intestines so restoring some kind of continuity[4]. It, however, became clear that in most infants recognised to have BA there was no proximal dilated remnant to find irrespective of how high one dissected into the porta hepatis. There were described as "uncorrectable" BA.

This pessimistic situation really did not change until the work of a Japanese surgeon, Dr Morio Kasai became more widely appreciated. In the late 1950s Morio Kasai, first began simply to transect high in the porta hepatis and join this up to a mobilised Roux loop even if there were no visible ducts present. In a proportion this did enable restoration of bile flow and disappearance of jaundice achieving the long-term benefits[5-6].

Over time this Kasai portoenterostomy (KPE) using a Roux-en-Y loop for reconstruction has become the standard operative treatment for BA. Despite a technically well-executed KPE, many children still develop progressive liver failure and cirrhosis. In such cases, liver transplantation remains the only available salvage treatment and indeed BA remains the most common disease requiring liver transplantation in children. Early diagnosis and operation with an excellent operative technique are the most important factors to the success of KPE.

Epidemiology

Biliary Atresia is a rare disorder with an incidence of 1:17000 - 19000 live births in European series[1-7-8-9-10] and appears more common in East Asian Countries. The highest incidences are reported in French Polynesia where it is about 1:3000 live births[11-12] and Taiwan (1 in 5000)[13]. In Japan the incidence is reported to be about 1:9000 live births[14]. The reported incidence in regional North

American series appears closer to the European figures (e.g. 1 in 15 000 in the southeast region of the USA[15].

BA has a higher preponderance in females in those considered to have a "developmental" origin (vide infra) but is much nearer to equality in the majority with isolated BA.

Aetiological Timing

It is likely that a number of different mechanisms can lead to what we appreciate as BA in the post-natal infant – aetiological heterogeneity. Biliary epithelial cells begin their development during the 7th week gestation and arise from hepatoblasts whose arrangement become uniform and bi-laminar around the ingrowing portal venule system. This arrangement is known as the ductal plate (DP). After about one week the bile ducts begin to branch out and radiate from the porta hepatis towards the periphery to conclude their development a few days later. The disease may be caused by failure of the remodelling process at the hepatic hilum, with persistence of fetal bile ducts poorly supported by mesenchyme. As bile flow increases perinatally, bile leakage from these abnormal ducts may trigger an intense inflammatory reaction, with subsequent obliteration of the biliary tree[16].

Four different subtypes of BA can be distinguished based on clinical or laboratory features[17-18].

1. Those with other congenital anomalies, and typically the BA splenic malformation (BASM) syndrome;
2. Cystic BA, i.e. extrahepatic cystic development within an obliterated biliary tree;
3. CMV-IgM +ve associated BA;
4. Isolated BA i.e. no features of the above.

(1) and (2) have probably in-utero origins and can be regarded as "developmental" while (3) is acquired perinatally. Those infants in subgroup (4) could have either a developmental or post-natal aetiology.

The majority of the patients affected by BA present as the isolated form for which there is no evidence of a specific cause. In some patients there is clear evidence of a developmental defect. For example, in those with BASM, the biliary malformation begins early in utero, probably at the embryonic stage and it has been associated with a higher incidence of maternal diabetes[19].

Other infants may develop a normal biliary tree which is then damaged secondarily. Several studies have attributed a key role to viral infection, especially REOvirus type 2 and cytomegalovirus (CMV). More recently two different studies from King's College Hospital, London,

United Kingdom, have shown that CMV-associated (IgM+ve) BA may represent a distinct entity[20-21]. Among all the different groups, CMV-associated BA appears to be the only with a confirmed and clear pathogenic hypothesis.

Redkar et al. showed that some cases of Cystic BA can be visualised antenatally[22] as it can be detected by ultrasound during prenatal scanning in about 40% of infants. Later in 2008, Caponcelli et al. showed that about 10% of all cases of BA have a cystic component and should be considered as a separate subtype of BA with its origin in prenatal life[23-24].

Pathologically BA is divided according with the extent of the fibrotic obliteration or absence of parts of the biliary tree (Fig. 1).

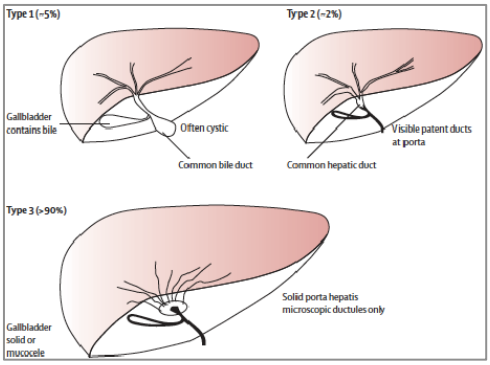

Fig1.: Classification of BA[1]

Cytokeratins are intermediate filaments expressed by epithelial cells in different combination depending on the epithelial cell subtype. Nineteen different types of CK were originally described[25]. In 1990 cytokeratin 20 (CK20) was added[26].

Biliary epithelial cells begin their development during the 7th week gestation and arise from hepatoblasts whose arrangement become uniform and bi-laminar around the ingrowing portal venule system. This arrangement is known as the ductal plate (DP). After about one week the bile ducts begin to branch out and radiate from the porta hepatis towards the periphery to conclude their development a few days later. In the same week of gestation, CK20 first appears in the developing intestinal mucosa[27].

Prior to the identification of CK20, only CK7, CK8, CK18 and CK19 were considered “liver related” cytokeratins. In 1998, Faa et al. showed, in rat models, that CK20 was expressed in bile duct cells and considered that CK20 was a marker of liver and bile duct maturation[28]. However, in the human, Sasaky et al. considered CK20 a marker of immaturity of bile-duct cells based on a study published in 2001. They showed that in fetal human liver CK20 was widely expressed on the developing bile-

duct but in older fetuses its expression diminished. Moreover CK20 was not expressed in "non-BA liver" while in some BA cases it was observed in intrahepatic bileduct cells or in the hepatocytes of the periportal zone[29].

In 2011, Zen et al. characterized the extent and prevalence of intestinal features (CK-20 expression) in otherwise normal gallbladder epithelium of infants and children. The authors showed that these features are physiologically present in gallbladder epithelium of children, particularly those younger than 6 years[30].

Symptoms and Screening

Jaundice is the key feature of BA. This together with pale stools, dark urine in an otherwise healthy infant are alarm signs which, if persistent (> two weeks), must be investigated.

Diagnostic Tools and Differential Diagnostics

Pregnancy is uneventful in more than 90% of cases of BA. All neonates with BA will have conjugated jaundice. Benign physiologic jaundice is characterized by an increasing fraction of unconjugated bilirubin. In contrast, all cases of BA will have persistent (>two weeks) conjugated bilirubin level >20 μmol/L, or a proportion of conjugated bilirubin >20%, and need urgent investigation[31]. Persistent acholic stools and dark urine in an otherwise healthy infant are alarm signs which and need to be investigated. More recent observations would suggest that virtually all cases of BA can be detected by conjugated bilirubin measurement in the 1st or 2nd day of life. This is an important point, having implications about onset and the value of screening[31].

Many infantile diseases present with cholestasis and aside from invasive procedures (e.g. operative cholangiography, needle biopsy) there are no specific diagnostic test which might exclude BA.

Although the serum gamma-glutamyl transferase (γGT) cannot be considered a diagnostic marker of BA, it is usually higher than in other intrahepatic cholestatic diseases[33-34]. Serum alkaline phosphatase also appears to be higher in BA than other cholestatic diseases. Serum cholesterol may be high, while triglycerides appear to be within the normal range. In almost all patients there is an increasing concentration of the hepatocellular liver enzymes (aspartate aminotransferase and alanine aminotransferase), reflecting on-going liver damage.

Abdominal ultrasound is the first specific investigation and may show an enlarged liver, absence of intrahepatic biliary dilation, and the absence or contraction of the gallbladder (usually after a 4 h

fast). In about 20%, the gallbladder and common bile duct are patent and visible on ultrasound. The value of more specific signs of BA on ultrasound is controversial. But, these may include a hyperechogenic liver hilum and the triangular cord sign[34].

Cholescintigraphy using a variety of technetium-labelled imino-diacetic acid derivatives (HIDA-scan) should be performed following 3 to 5 days oral phenobarbital and usually it shows a good hepatic uptake but an absent or reduced intestinal excretion of the isotope in the following 24 hours. Although cholescintigraphy is highly sensitive for BA, its specificity is low[35], in fact similar findings are sometimes reported in infants with severe intrahepatic cholestasis such as Alagille syndrome[36]. In contrast, the presence of the isotope in the intestine definitively excludes BA. However this diagnostic tool is still used in some Italian Centres but it has been abandoned in other Countries.

The role of magnetic resonance cholangiopancreatography (MRCP) is still debated due to technical difficulty in identification of the obliterated ducts. MRCP is useful in the evaluation of a cystic structure within the porta hepatis[37].

Liver histology obtained by percutaneous liver biopsy has been the usual diagnostic method in most specialist centres and it has approximately 90- 100% sensitivity and 80-85% specificity for biliary obstruction. The histological features are bile duct proliferation (Fig. 2), a small cell infiltrate (Fig. 6), ductular thrombosis, portal tract fibrosis (Fig. 6), ductular bile plugs
(Fig. 6) and absence of sinusoidal fibrosis. The presence of giant cell transformation might be confusing as this finding can be caused by other neonatal liver disease[39].

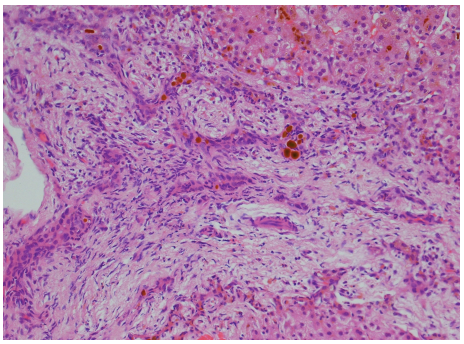

Fig. 2: Biliary atresia. An expanded portal tract showing fibroplasia,
a ductular reaction and ductular bile plugs, approximately 8 weeks after birth

If BA cannot be excluded after appropriate imaging studies then operative cholangiography (which can be undertaken either by laparoscopy or laparotomy) can be performed and represents the definitive investigation in the diagnosis of BA[1].

Treatment

The gold standard surgical approach for biliary atresia is the portoenterostomy procedure, as first described by Morio Kasai[5].

Many surgeons then divide the suspensory ligaments of the liver to allow complete mobilization in order to maximise exposure of the porta hepatis.

The fibrotic remnant of the extrahepatic bile ducts is dissected free, dividing first the common bile duct to allow it to be tracked back to the porta hepatis. It is then transected at the level of the liver capsule.

This transected portal plate is then anastomosed to a retrocolic 40cm jejunal Roux loop to restore biliary continuity.

The goals of the operation are to restore the bile flow to the intestine, reduce jaundice and halt on-going liver damage.

Several factors (experience of the surgeon, age at surgery, extent of liver damage) affect the success of Kasai portoenterostomy. Outcome is best defined as the achievement of a normal bilirubin (<20 umol/L or <2 mg/dL) value within 6 months of the procedure[40].

There is a pronounced effect of the age at surgery in the "developmental" variants of BA (i.e. cystic BA and BASM). However for those with isolated biliary atresia the effect is less clear (up to 90 days of age)[1]. Nevertheless the age at surgery probably remains the most widely quoted prognostic variable.

Histological features have a controversial prognostic value but some older authors consider ductule size as important[41-42].

A British survey showed the importance of surgical technical experience: the patients who underwent Kasai portoenterostomy at centres treating one case per year had significantly worse outcome than patients who underwent surgical treatment at centres performing > five cases per year.

This was true for 5-year native liver survival and overall survival[45]. The positive effects of the centralization program were published some years later[43-44].

Some children have initial restoration of bile flow after Kasai portoenterostomy but subsequently

develop progressive fibrosis and cirrhosis probably because of the persistence of the intrahepatic inflammatory processes. This has been shown to be present for at least 6 months after the procedure[45].

One of the most common complications after KPE is postoperative cholangitis and much of the postsurgical management is aimed at treating this complication. Its precise pathogenesis is still unclear and the incidence had not strong improvement despite the overall improvements of postoperative management. Although the use of postoperative prophylactic antibiotics postoperative is widely accepted, a recent systematic review showed that there are not satisfactory evidences supporting their use[46].

Complications

Ascending cholangitis is the more frequent complication after Kasai portoenteorostomy especially in the first postoperative year. This complication is due to the restoration of direct communications between intrahepatic bile ducts and the intestine. Early identification of cholangitis is very important in order to limit liver fibrosis, to prevent the loss of remaining patent bile ducts and to preserve the native liver function.

Portal hypertension and esophageal varices are two serious complications after Kasai portoenterostomy and they are due to the increasing of the liver fibrosis which raises the portal venous pressure. Progressive hepatosplenomegaly, gastrointestinal bleeding, ascites, encephalopathy, and hepato-pulmonary syndrome may be signs of portal hypertension.

Developing fibrosis and cirrhotic nodules is the natural progression of the liver affected by biliary atresia. Perhaps, one of the most dangerous complications of liver cirrhosis is the development of hepatocellular carcinoma. Fortunately only a small percentage of children with biliary atresia develop this kind of neoplasm and, in absence of the extrahepatic involvement, liver transplantation is the effective treatment[47].

Liver Transplantation

Biliary atresia is the most frequent reason for liver transplantation in children and this has completely changed the life prospects of these patients.

The presence of complications and the success of Kasai portoenterostomy influence the indications for liver transplantation.

Liver failure, persistent jaundice, recurrent cholangitis, variceal bleeding episodes, intractable

ascites and failure to thrive can be considered the most important indication for liver transplantation[48].

Transplantation is usually performed in the first 2 years of life in infants in whom bile drainage has not been achieved.

Outcomes

Several factors may influence the outcome of patients with biliary atresia. Although certainly Kasai portoenterostomy has strongly improved the prognosis of infants with biliary atresia, in literature a wide discrepancy exists in reported long-term postoperative results[49].

Age at surgical intervention remains a critical issue[50] and it reflects on the effectiveness of the referring primary care system and efficacy of the diagnostic process[51].

It is relatively uncontested that the more portoenterostomies you do as a Centre, the better the outcom even if centralization of patients has not been widely accepted.

Liver and biliary remnant histology may influence the outcomes but the evidence is poor and sometimes controversial. Some authors reported a worse native liver survival in patients with advanced liver fibrosis[52-53]. Certainly the extensive bile ductular proliferation is associated with poor prognosis[54].

Cytokeratins

Cytokeratins (CK) are a group of intermediate filament family proteins including more than 20 different gene products. They are characteristic for epithelial cells and give cell stability and intracellular organization. Moll et al. described a total of 19 cytokeratins[24] and later in 1990 he described cytokeratin 20 as a newer type of cytokeratin[26]. Since their discovery, cytokeratin expression has been well studied in human tissues and in animal models. In animal models, bile duct cells express CK20 in adult rat liver but this finding is absent in hepatocytes[25].

In humans while intrahepatic bile duct epithelial cells express CK8, CK18, CK7 and CK19, the normal adult human liver parenchymal cells express only CK8 and CK18[55].

CK20 first appears in the developing intestinal mucosa at embryonic week 8th[27] and it regulates the cell proliferation and differentiation along the crypt-villus axis[57]. Its expression seems to be entirely confined to the gastro-intestinal epithelium, to the urothelium and to Merkel cells[59-60].

Although theoretically the human liver and human bile ducts should not express CK20, a recent paper from Zen et al showed the presence of cells which express CK20 in children gallbladder[30].

Faa et al. investigated the immunoreactivity for CK20 in fetal and neonatal rat liver and induced bile ductular proliferation suggesting that CK20 should be considered, in the rats, as a ‘‘maturation’’ marker of biliary ducts[28]. This study also showed a strong CK20 expression in portal tracts of the hilar region and an increase in CK20 expression after birth. These findings confirmed the previous hypothesis that the process of maturation of the biliary tree starts at the liver hilum, from where it spreads through the liver[61], and that intrahepatic bile duct development may continues after birth[62].

Sasaki et al. examined more recently the degree of maturation and differentiation in the liver of children affected by biliary atresia comparing the findings with those in fetal and paediatric non-biliary atresia liver[29].

CK20 expression was observed more frequently in the early stage of foetal development and particularly in relation to the ductal plate and decreased later during fetal development. The authors therefore considered CK20 a marker of bile-duct cell immaturity, in contrast with the previous findings by Faa et al.

A recent paper quantitated the distribution of fibrosis and the extent of the transected biliary ductules. These variables had a relationship with liver biochemistry at the time of KPE. In this paper the authors found a homogeneous expression of CK7 in the biliary epithelium of the portal plate which was not related to the prognosis[63].

Aim of Study

The aim of this study was to analyse the expression of CK-20 in biliary remnants and liver specimens of patients who had undergone KPE. Our aim was to identify a relationship between CK-20 pattern in biliary remnant and liver specimens with other biochemichal variables both before and after KPE in order to establish if CK-20 expression could be prognostic marker.

Methods

The Department for Woman’s and Child’s Health of the University Hospital of Padova is a tertiary-referral Institution in Italy for the management of infants with liver disease since 1990.

We selected the liver and porta hepatis samples of infants with histologically-proven BA who underwent KPE between December 1999 and April 2017. Porta hepatis and liver samples were formalin-fixed and paraffin-embedded according to the standard protocol for diagnostic routine in use in the liver histopathology laboratory. The sections were treated with monoclonal mouse anti-cytokeratin 20 antibody (Ks 20.8, Cell Marque, 6600 Sierra College Blvd) (Fig 3A, 3B), obtained

from the supernatant and diluted in physiological solution to pH 7.3-7.7 (dilution 1:50).

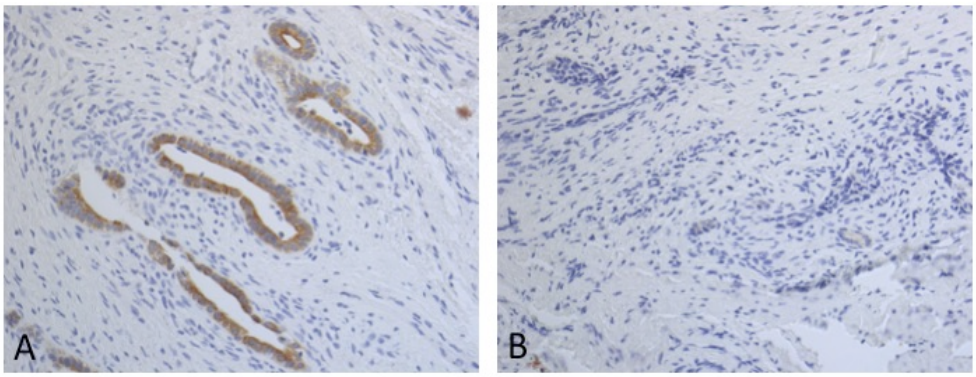

Fig. 3: A. positive expression of anti-CK20 antibodies in the tubular-glandular structures of the porta hepatis; **B.** negative pattern of anti-CK20 antibodies in the tubular-glandular structures of the hepatis porta

The samples were evaluated by the same pathologist who defined the presence or absence of CK20 staining in the bile ducts in liver and porta hepatis samples, the intensity (from 1 to 3, corresponding to low-medium-high intensity) and the distribution of CK20 on the sample from 0 to 100.

Patients without biliary epithelium on the sample were excluded. The distribution of the variable has been categorized according to the quartiles, as indicated in Table 1.

	Distribution of CK20 (liver)	**Distribution of CK20 (porta hepatis)**
25° percentile	0%	0%
50° percentile	10%	2%
75° percentile	45%	50%

Table 1: Percentiles of CK20 distribution

The distribution of CK20 in liver biopsy has been categorized and indicated as follows:

- 0: distributions belonging to the first two quartiles (<10% of the field)
- 1: distributions of CK20 belonging to the third quartile (10-45% of the field)
- 2: distributions of CK20 belonging to the fourth quartiles (≥ 45% of the field)

The distribution of CK20 in the biopsy of porta hepatis has been categorized as follows:

- 0: distributions belonging to the first two quartiles (<2% of the field). In this group no patient has a distribution equal to 1% and therefore "0" corresponds to patients CK20 negative (distribution with value 0%);
- 1: distributions belonging to the third quartile (<50% of the field) – named as *multifocal pattern*
- 2: distributions belonging to the fourth quartile (≥ 50% of the field) – named as *diffuse pattern*

Plasma concentrations of total bilirubin at onset, at 1, 3, 6 months post-Kasai (BT0, BT1, BT3, BT6), alanine transferase (ALT), aspartate transferase (AST) and γ-glutamyl transferase (γGT) were also measured, AST to platelet ratio index (APRi) was calculated.

The immunohistochemical findings (expression of CK20) with prognosis (established by success of Kasai procedure (defined as clearance of jaundice (< 20umol/L) within three months after KPE) and by native liver survival), and with biochemical liver function at presentation were compared.

Statistical analysis

Variables were analyzed with chi-square tests or Wilcoxon test. Native Liver Survival and overall survival analysis were performed using Kaplan-Meyer, Log Rank and Cox regression methods. Results were considered significant with $p < 0.05$.

Results

48 patients were treated with KPE or primary liver transplant at the University Hospital of Padova, Italy, between December 1999 and July 2018: 10 of them cleared their jaundice at 3 months, 29 patients were liver transplanted after KPE and 9 died (6/9 waiting LT, 3/9 after LT) (Figure 4). Expression, distribution and intensity were evaluated in samples of porta hepatis and liver (Table 2).

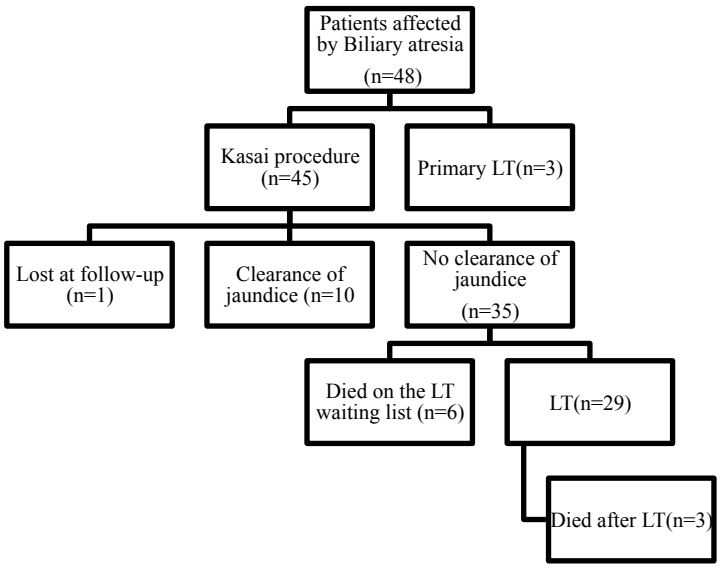

Figure 4. Outcomes of patients affected by BA

CK20	**n**	**%**
Expression (liver)	49	100%
CK20+	30	61.2%

Expression (porta hepatis)	48	100%
CK20 +	22	45.8%
Not evaluable	2	4.2%
Pattern of expression CK20	43	100%
0 (CK20- L, CK20-PH)	8	18,6%
1 (CK20-L, CK20+PH)	10	23,25%
2 (CK20+L, CK20-PH)	10	23,25%
3 (CK20+L, CK20+PH)	15	34,89%
Intensity (liver)	49	100%
0	19	38.8%
Low	19	38.8%
Medium	6	12.2%
High	5	10.2%
Distribution (liver)	49	100%
<10%	23	46,93%
10-45%	20	40,82%
>45%	6	12,24%
Distribution (porta hepatis)	46	100%
0%	22	47,82%
2-50%	13	28,26%
>50%	11	23,91%

Table 2. *Expression, intensity and distribution of CK20*

The expression of CK20, the distribution and the intensity of staining on the liver biopsy were not associated with a successful Kasai (p=0.69). Similarly, univariate analysis did not show any significant association with native liver survival for the expression (p = 0.91) and for the distribution of CK20 (p = 0.30).

The expression of CK20 at the porta hepatis was associated, with border-line significance (p=0.10), to a successful Kasai procedure. Interestingly, considering the distribution pattern of CK20 (distribution: 0%, <50% or ≥50%) (instead of the dichotomous expression of CK20), in our series the number of successful Kasai increases as the distribution(p = 0.048). By contrast the intensity of expression (0,1,2,3) was not associated to the success of Kasai procedure (p=0.18).

The expression of CK20 at the porta hepatis was associated to native liver survival (p = 0.046). This finding confirmed that the presence of CK20 reduced the risk of LT by 2,04 in the time unit. Moreover, the diffuse pattern of CK20 expression (≥50% distribution) at the porta hepatis was associated to an enhanced native liver survival (p = 0.015; HR = 0.62) (Fig.6). Univariate data analysis also suggested a borderline association (p = 0.086) between a high intensity of expression (3+) and the native liver survival.

Furthermore, even considering the expression of CK20 in the liver and in the porta hepatis together we did not find any significant correlations in terms of Kasai success and native liver survival (p = 0.33). (Table 3,4)

CK20	**Unsuccessful Kasai**	**Successful Kasai**	**χ2**	**p**
CK20 + (liver)	22 (59,45%)	6 (66,62%)	0,16	0,69
CK20 + (porta hepatis)	17 (47,22%)	7 (77,78%)	2,70	0,10
Intensity CK20 (liver) 0 low medium high	15 (40,54%) 14 (37,8%) 3 (8,11%) 5 (13,51%)	3 (33%) 3 (33%) 3 (33%) 0 (0%)	4,48	0,18
Intensity CK20 (porta hepatis) 0 low medium high	19 (52,78%) 9 (25%) 3 (8,33%) 5 (13,89%)	2 (22,22%) 2 (22,22%) 1 (11,11%) 4 (44,44%)	4,88	0,18
Distribution CK20 (liver) 0 (<10%) 1 (<45%)	19(51,35%) 15 (40,54%) 3 (8,11%)	3 (33,33%) 3 (33,33%) 3 (33,33%)	4,12	0,13

2 (>45%)				
Distribution CK20 (porta hepatis) 0 (0%) 1 (2-50%) 2 (>50%)	 19 (52,77%) 11 (30,56%) 6 (16,67%)	 2 (22,22%) 2 (22,22%) 5 (55,55%)	6,06	0,048
CK20 pattern 0 1 2 3	 23,52% 20,58% 23,52% 32,35%	 0% 33% 22,22% 44,44%	2,92	0,4

Table 3. Clearance of jaundice (Chi-quadro test)

	p	**HR**	**IC (95%)**
CK20 (liver)	0,9071	1,04	0,524
CK20 (porta hepatis)	0,046	0,49	0,242
Distribution CK20 (liver)	0,30		
Distribution CK20 10-45% (liver)	0,31	1,45	0,70
Distribution CK20 ≥ 45% (liver)	0,38	0,57	0,17
Distribution CK20 (porta hepatis)	0,053		
DistributionCK20 ≥50% (porta hepatis)	0,015	0,29	0,11
Distribuzione CK20 2-50% (porta hepatis)	0,47	0,74	0,34

Intensity CK20 (porta hepatis)	0,24		
Low	0,49	0,74	0,32
Medium	0,18	0,36	0,083
High	0,081	0,37	0,12

Table 4. Native liver survival analysis (Cox regression)

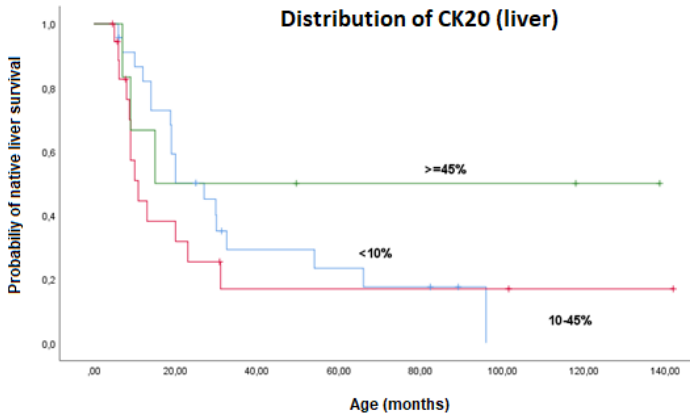

Figure 5. Native liver survival stratified for the distribution of CK20

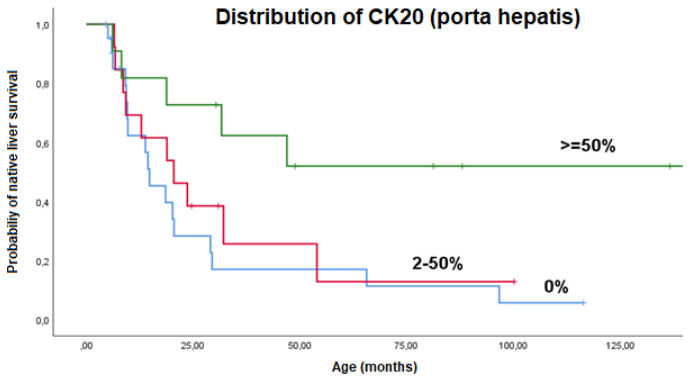

Figure 6. Native liver survival stratified for the distribution of CK20

	CK20- (Median)	**CK20+ (Median)**	**p**
APR index	1,33 (0,41-12,10)	0,96 (0,17-9,7)	0,10
BT0	162 (108-296)	162,8 (86-377)	0,54

BT1	135 (7-354)	132 (7-125)	0,64
BT3	179 (2,7-489)	135 (3-1506)	0,62
BT6	186 (3,5-392)	149 (3-749)	0,52
GGT0	485 (73-1688)	306 (73-1688)	0,13

Table 3. Comparison between patients expressing CK20 and patients not expressing CK20(Wilcoxon test)

We compared the expression of CK20 at the porta hepatis and the biochemilcal data. Although there were no significant results, our data showed that patients expressing CK20 at the porta hepatis had lower laboratory findings.

Discussion

CK20 is a marker of gastrointestinal and urothelial cells, discovered in 1990 and defined as "a biliary duct type"[6]. Physiologically, CK20 is not expressed in biliary epithelial cells in adult humans (who express CK7, CK8, CK9, CK18). A few studies have examined the expression of CK-20 in animals and humans but with contrasting results: in mice, CK-20 has been considered a marker of ‘‘maturation’’ in the biliary tree[6], by contrast CK-20 expression was considered a “marker of immaturity” in human bile ducts[4]. CK-20 was also detected in some intrahepatic bile-duct cells of children affected by BA[4]. More recently, Zen et al. described the expression of intestinal features in gallbladder epithelium of children, particularly in those younger than 6 years[5].

In literature the prognostic role of CK20 has never been explored and represents the aim of our study.

In 2015, an unpublished paper from King’s College Hospital of London investigated the expression pattern of CK20 at porta hepatis of 56 patients treated for BA affected by BA in order to evaluate its possible use as marker of development of the biliary tree or as a prognostic one. Clearance of jaundice post-KPE was less frequent in CK-20+ infants (56% vs. 80%; p= 0.28) and the need for liver transplant within 5 years was higher in the CK-20 + (porta hepatis) group (39% vs. 20%; p= 0.29) [Rif. *Unpublished data. Cytocheratin-20 expression in Biliary Atresia: a possible developmental*

marker. La Pergola E., Quaglia A., Davenport M. Presented at the British Association of Paediatric Surgeon Congress, London, 2016).

Our group has repeated the study at the University Hospital of Padova, Italy, evaluating also the NLS and the prognostic role of the intensity and distribution of CK20 in liver and porta hepatis samples.

In our population, the expression, distribution and intensity of CK20 in the liver specimens had not a prognostic value. By contrast, the CK20 diffuse distribution (> 50%) at the porta hepatis was associated with a better prognosis in terms of success of KPE procedure (p=0.048) and native liver survival (p=0.015). Patients with multifocal distribution pattern (<50%), in our serie, had a worse prognosis, overlapping those without expression of CK20 (CK20 negative).

Considering the poor literature and the limits of our study we can not explain these findings or understand if CK20 may be a marker of maturity or immaturity of the biliary epithelium. Assuming that CK20 could be a marker of bile duct maturity (as suggested by Faa et al.), the best prognosis could perhaps be explained by a conformation that makes the bile ductules more responsive to Kasai procedure. Zen suggested that CK20 is physiologically present in children, particularly those aged less than 6 years. Since gallbladder arises from the pars cystica of the hepatic diverticulum, we could suppose that this pattern of expression may be represented in all extrahepatic biliary tract, including porta hepatis: CK20 in porta hepatis could be physiologically present and its expression in extrahepatic biliary tract would not represent a pathological marker. To investigate this hypothesis, a large-scale prospective study of CK20 expression in patients with BA and controls would be necessary. The protective role of CK20 could perhaps be explained by the trend of anti-CK20 antibody to adhere to patent ducts but to validate this hypothesis, an extensive study of hepatic morphology is necessary. If our results were confirmed CK20 could be a prognostic marker to be evaluated at the moment of Kasai procedure. Although this factor will not probably modify the therapeutic algorithm for primary LT, it could have a clinical application. The evaluation of CK20, along with other clinical and laboratory findings, could be useful for the risk classification of the patients and them management (i.e. optimize the LT waiting list, set up the follow-up programs with supporting therapies and to manage the complications of liver disease). We believe that the possibility to identify a prognostic markers is important to inform and support the families (i.e. inform them for a perspective of liver transplantation).

Considering that liver and porta hepatis samples are routinely obtained at Kasai procedure, the pathologists could easily perform a morphological and immunohistochemical study as a routinary investigation.

Moreover the anti-CK20 antibody is used to mark urothelium and intestinal epithelium and is widely available. The cost of immunohistochemical analysis is elevated even if in considering the rarity of BA, we believe that the cost-benefit ratio is in favour of applying CK20 to patient management

Conclusion

To the best of the knowledge, this is the first study that finds evidences supporting the CK20 distribution pattern at the porta hepatis as a prognostic marker in BA. Larger multicentric studies are necessary and should be routinarie in BA even to validate the role of CK20 as prognostic marker.

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