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Th17-associated cytokines multiplex testing indicates the potential of macrophage inflammatory protein-3 alpha in the diagnosis of biliary atresia

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ABSTRACT

Background & aims: Biliary atresia (BA) is a neonatal obliterative cholangiopathy with high prevalence in south China. Accurate identification of BA among infants with obstructive jaundice is still difficult by noninvasive diagnostic tools. Th17 cells have been reported closely related with the development of BA, which suggest that Th17-associated cytokines were potential biomarkers for the diagnosis of BA patients.

Methods: In the training study, 76 infants who were divided into 2 groups, including BA group (n = 31) and non-BA jaundice group (n = 45). Clinical and routine laboratory data were collected from all subjects. Totally 25 Th17-associated cytokines were tested and compared between groups. The diagnostic value of each differential cytokine was evaluated by the area under the receiver operating characteristic curve (AUC). The best potential diagnostic biomarker was further validated in a cohort including 68 jaundice infants from our partnering institution in a blinded fashion.

Results: Data from the training study showed that gamma-glutamyl transferase (GGT) and clay stool would be helpful in the identification of BA patients in jaundice subjects. Th17-associated cytokines assay indicated that IL-17F, IL-10, macrophage inflammatory protein-3alpha (MIP3a), IL-22, IL-13, IL-33, IL-6, IL-17E, IL-27, IL-31, TNF-a and TNF-b were differentially expressed in BA patients, and the AUC of MIP3a was higher than other markers. MIP3a alone or combined with other laboratory data would significantly increase the diagnostic accuracy of BA. The diagnostic value of MIP3a was further confirmed in our validation study.

Conclusion: MIP3a alone or combined with other laboratory data would significantly increase the diagnostic accuracy of BA.

1. Introduction

Biliary atresia (BA) is a neonatal obliterative cholangiopathy characterized by a destructive inflammatory and fibrosclerotic process affects both intrahepatic and extrahepatic bile ducts [1]. The disease occurs on all continents, with variable geographical frequency. However, in south China, the disease prevalence is about 1/5000, which is much higher than other area [2,3]. If untreated, progressive liver cirrhosis leads to liver failure in 2 years. Liver transplantation is a curative treatment for patients with BA. However, due to the lack of donors, the timely Kasai portoenterostomy (KPE) is still a first line treatment for establishing the bile drainage in patients before 60 days [4,5]. Currently, identification of BA among infants with obstructive jaundice is still difficult by noninvasive diagnostic tools, such as Ultrasound, SPECT and MRI [6,7]. For example, the abdominal ultrasound of BA has a high specificity but needing experienced doctors [8]. Many infants with BA lost chance to have KPE because of delayed diagnosis [9]. In addition, it remains difficult for the clinician to identify cases that

Abbreviations: BA, biliary atresia; GGT, gamma-glutamyl transferase; AUC, area under the receiver operating characteristic curve; MIP3a, macrophage inflammatory protein-3alpha; KPE, Kasai portoenterostomy; TP, total protein; ALT, alanine aminotransferase; AST, aspartate aminotransferase

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should undergo intraoperative cholangiography. Exposing a newborn with cholestasis to unnecessary anesthesia and surgery is traumatic for both the baby and family.

The etiology of BA is multifactorial including defective embryogenesis, genetic factors, environmental toxins, viral infection, and abnormal inflammation and autoimmunity [2,3,10]. Among all proposed factors linked to the pathogenesis of biliary atresia, the immune system plays a central role which was evidenced by the infiltration of the liver by inflammatory cells and overexpression of cytokines and/or chemokines in BA patients at the time of diagnosis [2,11]. Recently, a novel T cell subject-Th17 has been reported closely related with the development of BA in both patients and animal models [12–14], which suggest that Th17 related cytokines were potential biomarkers for the early screening and diagnosis of BA patients [15]. Many of these cytokines have not been well studied in BA patients yet. Systematic study the Th17 cytokines in BA patients would help to identify more valuable markers for disease screening and diagnosis. The aim of this study was to evaluate the value of Th17-associated cytokines in the identification of BA patients.

2. Subjects and methods

2.1. Study design and population

Our study was divided into 2 parts. For the training study, we aimed to screening the potential diagnostic biomarker in Th17-associated cytokines. 76 jaundice infants suspected with BA referred to the Pediatric Surgery Department and Pediatric Department of the First Affiliated Hospital of Sun Yat-sen University from 2016 to 2017 were divided into two age and sex matched groups: BA group (n = 31) and non-BA jaundice group (n = 45). The diagnosis of BA was made by intraoperative cholangiography (n = 31). The diagnosis of the non –BA jaundice group were neonatal sepsis (n = 3), idiopathic neonatal hepatitis (n = 24), cytomegalovirus hepatitis (n = 5), cholestasis syndrome (n = 5), progressive familial intrahepatic cholestasis (n = 2), neonatal hemolytic jaundice (n = 6).

For the validation study, 68 jaundice infants from the Pediatric Surgery Department of Guangzhou women and children's medical center were enrolled and tested with our rule developed from the training study from 2017 to 2018. The diagnosis of BA was made by intraoperative cholangiography (n = 30). Cases without BA at intraoperative cholangiography and those with other causes of cholestasis were included in the non-BA group (n = 38; idiopathic neonatal hepatitis 20, cytomegalovirus hepatitis 5, Niemann–Pick 2, cystic fibrosis 3, congenital toxoplasma 1, glycogen storage disease 2, hemolytic jaundice 3 and progressive familial intrahepatic cholestasis 2). The serum samples were distributed as blinded aliquots to the clinical laboratory department of the First Affiliated Hospital of Sun Yat-sen University for cytokine detection. A written informed consent was signed by parents of each infant. The study was approved by the research ethics committee of the Sun Yat-sen University and conforms to the 1975 Declaration of Helsinki and its later amendments.

2.2. Etiological diagnosis

Full history taking, thorough clinical examination, routine laboratory investigations were performed in all subjects and a set of specific investigations according to the expected etiology, and liver biopsy for indicated cases were performed. Diagnosis of BA was confirmed by operative cholangiography findings prior to surgery.

2.3. Blood sample collection

Blood samples were collected from all subjects in serum separator tubes and were centrifuged, aliquoted, and frozen within 4 h. The samples were stored at −80 °C until analysis. All blood samples were collected before surgery or other invasive operations.

2.4. Routine laboratory test

A routine laboratory cholestasis test panel including bilirubin, gamma-glutamyl transferase (GGT), total protein (TP), albumin, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined in all subjects using a chemistry analyzer (AU5481, Beckman Coulter, CA, USA) according to the manufacture's instruction. Cytomegalovirus (CMV) and Hepatitis Virus B were determined by realtime PCR on ABI7500 thermocycler with commercial diagnostic kits (DAAN diagnostic corporation, Guangzhou, China).

2.5. Th17 cytokinesis multiplex assays

In the training study, the concentrations of Th17 cytokines were determined in serum samples of all subjects using the human Th17 Magnetic Bead Panel (EMD Millipore; Billerica, MA, USA) with a MAGpix™ instrument (MAGpix™ Luminex, CA, USA) according to the manufacturer's instructions. Totally 25 cytokines were analyzed including: GM-CSF, IFN-γ, IL-1β, IL-2(p70), IL-4, IL-5, IL-6, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17A, IL-25, IL-17F, IL-21, IL-22, IL-23, IL-27, IL-28A, IL-31, IL-33, macrophage inflammatory protein-3alpha (MIP3a or CCL20), TNF-a and TNF-b. The raw data were analyzed using MILLIPLEX Analyst V5.1 software (EMD Millipore; Billerica, MA, USA). Standard curves were generated from lyophilized standards provided with kit. The concentration for each analyte in each sample was determined via interpolation from each corresponding standard curve. All samples were analyzed in duplicate and batched to reduce potential inter-assay variability.

2.6. MIP3a ELISA assay

In the validation study, the concentration of MIP3a in the serum from subjects was determined using a sandwich ELISA kit (DM3A00; R& D Systems), according to the manufacturer's protocol. All samples were analyzed in duplicate and batched to reduce potential inter-assay variability.

2.7. Statistical analysis

Statistical analysis was performed using SPSS software version 20.0 for Windows (IBM Corp., Armonk, NY) and MedCalc 12.7.0.0 (MedCalc Software, Mariakerke, Belgium). Data distributions were assessed by the Kolmogorov-Smirnov test for normality. Data were expressed as mean ± standard deviation (SD) or median (range) values depending whether they had a Gaussian distribution. The statistical difference was calculated using analysis of variance, Chi-square test, Student's *t* test or the Mann-Whitney *U* test appropriately. The diagnostic value of serum markers was assessed by calculating the area under the receiver-operating characteristic (AUC) curve. The cutoff value for optimal clinical performance was determined from the ROC curves by the maximum of Youden index(sensitivity + specificity − 1). In this study, an AUC higher than 0.8 was considered as useful. The diagnostic performance was measured as sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). A two-tailed P value of less than 0.05 was considered statistically significant.

3. Results

3.1. Study population characteristics

In the training study, 76 infants were divided into the BA group (n = 31) with ages of 56.23 ± 16.02 days, the Non-BA jaundice group (n = 45) with ages of 54.84 ± 15.43 days (Table 1). Both groups were sex and age matched (P = 0.654 and 0.889 respectively). Our data

Table 1
Patients' demographic, clinical and laboratory data of all subjects.

	Training study			Validation study		
Index	BA group (n = 31)	Non-BA jaundice group (n = 45)	p value	BA group (n = 30)	Non-BA jaundice group (n = 38)	p value
Age (days)	56.23 ± 16.02	54.84 ± 15.43	0.889	59.30 ± 15.02	62.21 ± 16.91	0.765
Sex (male/female)	14/17	20/25	0.654	13/17	17/21	0.698
Clay stool[#]	29	30	< 0.001	30	23	< 0.001
Hepatomegaly	15	16	0.369	14	15	0.531
Splenomegaly	14	12	0.234	12	13	0.421
Ascites	10	12	0.214	9	11	0.214
Total bilirubin (mg/dL)	19.69 ± 5.23	16.01 ± 6.12	0.325	18.72 ± 8.21	17.23 ± 7.56	0.738
Direct bilirubin (mg/dL)	13.32 ± 3.49	10.25 ± 4.26	0.541	14.21 ± 5.21	12.25 ± 6.04	0.623
Total protein (g/dL)	5.21 ± 0.54	4.91 ± 0.62	0.214	5.31 ± 0.54	4.91 ± 0.62	0.237
Albumin (g/dL)	3.1 ± 0.48	3.6 ± 0.34	0.112	3.4 ± 0.72	3.7 ± 0.84	0.321
AST (U/L)	251.57 ± 121.99	241.22 ± 69.41	0.253	236.71 ± 110.63	221.22 ± 86.4	0.152
ALT (U/L)	150.31 ± 94.13	143.25 ± 76.35	0.682	145.21 ± 90.22	135.17 ± 74.35	0.714
GGT (U/L)[#]	648.02 ± 593.36	296.11 ± 236.23	0.041	701.03 ± 533.21	278.11 ± 127.21	< 0.001
Cytomegavirus (positive/negative)	10/21	16/29	0.598	8/22	10/28	0.362
Hepatitis virus B (positive/negative)	3/28	5/40	0.267	2/28	4/34	0.279

[#] Indicated a statistic significance between BA group and Non-BA jaundice group.

indicated that hepatomegaly, splenomegaly, TB, DB, TP, ALB, AST, ALT were comparable between groups ($P > 0.05$). However, comparison between the two groups indicated that GGT and Clay stool showed significant difference ($P = 0.041$ and $P < 0.001$ respectively). Similar findings were observed in the validation study (Table 1), Clay stool was seen in almost all BA infants (training study: 96%; validation study: 100%) and these patients tended to have higher GGT than other jaundice disease. As Cytomegalovirus (CMV) and Hepatitis Virus B were common infections in infants and may influence cytokine levels in the serum, we also reported the virus status of all patients and no significant difference was found between BA and non-BA subjects (Table 1).

3.2. Th17 cytokines screening in the training study

Totally 25 cytokines related with Th17 pathway were tested in the training study. The mean and SD of each cytokine in two groups was listed in Table 2. Among the tested cytokines, 12 cytokines showed significant difference between BA and non-BA infants, including IL-17F, IL-10, MIP3a, IL-22, IL-13, IL-33, IL-6, IL-17E, IL-27, IL-31, TNF-a and TNF-b (Table 2).

3.3. Diagnostic performance of Th17-associated cytokines in the training study

IL-17F, IL-10, MIP3a, IL-22, IL-13, IL-33, IL-6, IL-17E, IL-27, IL-31, TNF-a and TNF-b were further analyzed by ROC curves (Table 3). In our subjects, the AUCs of MIP3a, IL-13 and TNFa were higher than 0.8. The cutoff value for optimal clinical performance was determined from the ROC curves by the maximum of Youden index. At the level of > 435.14 pg/ml, the sensitivity and specificity of MIP3a was 90.40% and 80.0% respectively. At the level of > 815.12 pg/ml, the sensitivity and specificity of IL-13 was 66.67% and 90.91% respectively. At the level of > 148.32 pg/ml, the sensitivity and specificity of TNFa was 71.43% and 87.27% respectively. The AUC of IL-17F, IL-10, IL-22, IL-33, IL-6, IL-17E, IL-27, IL-31, and TNF-b were less than 0.8 (Table 4). We also evaluated the diagnostic value of GGT and Clay stool in our subjects. The AUC of GGT and Clay stool was 0.802 and 0.792 respectively.

3.4. MIP3a based combination test

In the training study, we aimed to screening the valuable marker for the diagnosis of BA. Theoretically, combing multiple cytokines would be beneficial in increasing the diagnostic accuracy. However, multiplex assay would increase the economic burden and was not widely available in clinical laboratory. So we tested whether single marker alone or combined with clay stool and GGT would increase the diagnostic accuracy. First, we performed a multiple regression to determine which cytokines contribute most to the diagnosis of BA. The results were similar with ROC curve analysis (Table 3) which showed that MIP3a was better than other cytokines in the diagnosis of BA. We further tested the diagnostic accuracy of clay stool + GGT, clay stool + MIP3a, GGT + MIP3a and clay stool + GGT + MIP3a in our subjects (Table 4). Our results showed that The AUC of clay stool + GGT, clay stool + MIP3a, GGT + MIP3a and clay stool + GGT + MIP3a was 0.824, 0.918, 0.880 and 0.892 respectively,

Table 2
Th17 cytokines profiling in training study.

Index	BA group (n = 31)	Non-BA jaundice group (n = 45)	p value
IL17F (pg/ml)	605.39 ± 439.10	353.07 ± 72.03	0.037*
GMCSF (pg/ml)	1376.99 ± 796.45	1244.58 ± 448.36	0.557
IFNγ (pg/ml)	235.49 ± 160.03	223.06 ± 90.99	0.091
IL10 (pg/ml)	53.59 ± 19.79	43.21 ± 17.27	0.002*
MIP3a (pg/ml)	697.52 ± 135.42	353.31 ± 157.55	< 0.001*
IL12 (pg/ml)	153.41 ± 74.30	143.88 ± 35.37	0.434
IL13 (pg/ml)	930.91 ± 313.42	581.94 ± 142.11	< 0.001*
IL15 (pg/ml)	115.74 ± 59.99	119.83 ± 36.36	0.091
IL17A (pg/ml)	141.51 ± 71.64	127.27 ± 36.06	0.061
IL22 (pg/ml)	1982.79 ± 265.4	1214.78 ± 233.58	0.003*
IL9 (pg/ml)	130.12 ± 57.30	113.55 ± 34.40	0.485
IL1b (pg/ml)	70.62 ± 31.08	68.69 ± 18.58	0.892
IL33 (pg/ml)	359.58 ± 190.82	283.83 ± 55.20	0.042*
IL2 (pg/ml)	103.64 ± 62.12	91.88 ± 35.90	0.607
IL21 (pg/ml)	389.8 ± 126.31	332.77 ± 62.50	0.086
IL4 (pg/ml)	854.17 ± 578.60	632.83 ± 210.08	0.054
IL23 (pg/ml)	72289 ± 2974.51	45009.07 ± 4541.69	0.201
IL5 (pg/ml)	95.28 ± 37.56	81.41 ± 20.47	0.140
IL6 (pg/ml)	136.53 ± 65.55	95.72 ± 32.70	0.005*
IL17E (pg/ml)	6065.49 ± 2978.05	3572.36 ± 758.49	0.006*
IL27 (pg/ml)	4048.35 ± 2622.05	2841.29 ± 429.27	0.031*
IL31 (pg/ml)	2638.56 ± 1858.56	1571.56 ± 261.14	0.013*
TNFa (pg/ml)	159.04 ± 33.43	104.53 ± 21.50	< 0.001*
TNFb (pg/ml)	1373.58 ± 493.78	1036 ± 197.63	0.038*
IL28a (pg/ml)	7061.86 ± 2218.79	5849 ± 1058.03	0.065

* Indicated for $p < 0.05$.

Table 3
Th17 related cytokines, GGT and clay stool in diagnosis of BA patients in the training study.

	AUC (95%CI)	Cut off	Sensitivity	Specificity	PPV	NPV
IL17F	0.707 (0.558–0.856)	> 486.25 (pg/ml)	57.14	89.09	66.70	84.50
IL10	0.737 (0.606–0.869)	> 51.17 (pg/ml)	61.90	83.64	59.10	85.20
MIP3a	0.897 (0.806–0.955)	> 435.14 (pg/ml)	90.48	80.00	63.30	95.70
IL13	0.812 (0.706–0.893)	> 815.12 (pg/ml)	66.67	90.91	73.70	87.70
IL22	0.765 (0.653–0.854)	> 1327 (pg/ml)	85.71	69.09	51.40	92.70
IL33	0.669 (0.552–0.773)	> 314.1 (pg/ml)	61.90	78.18	52.00	84.30
IL6	0.747 (0.634–0.840)	> 112.76 (pg/ml)	71.43	74.55	51.70	87.20
IL17E	0.758 (0.646–0.849)	> 4404 (pg/ml)	71.43	89.09	71.40	89.10
IL27	0.690 (0.573–0.791)	> 3201 (pg/ml)	57.14	87.27	63.20	84.20
IL31	0.739 (0.625–0.833)	> 2135 (pg/ml)	57.14	94.55	80.00	85.20
TNFa	0.813 (0.707–0.893)	> 148.32 (pg/ml)	71.43	87.27	68.20	88.90
TNFb	0.709 (0.594–0.808)	> 1280 (pg/ml)	57.14	87.27	63.20	84.20
GGT (U/L)	0.802 (0.731–0.885)	> 279 (IU/L)	86.35	72.53	63.30	91.30
Clay stool	0.7920 (681–0.875)	Positive	93.50	34.33	49.15	88.24

PPV: positive predictive value; NPV: negative predictive value.

the combination of MIP3a would significantly increase the diagnostic accuracy of BA in jaundice infants.

3.5. Diagnostic performance of MIP3a and combination test in validation study

In the validation set, we tested whether MIP3a alone or combination with clay stool and GGT would increase the diagnostic accuracy of BA in a partnering institution. As indicated by ROC curve (Table 5), the AUC of MIP3a, clay stool + GGT, clay stool + MIP3a, GGT + MIP3a and clay stool + GGT + MIP3a was 0.881, 0.824, 0.918, 0.880 and 0.892 respectively. The diagnostic value of MIP3a in the validation study is similar with the training study. And when MIP3a was combined with clay stool or GGT, the diagnostic specificity was significantly increased.

4. Discussion

BA is a multifaceted liver disease of complex pathogenesis, which has devastating consequences to child health worldwide [5]. The fibroinflammatory obstruction of the extrahepatic biliary tree leads to progressive jaundice and rapid fibrosis if not treated in a timely fashion [2,11]. However, making a definite diagnosis of BA is not easy as currently no single test could confirm the diagnosis preoperatively [7,9]. In addition, diagnosis is difficult to reach in developing countries due to the lack of specified diagnostic modalities, such as hepatobiliary scintigraphy, duodenal tube test and liver biopsy [2]. Serum biomarkers would be preferred than magnetic resonance or cholangiography in the screening and diagnosis of BA patients since that they are non-invasive, cost-effective and non-radiative. In addition, serum biomarkers were more promising in identifying BA patients earlier than clinical imaging.

Recent studies indicated that CD4 + Th17 cells and Th17-associated cytokines play an important role in the immune mediated injury against intrahepatic bile duct epithelial cells [12–14]. In this study, we aimed to evaluate whether Th17-associated cytokines can be applied in the differential diagnosis of BA in developing countries.

Our training study enrolled 76 infants who were further divided into two groups: BA groups, non-BA jaundice group. Our results indicated that the age and sex were comparable between groups. However, the comparison between BA groups and non-BA jaundice group showed only GGT and clay stool were significantly different. In our study, clay stool was a sensitive marker for the diagnosis of BA, but lack of specificity. GGT has been reported as a useful marker in the screening of BA patients, however, the accuracy varied from 75.1% to 86.8% in different studies [16–18]. Our results were consistent with previous studies [16,17,19,20].

We further parallel tested 25 Th17-associated cytokines in different group. The selection of these cytokines was according to the manufacture's suggestion, which were premixed in the assay panel. These cytokines were closely related with Th17 cell induction, activation, migration and secretion. In all 25 cytokines tested, 12 cytokines showed significant difference between two groups, including IL-17F, IL-10, MIP3a, IL-22, IL-13, IL-33, IL-6, IL-17E, IL-27, IL-31, TNF-a and TNF-b. In BA group. These cytokines were significant higher than in non-BA jaundice group. We noticed that Yang et al have reported elevated IL-17A in BA infants [21], however, the values of IL-17Awas not very good in differentiating BA from non-BA. We considered that the discrepancy might originate from different samples (serum vs PBMC) and detection methods (Luminex vs realtime PCR) used in the study. Since that our serum samples were obtained at the time point of Kasai-procedure, it would be difficult to make conclusions about the initial triggers of Th17 cell or related immunity in BA. However, the differential profile of

Table 4
Diagnostic performance of MIP3a combined with other markers in the training study.

Index	AUC (95%CI)	Sensitivity	Specificity	PPV	NPV	P value[#]	P value[☆]
MIP3a	0.897 (0.806–0.955)	90.48	80.00	63.30	95.70	…	…
clay stool	0.792 (681–0.875)	93.50	34.33	49.15	88.24	< 0.001*	0.012*
clay stool + MIP3a	0.928 (0.833–0.969)	90.32	93.33	90.30	93.30	0.156	
GGT	0.802 (0.731–0.885)	86.35	72.53	63.30	91.30	< 0.001*	0.026*
GGT + MIP3a	0.880 (0.785–0.943)	87.10	88.89	84.40	90.90	0.652	
clay stool + GGT	0.824 (0.720–0.902)	87.10	77.78	73.00	89.70	0.045*	0.035*
clay stool + GGT + MIP3a	0.892 (0.801–0.952)	80.65	97.78	96.20	88.10	0.865	

The cut-off value for MIP3a was > 435.14 pg/ml. The cut-off value for GGT was > 279 IU/L.
Comparison of AUC was analyzed by MedCal software using Delong's method.
* Indicated for $p < 0.05$; PPV: positive predictive value; NPV: negative predictive value.
[#] Comparison of AUCs between MIP3a and other index.
[☆] Comparison of AUCs between with and without MIP3a combination.

Table 5
Diagnostic performance of MIP3a in the validation study.

Index	AUC (95%CI)	Sensitivity	Specificity	PPV	NPV	P value[#]	P value[☆]
MIP3a	0.881 (0.790–0.960)	86.67	83.33	83.90	86.20	…	…
clay stool	0.720 (0.568–0.812)	100.00	40.00	62.50	100.00	< 0.001[*]	0.021[*]
clay stool + MIP3a	0.893 (0.795–0.962)	85.21	93.33	92.90	87.50	0.156	
GGT	0.763 (0.693–0.951)	86.67	53.33	65.00	80.00	< 0.001[*]	0.036[*]
GGT + MIP3a	0.833 (0.774–0.952)	80.00	86.67	85.70	81.20	0.652	
clay stool + GGT	0.817 (0.621–0.853)	93.33	70.00	75.70	91.30	0.045[*]	0.040[*]
clay stool + GGT + MIP3a	0.883 (0.795–0.962)	80.00	96.67	96.00	82.90	0.865	

The cut-off value for MIP3a was > 435.14 pg/ml. The cut-off value for GGT was > 279 IU/L.
Comparison of AUC was analyzed by MedCal software using Delong's method.
[*] Indicated for $p < 0.05$; PPV: positive predictive value; NPV: negative predictive value.
[#] Comparison of AUCs between MIP3a and other index.
[☆] Comparison of AUCs between with and without MIP3a combination.

cytokines in BA patients suggested an important role of Th17 cells involvement in BA and supported previous studies [12,13,22,23]. In addition, these cytokines were of potential value in the differential diagnosis of patients with obstructive jaundice.

We evaluated the performance of differential cytokines in the diagnosis of BA infants by ROC curves. And since that clay stool and GGT has been reported useful, these markers were also tested and compared. Our results showed that the AUC of MIP3a, was higher than other markers tested in our study. The sensitivity and specificity was 90.48% and 80.00% respectively in identifying BA patients from jaundice infants. The diagnostic performance of MIP3a was similar with hepatic scintigraphy (AUC: 0.899) and magnetic resonance cholangiography (AUC: 0.917) [20,24], however, detection of MIP3a would be non-invasive, non-radiative and more cost-effective. When compared with other biomarkers previously reported, MIP3a also showed advantage in sensitivity or specificity [16–18,25].

MIP3a, also known as cysteine motif chemokine ligand 20 (CCL20), is widely expressed in a variety of human endothelial cells and by different types of immune cells [26,27]. Previous study have showed that MIP3a overexpression caused by the biliary innate immune and cytokine response is closely related with recruitment of immune cells and subsequent inflammatory damage in bile ducts [28,29]. Further more, Th17 cell positioning near bile ducts is dependent on cholangiocyte-secreted CCL20 [30]. Gene expression signature study has also indicated that CCL20 was highly enriched in liver tissue from BA infants. The expression of CCL20 in BA was much higher than in negative control and disease control [31]. These results also support our findings, since that higher level of MIP3a was detected in BA patients and MIP3a accurately identified BA patients in our training study. In addition, the elevated MIP3a in serum would originate from fibroinflammatory liver tissue of BA infants. So MIP3a was selected for further evaluation. Our date showed that the addition of MIP3a would increase the AUCs of the routine laboratory data (clay stool and GGT). Both clay stool and GGT were lack of specificity in the diagnosis of BA, while the addition of MIP3a increase the specificity significantly. Generally, combining test would increase the diagnostic specificity and decrease the sensitivity. However, with the current cut-off value, both the specificity and sensitivity of MIP3a were excellent, so the combing test showed significant increase in specificity and minor changes in sensitivity.

In order to validate the diagnostic value of MIP3a, 68 jaundice infants from our partnering institution (Guangzhou women and children's medical center) were recruited and tested. In the validation study, MIP3a was detected by ELISA considering that the method is simple and widely available in most laboratory. The results were similar with our training study. In summary, our results showed that MIP3a alone or combined with clay stool and GGT would facilitate the right diagnosis of BA.

Limitations of our study should be noted. First, our study was conducted in well-defined subjects and the results should be carefully interpreted in patients complicated with other disease. Second, our data was from two medical centers in south China and which deserves further verification in broad range or in independent cohorts to increase the clinical value. Last, our samples were collected at the time point of Kasai-procedure, whether MIP3a is suitable for BA early screening in general population remains unclear.

5. Conclusions

The present study, with the use of infant samples obtained rigorously, analyzed the preoperative profiles of Th-17 associated cytokines and evaluated the diagnostic performance of these cytokines in the screening and differential diagnosis of BA infants. Our result indicated that MIP3a alone or combined with other laboratory data would significantly increase the diagnostic accuracy of BA.

Author contribution

Luyao Zhou and Junjian Lyu conceived and designed the study. Peisong Chen drafted the manuscript. Zhihai Zhong, Hong Jiang and Huadong Chen were involved with data generation, collection, assembly, analysis, and/or interpretation.

Conflict of interest

The authors declared that there is no conflict of interest.

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