Cytokeratin 7 and Cytokeratin 19 Expressions in Oval Cells and Mature Cholangiocytes as Diagnostic and Prognostic Factors of Cholestasis

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ABSTRACT

Background: *The activity of liver progenitor cells as bipotent liver stem cells, such as the oval cells, has been observed. The presence of oval cells and mature cholangiocytes forming hepatobiliary ductules may be applied to distinguish extrahepatic and intrahepatic cholestasis of the infants.*

Method: *This cross sectional study was performed on 40 paraffin-embedded sections consisting of 2 groups of 20 cases with extrahepatic and intrahepatic cholestasis of the infants from histophatological examination in Cipto Mangunkusumo hospital Jakarta between January 2000 and September 2011. The liver fibrosis grading was reevaluated by hematoxylin and eosin and also trichrome staining. The specimens were tested by immunohistochemical staining for cytokeratin (CK) 7 and CK 19 expressions in oval cells and mature cholangiocytes. The correlation between CK7/CK19 expressions in oval cells and liver fibrosis were analyzed by Spearman's correlation test.*

Results: *Expressions of CK7 and CK 19 on oval cells and mature cholangiocytes performed in hepatobiliary ductules, were significantly higher in extrahepatic than intrahepatic cholestasis with $p < 0.05$. CK7 and CK19 expressions in oval cells showed strong correlation with the degree of liver fibrosis with $r = 0.793$; $p < 0.05$ for CK 7 and $r = 0.827$; $p < 0.05$ for CK 19.*

Conclusion: *Expressions of CK7 and CK19, in oval cells and mature cholangiocytes, were higher at extrahepatic than intrahepatic cholestasis. Expressions of CK7 and CK19 in oval cells were directly proportional to the degree of liver fibrosis in cholestasis of the infants.*

Keywords: *cholestasis, oval cell, cholangiocytes, fibrosis, CK7, CK19*

ABSTRAK

Latar belakang*: Aktivitas sel progenitor hati sebagai sel punca hati yang bersifat bipotential yang dikenal sebagai sel oval sudah diteliti. Adanya sel oval dan kolangiosit matur yang membentuk duktulus hepatobiliaris dapat digunakan untuk membedakan kolestasis ekstrahepatik dan intrahepatik pada bayi.*

Metode*: Studi potong lintang dilakukan pada 40 blok parafin yang terbagi menjadi 2 kelompok yaitu 20 kasus kolestasis ekstrahepatik dan 20 kasus kolestasis intrahepatik pada bayi yang tercatat di laboratorium patologi anatomi rumah sakit Cipto Mangunkusumo pada bulan Januari 2000 sampai September 2010. Derajat fibrosis hati dinilai dengan pewarnaan hematoksilin dan eosin, dan juga trikrom. Kemudian dilakukan pewarnaan imunohistokimia untuk menilai ekspresi cytokeratin (CK) 7 dan CK 19 pada sel oval dan kolangiosit matur. Korelasi antara ekspresi CK7/CK19 pada sel oval dan fibrosis hati dianalisis dengan uji korelasi Spearman's.*

Hasil*: Ekspresi CK7 dan CK 19 pada sel oval dan kolangiosit matur yang membentuk duktulus biliaris hati lebih tinggi derajatnya pada kolestasis ekstrahepatik dibandingkan dengan intrahepatik dengan nilai $p < 0,05$. Ekspresi CK7 dan CK19 pada sel oval menunjukkan korelasi kuat dengan derajat fibrosis hati dengan nilai $r = 0,783$; $p < 0,05$ untuk CK7 dan $r = 0,827$; $p < 0,05$ untuk CK19.*

***Kesimpulan**: Ekspresi CK7 dan CK19 pada sel oval dan kolangiosit matur lebih tinggi derajatnya pada kolestasis ekstrahepatik dibandingkan intrahepatik. Ekspresi CK7 dan CK19 pada sel oval berbanding lurus dengan derajat fibrosis hati pada kolestasis bayi.*

***Kata kunci**: kolestasis, sel oval, kolangiosit, fibrosis, CK7, CK19*

INTRODUCTION

Cholestasis of the infants is a rare disease but still remains as an important problem since it frequently leads to severe and terminal liver disease and also death in children. Determination of types of cholestasis in infants is important, because it correlates to the determination of treatment and predict the prognosis.[1-7] There are still some similarities and overlapping microscopic features between extrahepatic and intrahepatic cholestasis, causing difficulties in histopathologic diagnosis when using common routine staining.[1,3-8] Severe liver damage to liver cirrhosis could be found in infants with cholestasis since 3 months of age; therefore, early diagnosis and rapid treatment might provide optimal results and increased life expectancy of the patients. If there is any occurrence of liver cirrhosis, liver transplantation would be the only ultimate option.[1-8] The success rate of surgical treatment performed in infant with cholestasis also depends on many factors, including the types of obstruction and involvement of liver progenitor cells in liver regeneration.[1,3-10]

Liver progenitor cell plays an important role on various pathological conditions in the liver. The presence one of such cells has been recognized as oval cell. Oval cells, which have been known as a facultative bipotent liver stem cells, are capable to differentiate between hepatocytes and cholangiocytes.[11-15] Oval cells do not exist or very rare in normal liver and it will multiply if there is any injury or severe inflammation in liver tissue, including in cholestatic liver. The activity of liver progenitor cells and study of liver stem cells have been observed but specific markers have not been found. Progenitor cells or ductular markers, such as cytokeratin (CK)-7 or CK19, were expressed on oval cells and also mature cholangiocytes in hepatobiliary ductules or known as ductular reactions.[16-19] The presence of oval cells and mature cholangiocytes forming hepatobiliary ductules may be applied to distinguish between extrahepatic or intrahepatic cholestasis. Both of these markers are expected to assist histopathologic diagnosis accuracy on difficult cases of cholestasis in infants.

The aim of this study was to learn the differences regarding the degree of CK7 and CK19 expressions on oval cells and hepatobiliary ductules in infant's liver biopsy between extrahepatic and intrahepatic cholestasis. Interestingly, the study was also intended to recognize the association of their expressions on oval cells with liver fibrosis grading. Markers of CK7 or CK19 are expected to be applied for diagnostic purpose and prognostic prediction of cholestasis in infants.

METHOD

This study used cross sectional design with the sample population was liver tissue specimens consisting of formalin-fixed paraffin-embedded sections, which had been diagnosed as extrahepatic or intrahepatic cholestasis. The sections were derived from pediatric patients aged 0 to 12 months, who were clinically diagnosed with cholestasis. All specimens were referred to histopathological examination in Cipto Mangunkusumo hospital Jakarta, between January 2000 and September 2010. The samples were collected by consecutive sampling. Each specimen should contain at least 5 portal areas and consequently was regarded as representative sample. Such samples were included in the study. If there was no clinical data about the patient's age (in months) or the liver biopsy was not representative, the sample would be excluded. The amount of samples in this study was 40 liver tissue biopsies of the infants on paraffin-embedded section consisting of 2 groups of 20 cases of extrahepatic and intrahepatic cholestasis.

Age in months and sex of patients were recorded. Histological liver fibrosis grading was reevaluated by hematoxylin and eosin as well as the trichrome staining as: F0/none, F1/portal fibrosis without septal involvement, F2/portal fibrosis with mild septal fibrosis, F3/portal fibrosis with severe septal fibrosis, and F4/cirrhosis. The subtype of histopathological diagnosis of cholestasis was also recorded. Histopathologic diagnosis of extrahepatic cholestasis was made based on the amount proliferative characteristics of hepatobiliary duct/ductular, in all of portal areas, which included portal widening and bile plug. Histopathologic diagnosis of intrahepatic cholestasis was made based on normal or the amount of hepatobiliary ductular non-proliferative characteristics (in idiopathic neonatal hepatitis) or less than normal or paucity of hepatobiliary ductular amount (in Alagille syndrome), along with giant cell hepatocytes and extramedular hemopoietic.[1,3,7]

Immunohistochemical studies were conducted by immunoperoxidase system and using primary antibodies, consist of mouse anti-human CK7

monoclonal antibody (1 : 150, Dako) and mouse anti-human CK19 monoclonal antibody (1 : 200, Novocastra). Antigen was retrieved from deparaffinized and rehydrated tissue by microwave oven in sodium citrate buffer solution with a pH of 6.5. Diaminobenzidine was used as chromogen and the sections were counterstained with hematoxylin. As a negative control, non immunized mouse immunoglobulin was substituted for the primary antibody. Expressions of CK7 and CK19 in oval cells was calculated by the number of oval cells which had small-sized single cell (about 10 μm, less than the size of cholangiocyte or hepatocyte and cluster of maximally 4 small-sized cells without forming of lumen hepatobiliary ductules). The cell appeared with brown positive stain on the membrane and cytoplasm in sinusoid zone 1 and it was limited to the plate at 5 periportal same areas as determined by observer; using light microscope with 200 x magnification, each case was subsequently scored as grade I, II or III. The determination was: grade I/ average range 0-10, grade II/average range 11-50, and grade III/average range > 50. Expressions of CK7 and CK19 in mature cholangiocytes performed on hepatobiliary ductules was determined by calculating cluster of 4 or more mature cholangiocytes with forming lumen of hepatobiliary ductules, which had brown positive stain on the membrane and cytoplasm in 5 portal same areas as had been determined by observer using light microscope with 200 x magnification. If there were many clusters of mature cholangiocytes with many lumens, 1 lumen would be calculated as 1 ductule. The average value of each case was calculated and was scored as score I or less than normal by average range < 0.5, score II or normal by average range 0.5-2 and score III or more than normal by average range ≥ 3. To unify our perception and to avoid bias, the calculation of cells was conducted by 2 observers before evaluation for quality control. Independent and blind interpretation was performed by each observer.[1,7,18]

In this study, unpaired t-test was performed to evaluate the significance of CK7/CK19 expressions in oval cells and mature cholangiocytes forming hepatobiliary ductules between 2 groups. The correlation between CK7/CK19 expressions in oval cells and liver fibrosis grading was analyzed by Spearman correlation test and the value of $p < 0.05$ was considered as statistically significant. Interrater agreement between 2 observers, was analyzed by MedCalc version 9.3.9, at least 10% of all samples studied.

RESULTS

Based on clinicopathological parameters, most of patients in intrahepatic cholestasis group were male aged under 3 months with subtype of neonatal hepatitis, fibrosis grading F0 and mild degree of CK7/CK19 expression in oval cells. On the other hand, most patients in extrahepatic cholestasis group were female and all patients had fibrosis grading of F4 and moderate to severe degree of CK7/CK19 expressions in oval cells with CK7/CK19 expressions in mature cholangiocytes performed on hepatobiliary ductules were more than normal. There were statistically significant results between 2 groups of CK7/CK19 expressions in oval cells and mature cholangiocytes that were performed on hepatobiliary ductules as shown on Table 1.

Table 1. Data distribution of intrahepatic and extrahepatic cholestasis

Variable	Intrahepatic	Extrahepatic	p
	n (%)	n (%)	
Sex			
Male	18 (90)	6 (30)	
Female	2 (10)	14 (70)	
Age (months)			
< 3	13 (65)	10 (50)	
≥ 3	7 (35)	10 (50)	
Subtype of cholestasis			
Neonatal hepatitis	16 (80)	-	
Progressive familial intrahepatic	2 (10)	-	
Alagille syndrome	2 (10)	-	
Extrahepatic biliary atresia	-	20 (100)	
Fibrosis grading			
F0	15 (75)	-	
F1	3 (15)	-	
F2	1 (5)	-	
F3	1 (5)	-	
F4	-	20 (100)	
Expression of CK7 in oval cells	(range 0-41)	(range 9-146)	< 0.05
Grade I	11 (55)	1 (5)	
Grade II	9 (45)	9 (45)	
Grade III	-	10 (50)	
Expression of CK19 in oval cells	(range 6-54)	(range 21-157)	< 0.05
Grade I	4 (20)	-	
Grade II	15 (75)	11 (55)	
Grade III	1 (5)	9 (45)	
Expression of CK7 in mature cholangiocyte	(range 0-34)	(range 1-115)	< 0.05
Less than normal	5 (25)	-	
Normal	7 (35)	-	
More than normal	8 (40)	20 (100)	
Expression of CK19 in mature cholangiocyte	(range 0-32)	(range 7-139)	< 0.05
Less than normal	2 (10)	-	
Normal	8 (40)	-	
More than normal	10 (50)	20 (100)	

Strength of agreement between 2 observers was fair until very good with weighted kappa value as shown in Table 2. There were positive correlations between CK7/CK19 expressions in oval cells and staging of liver fibrosis, with p value and correlation coefficient are shown on Table 3.

Table 2. Strength of agreement between 2 observers

Variable	Weighted Kappa	Value
Degree of CK7 expression in oval cell	1	Very good
Degree of CK19 expression in oval cell	0.333	Fair
Degree of CK7 expression in mature cholangiocyte	1	Very good
Degree of CK19 expression in mature cholangiocyte	0.727	Good

Table 3. Correlation between CK7/CK19 expressions in oval cells and liver fibrosis

Variable	p	Correlation coefficient
CK7 expressions in oval cell and staging fibrosis	< 0.05	0.793/strong
CK19 expressions in oval cell and staging fibrosis	< 0.05	0.827/very strong

Morphologic features of CK7/CK19 expressions in oval cells and mature cholangiocytes performed on hepatobiliary ductules or the ductular reactions in cholestatic liver of the infants are shown in Figure 1 and 2.

Sensitivity, specificity, and criteria or cut off point of CK7/CK19 expressions in oval cells and mature cholangiocytes performed on hepatobiliary ductules that differentiate the extrahepatic and intrahepatic cholestasis of the infants are shown on Table 4.

Table 4. Sensitivity, specificity, and criteria of CK7/CK19 expressions in oval cells and mature cholangiocyte forming hepatobiliary ductules or ductular reactions

Variable	Sensitivity	Specificity	Criteria
CK7 expression in oval cell	90.0	90.0	≤ 26.2
CK19 expression in oval cell	90.0	95.0	≤ 29.6
CK7 expression in mature cholangiocyte	95.0	95.0	≤ 8.8
CK19 expression in mature cholangiocyte	90.0	100.0	≤ 6

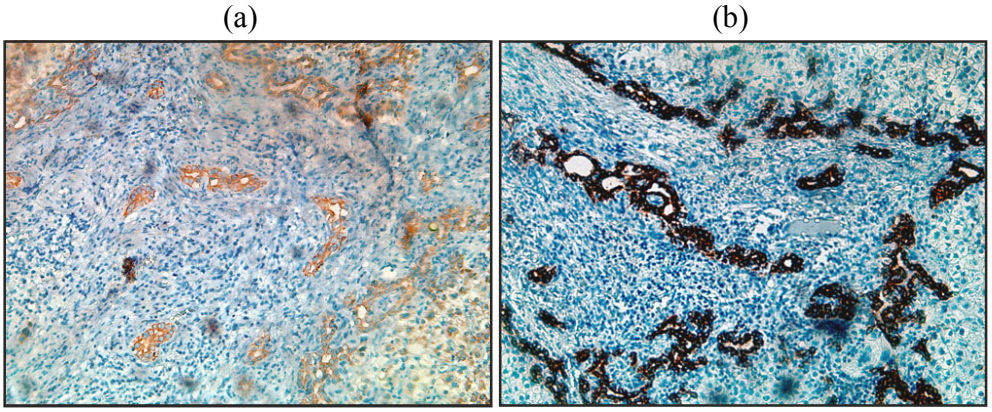

Figure 1. Degree of CK7 (a) and CK19 (b) expressions in oval cells by score III/severe and mature cholangiocyte forming ductular reactions by score III/more than normal of extrahepatic cholestasis with 200 x magnification in the 1 of the same portal area

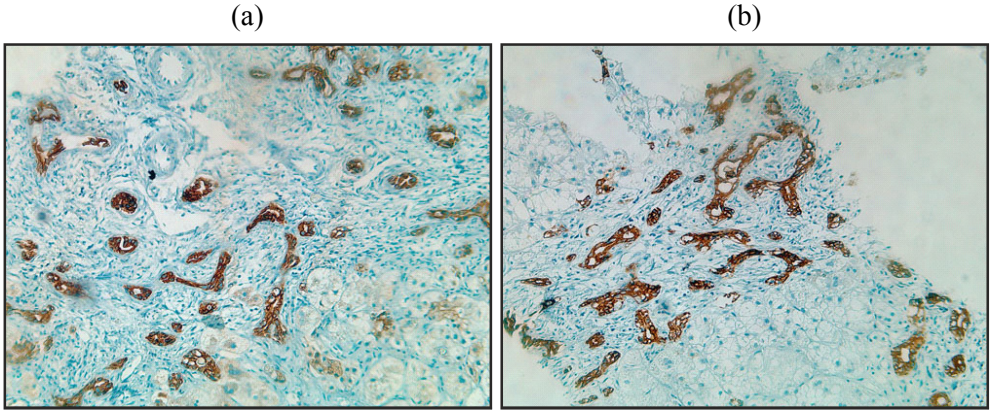

Figure 2. Degree of CK7 (a) and CK19 (b) expressions in oval cells by score II/mild-moderate and mature cholangiocyte forming ductular reactions by score III/more than normal of intrahepatic cholestasis with progressive familial intrahepatic cholestasis (PFIC) subtype and 200 x magnification in the same portal area

DISCUSSION

According to literatures, the prevalence of intrahepatic cholestasis is higher than extrahepatic and most patients are female in extrahepatic cholestasis group.[1-6,20] This study demonstrated similar results. Assessment of hepatobiliary duct/ductules and degree of liver fibrosis are several parameters that can be used to distinguish cases of intrahepatic or extrahepatic cholestasis as mentioned by Nayak et al.[21]

Another study demonstrated that CK 19 expressing the intrahepatic liver stem cells and mature cholangiocytes in liver tissue can also be used as a marker, which was reported by Theise et al and Hwang et al. However, they compared the normal liver with massive necrosis liver and also using other markers such as c-kit, alpha-fetoprotein (AFP), hepatocyte paraffin-1 and CD133.[22,23] Difficulty in identifying the canals of Hering (coH) without immunohistochemical examination has also been suggested by Theise et al and identification would be easier by using trichrome staining; however, such staining could not be more sensitive than immunohistochemistry.[22]

In this study, the degree of CK7/CK19 expression in oval cells and mature cholangiocyte forming hepatobiliary ductules or ductular reactions were higher in extrahepatic than intrahepatic cholestasis group. Such ductular reaction may be caused by severe inflammatory reaction that may occur in cholestasis diseases. In addition, the involvement of liver progenitor cells as oval cells also will increase the ductular reaction. The existence of a very strong correlation between ductular reactions and liver fibrosis in cholestasis has also been argumented by Pereira et al.[20]

Progression of the disease in extrahepatic cholestasis is higher than intrahepatic group. Incidence of liver cirrhosis has been found in 3-months-old infants who had extrahepatic cholestasis without any treatment. In addition, the number of oval cells will also increase and it is associated with the progression of liver disease.[1-6,20] The presence of liver fibrosis and cirrhosis in patients with cholestasis may occur in less than 3 months of age. It could be found in extrahepatic biliary atresia, which has not been resolved. On the other hand, in intrahepatic cholestasis group progressive familial intrahepatic cholestasis (PFIC) may lead to cirrhosis; however, the onset of symptoms is usually more than 6 months and cirrhosis may develop along with the duration of the disease. Portmann et al in his study found that cirrhosis in PFIC may occur in advanced stages in patients aged between 17 to 60 months. The opposite condition has been found in Alagille syndrome, in which the ductular reaction is absent. The absence of such ductular reaction would not trigger liver fibrosis consequently; however, fibrosis may occur but it is usually mild.[1,20]

This study demonstrated that there were strong or very strong positive correlations between expression of CK7/CK19 in oval cells and liver fibrosis grading in infants with cholestasis as shown on Table 3. It means that expressions of CK7/CK19 in oval cells were directly proportional to the degree of liver fibrosis in infant with cholestasis. The ability such marker to detect the degree of liver fibrosis early and to monitor disease progression will be associated with diagnosis and management of the disease and also predict the prognosis. It could be explained that liver fibrogenesis in cholestatic diseases is also influenced by the interaction of liver progenitor cells and hepatic stellate cells.

The strength of agreement between 2 observers on CK7/CK19 expressions in oval cells and mature cholangiocytes of this study was fair until very good as shown on Table 2. It could be explained since 2 observers had tried to get same perceptions when observing the cells by using dual heal microscope prior to evaluation to avoid bias and have a good quality control; therefore, the data was more accurately evaluated.

Criteria and cut off point of CK7/CK19 expressions in oval cells and mature cholangiocytes performed in hepatobiliary ductules of this study are shown on Table 4. It could be used to assist histopathological diagnosis accuracy for difficult cases of cholestasis in infants, to differentiate the extrahepatic or intrahepatic cause, as well as to predict the prognosis. Sensitivity of CK7 and CK19 expressions in oval cells are similar; but specificity of CK19 expression in oval cell is higher than CK7. Therefore, CK19 would be a better marker than CK7 for oval cell. In contrast, the marker of CK7 expression in mature cholangiocyte is more sensitive than CK19; however, it is less specific than CK19 expressions.

CONCLUSION

The degree of CK7 and CK19 expressions in oval cells and mature cholangiocytes performed in hepatobiliary ductules are higher for extrahepatic compared to intrahepatic cholestasis. The degree of CK7 and CK19 expressions in oval cells are directly proportional to degree of liver fibrosis in cholestasis of the infants.

SUGGESTION

There are still various questions of stem cells which can be investigated for further studies in this case, including: (1) how important is the role of intrahepatic liver stem cells, such as small hepatocytes; (2) what are the role of stem cells including extrahepatic liver stem cells derived from mesenchymal, hematopoietic,

embryonic or bone marrow. Further studies could be conducted regarding these issues with more specific markers.

Further studies should be performed to determine the factors affecting stem cells in liver regeneration, such as the role of growth factor or inflammatory cytokine by more specific markers. Advanced studies in the future are necessary in order to assess standard liver regeneration mediated through hepatocyte by using markers for fetal and mature hepatocyte differentiation.

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