

# MiR-200a/ZEB1 Pathway in Liver Fibrogenesis of Biliary Atresia

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## I. INTRODUCTION

**B**A is a cholestatic liver disease, which is characterized by biliary obstruction and inflammation with progressive liver fibrosis [1]. Kasai portoenterostomy (PE) may re-establish bile flow and is regarded as the first of line treatment of BA [2]. However, most BA patients still require liver transplantation due to progressive liver fibrosis and cirrhosis [3]. The aspartate aminotransferase to platelet ratio index (APRI) could benefit the diagnosis for significant liver fibrosis of BA patients and preoperative APRI also could predict jaundice persistence after PE [4]. Nevertheless, the pathogenesis of liver fibrosis in BA remains to be investigated.

MicroRNAs (miRNAs) are short non-coding RNA molecules (about 22 nucleotides), which regulate the expression of diverse genes by binding to complementary sites of targeted mRNAs, causing translational repression or degradation [5], [6]. Accumulating studies indicated that miR-200a might have a critical effect on the fibrotic processes of many organs by regulating the process of EMT [7], [8]. EMT relates to a process in which epithelial cells lose cell-cell contacts and the unique protein expression patterns of epithelia and acquire the phenotypic characteristics of mesenchymal cells or fibroblast markers [9], [10]. The levels of miRNAs were found to be present in the serum and plasma stably, and the specific expression patterns of circulating miRNAs might be related to various diseases [11]. It was demonstrated that miR-200a was significantly increased in the serum of BA patients [12]. Meanwhile, our previous study has shown that miR-200a was markedly elevated in the plasma of BA patients using Next-Generation Sequencing. However, additional experiments are still needed to identify the role of miR-200a in EMT of BA liver fibrosis. In the present study, we explored miR-200a alteration in liver tissues using TSA-FISH and used RT-qPCR to examine the miR-200a expression in plasma. We also used immunohistochemistry (IHC) to detect the hepatic expression of ZEB1, E-cadherin and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA). We found miR-200a increased and EMT persisted in BA, whereas different stages of liver fibrosis had different conditions of EMT, which suggests miR-200a may participate in the fibrogenesis process of BA by regulating EMT.

**Abstract**—Objective: Biliary atresia (BA) is characterized by progressive liver fibrosis. Epithelial-mesenchymal transition (EMT) has been implicated as a key mechanism in the pathogenesis of organ fibrosis. MiR-200a has been shown to repress EMT. We aim to explore the role of miR-200a in the fibrogenesis of BA. Methods: We obtained the plasma samples and liver samples from patients with BA or controls to examine the role of miR-200a. Histological liver fibrosis was assessed using the Ishak fibrosis scores. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed to detect the expression of miR-200a in plasma. We also evaluated the expression of miR-200a in liver tissues using tyramide signal amplification fluorescence in situ hybridization (TSA-FISH). The expression of EMT related proteins zinc finger E-box-binding homeobox 1 (ZEB1), E-cadherin and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) in the liver sections were detected by immunohistochemical staining. Results: We found that the expression of miR-200a was both elevated in the plasma and liver tissues from BA patients compared with the controls. The hepatic expression of ZEB1 and  $\alpha$ -SMA were markedly increased in the liver sections from BA patients compared to the controls, whereas E-cadherin was downregulated in the BA group. Simultaneously, we noted that the hepatic expression of miR-200a, E-cadherin and  $\alpha$ -SMA were upregulated with the progression of liver fibrosis in the BA group, while ZEB1 was downregulated with the progression of liver fibrosis in BA patients. Conclusion: These findings suggest EMT has a critical effect on the fibrotic process of BA, and the interaction between miR-200a and ZEB1 may regulate EMT and eventually influence liver fibrogenesis of BA.

**Keywords**—Biliary atresia, liver fibrosis, MicroRNA, epithelial-mesenchymal transition, zinc finger e-box-binding homeobox 1.

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## II. MATERIALS AND METHODS

### A. Samples and Pathology

Plasma samples were collected from 25 patients with BA and 10 healthy control subjects. A total of 12 liver biopsies were obtained from BA patients at the time of Kasai PE. As controls, we obtained normal liver tissues from two child patients whose liver pathology were normal, including one core needle biopsy and one surgical wedge biopsy (Table I). The samples were collected at the time of diagnosis in Guangzhou Women and Children's Medical Center (Guangzhou, China). BA were identified by operative exploration, cholangiography, and histology [13]. The control plasma samples were from individuals who came in for a routine physical examination. The tissue specimens were fixed in formalin, embedded in paraffin, sliced, and stained with hematoxylin -eosin (H&E) and Masson's trichrome for light microscopy analyses. Histological liver fibrosis was assessed by Ishak (0-6) fibrosis scores by two experienced pathologists, blinded to the clinical patient data, until consensus was reached [14]. The Ethics Committee of Guangzhou Women and Children's Medical center approved this study.

TABLE I  
BASIC CHARACTERISTICS OF SAMPLES FROM SUBJECTS

|                   | BA plasma<br>(n=25) | Control plasma<br>(n=10) | BA liver<br>(n=12) | Control liver<br>(n=2) |
|-------------------|---------------------|--------------------------|--------------------|------------------------|
| Gender(M/F)       | 12/13               | 8/2                      | 7/5                | 2/0                    |
| Age at collection | 74±26 days          | 117±45 days              | 89±18 days         | 53±42 months           |
| Age at operation  | 81±25 days          | —                        | 89±18 days         | 53±42 months           |
| ALT (U/l)         | 161±118             | 31±7                     | 247±219            | 19±10                  |
| AST (U/l)         | 279±309             | 43±10                    | 418±671            | 50±14                  |
| γ-GT (U/l)        | 880±704             | 38±36                    | 849±429            | 45±21                  |
| TBIL (μmol/l)     | 158.4±53.3          | 8.6±7.0                  | 154.0±71.0         | 4.7±1.1                |
| DBIL (μmol/l)     | 122.8±39.4          | 2.9±2.7                  | 122.0±54.1         | 3.1±1.1                |

Data presented as number of patients or means ±standard deviation. ALT: alanine aminotransferase; AST: aspartate aminotransferase; γ-GT: γ-glutamyl transpeptidase; TBIL: total bilirubin; DBIL: direct bilirubin. Reference intervals: ALT (9-50 U/l), AST (5-60 U/l), γ-GT (13-57 U/l), TBIL (2-17 μmol/l), DBIL (0-7 μmol/l).

### B. RT-qPCR

The total RNA of plasma was extracted using the Plasma/Serum Circulating RNA Purification Kit (Norgen, 42800, Canada). OD260/280 ratios typically ranged from 1.8 to 2.0, showing high RNA purity. 5 μl of RNA was reverse transcribed with a miRNA-specific stem-loop primer using the PrimeScript® RT reagent Kit (Takara, RR037A, Japan). Polymerase chain reaction (PCR) was carried out using the SYBR® PrimeScript miRNA RT-PCR kit (Takara, RR716, Japan). All PCRs were performed by the Applied Biosystems 7300 System (ABI, USA). MiR-16 served as a control [15], [16]. The sequences of the primers are as follows: miR-200a: 5'- GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACT GGATACGACACATCG-3' (RT primer), 5'- GCGGTAACACTGTCTGGTAACG-3' (Forward), 5'- CGCAGGGTCCGAGGTATTC-3' (Reverse); miR-16: 5'- GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTG GATACGACCGCAA-3' (RT primer), 5'-

TGGCGTAGCAGCACGTAAT-3' (Forward), 5'- CAGTGCAGGGTCCGAGGTAT-3' (Reverse).

### C. TSA-FISH

Liver sections were first deparaffinized with xylol and hydrated using descending concentrations of ethanol. After washing twice in phosphate-buffered saline (PBS) for 10 min, slides were immersed in 0.2 N HCl for 20 min and incubated with Proteinase K (200 μg/ml in PBS) at 37 °C for 12 min. After digestion, the slides were washed twice (for 5 min each) in PBS, immersed in 3% H<sub>2</sub>O<sub>2</sub> for 30 min, and fixed in 4% paraformaldehyde for 15 min. The slides were then pre-hybridized with a hybridization buffer at 55 °C for 1 h in a humid chamber, followed by hybridization with a probe at 39 °C for 20 h. The slides were washed thrice in 2×SSC (for 8 min each), blocked with 1% bovine serum albumin for 1 h at 37 °C, and incubated with HRP-labeled anti-digoxigenin antibody (Bellancom, USA) diluted 1:100 at 37 °C for 90 min. For tyramide signal amplification, freshly prepared tyramide-Cy3 working solution (1 part tyramide and 1 part 0.15% H<sub>2</sub>O<sub>2</sub> mixed with 50 parts amplification buffer, invitrogen, USA) was applied to the sections for 15 min at 37 °C. Slides were washed thrice in PBS (for 8 min each) and mounted in 4',6-diamidino-2-phenylindole (DAPI). The miR-200a probe was purchased from Exonbio Lab (Guangzhou, China). The sequence of the probe is 5'- ACATCGTTACCAGACAGTGTTA-3', the 5' and 3' ends were modified with digoxigenin. The U6 probe with the sequence of the 5'- GAACGCTTCACGAATTTGCGTGTTCATCCTTGCGCA -3' served as a positive control, and a hybridization buffer was used as a negative control. Images of miRNA signals were captured by a Nikon 80i fluorescence microscope. The exposure time for all the images captured in the present study was 0.1 seconds. A total of five random images were used to calculate the mean gray value of a sample (× 200 magnification). The green signals of images were used to analyze the mean gray value using image J software.

### D. IHC

The liver sections were incubated with xylol and descending concentrations of ethanol. Endogenous peroxidases were removed with 3% H<sub>2</sub>O<sub>2</sub>. After antigen heat retrieval for 15 min in a citrate buffer (pH 6.0), sections were returned to room temperature and washed twice (for 5 min each) in PBS. The primary antibodies (rabbit anti-human ZEB1 polyclonal antibody, rabbit anti-human E-cadherin polyclonal antibody and rabbit anti-human α-SMA polyclonal antibody, 1:100 dilution, Bioss, Beijing, China) were then applied at 4 °C overnight in a humid chamber after blocking with 3% bovine serum albumin for 1 h at room temperature. The sections were rinsed in PBS and incubated with the secondary antibody (Gene Tech, Shanghai, China). Next, sections were washed twice (for 5 min each) in PBS, stained with 3, 3'-diaminobenzidine tetrahydrochloride (DAB), and counterstained with hematoxylin. The sections were visualized using a Nikon 80i fluorescence microscope. Five random

images were used to calculate the percentage of the positive staining area of a sample ( $\times 400$  magnification). Image Pro Plus 6.0 was used to determine the percentage of the positive staining area of ZEB1, E-cadherin and  $\alpha$ -SMA.

#### E. Statistical Analyses

Data are reported as the means  $\pm$  standard deviation or medians. The levels of miR-200a measured by RT-qPCR were presented as the fold change ( $2^{-\Delta\Delta Ct}$ ). When comparisons were made between the two groups, and the statistical significance was determined by Mann-Whitney U-test using the SPSS 13.0 software program. A P value less than 0.05 was considered statistically significant.

### III. RESULTS

#### A. Histopathological Examination

Widespread inflammation and fibrosis were noted in the hepatic portal area of BA group using H&E staining. We also observed evident bile canalicular hyperplasia, degeneration and necrosis, which were accompanied by increased liver fibrosis, damage of the lobular structure. Collagen fibers significantly increased in BA samples using Masson's trichrome staining (Fig. 1). According to H&E staining and Masson's trichrome staining, the liver fibrosis of BA samples was assessed by Ishak fibrosis scores (Table II).

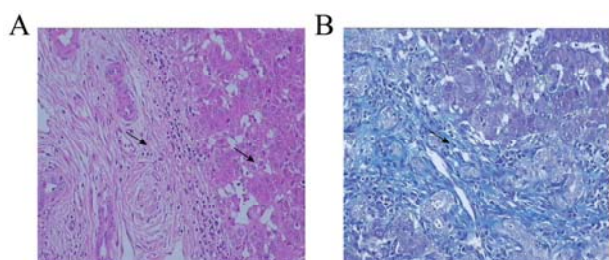


Fig. 1 Histopathological examination of liver sections from BA patients: (A) Extensive inflammation and fibrosis (arrow) were observed in the hepatic portal area. Hepatocytes (arrow) emerged degeneration and necrosis (H&E staining;  $\times 200$  magnification). (B) Collagen deposition (arrow) was marked in the portal tract and septum area (Masson's trichrome staining;  $\times 200$  magnification)

TABLE II

| ISHAK LIVER FIBROSIS SCORES OF BA PATIENTS |   |   |   |   |   |   |   |
|--|---|---|---|---|---|---|---|
| Ishak fibrosis scores                      | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
| BA cases                                   | 0 | 1 | 3 | 3 | 0 | 2 | 3 |

Footnote: Data presented as number of patients.

#### B. Expression of miR-200a in BA Plasma vs Controls

We quantitatively detected the expression of miR-200a in plasma. RT-qPCR showed difference of the miR-200a expression between the two groups (Fig. 2). We found miR-200a was markedly elevated in the BA group compared to the controls (3.25 vs 1.35,  $P < 0.01$ ).

#### C. Expression of miR-200a in Liver Tissues Refers to Liver Fibrosis in BA

We evaluated the expression of miR-200a in liver specimens. We found miR-200a was significantly upregulated in the BA group compared to the control group ( $11.92 \pm 2.18$  vs  $3.44 \pm 1.00$ ,  $P < 0.05$ ) (Fig. 3 (A)). According to the Ishak fibrosis scores, the BA specimens that were scored as 1, 2, and 3 were combined into the low graded fibrosis group, which were scored as 4, 5, and 6, were combined into the high graded fibrosis group. We found miR-200a increased prominently in the high graded fibrosis group compared to the low graded fibrosis group ( $13.37 \pm 1.78$  vs  $10.89 \pm 1.90$ ,  $P < 0.05$ ) (Fig. 3 (B)).

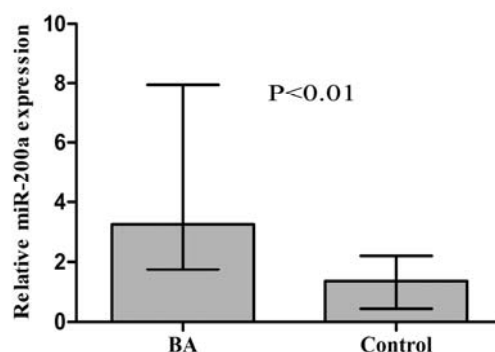


Fig. 2 Expression of miR-200a in plasma. MiR-200a was markedly elevated in the BA group compared to the control group. The bar represents the median, and the line across the whiskers represents the interquartile range

#### D. Expression of ZEB1, E-Cadherin and $\alpha$ -SMA in BA Liver Tissues vs Controls

We analyzed the expression of ZEB1, E-cadherin and  $\alpha$ -SMA in the liver sections (Fig. 4). Positive ZEB1 staining was detected predominantly in the cell nucleus. The percentage of the ZEB1 positive staining area in the BA group was 2.40%, while it was 0.21% in the control group ( $P < 0.05$ ). The expression of E-cadherin was markedly decreased in the BA group compared to the control group (8.93% vs 23.14%,  $P < 0.05$ ). The BA group also had much higher  $\alpha$ -SMA expression compared to the controls (8.66% vs 0.76%,  $P < 0.05$ ).

#### E. Expression of ZEB1, E-Cadherin and $\alpha$ -SMA Refer to Liver Fibrosis in BA

The same as the former, the BA specimens were divided into two groups. As shown in Fig. 5, the expression of E-cadherin and  $\alpha$ -SMA were remarkably increased in the high graded fibrosis group compared to the low graded fibrosis group (15.17% vs 3.80%,  $P < 0.05$ , 23.73% vs 3.93%,  $P < 0.05$ , respectively), whereas the expression of ZEB1 was prominently decreased in the high graded fibrosis group compared to the low graded fibrosis group (0.74% vs 3.62%,  $P < 0.05$ ).

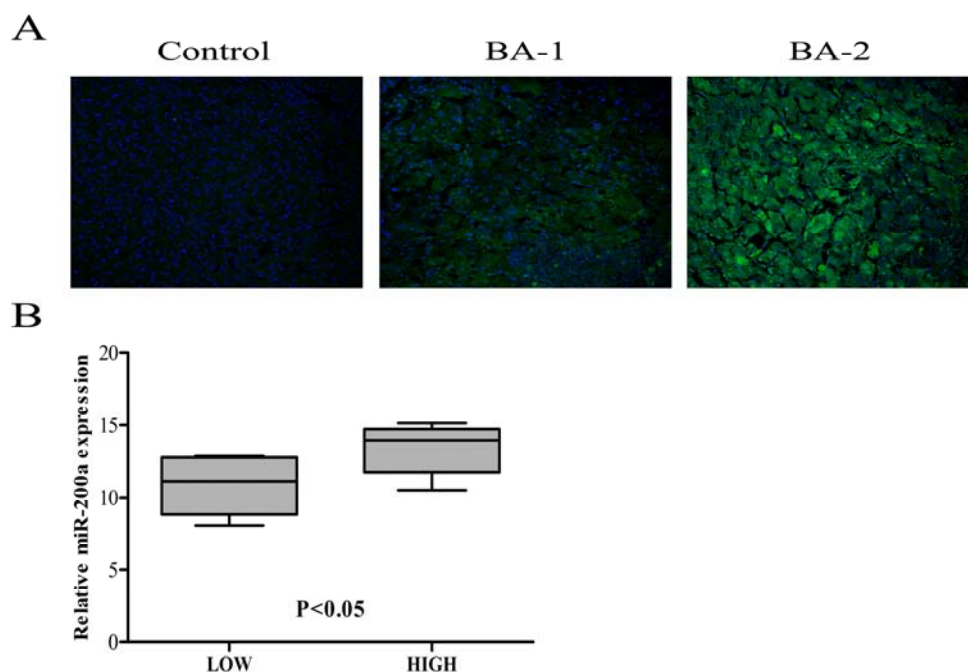


Fig. 3 Expression of miR-200a in BA liver tissues. BA-1 represents the low graded fibrosis group and BA-2 represents the high graded fibrosis group. (A) The BA group exhibited much higher expression of miR-200a than the control group ( $\times 200$  magnification). (B) MiR-200a significantly increased in the high graded fibrosis group compared to the low graded fibrosis group. The box represents the interquartile range, line across the box median, and the whiskers 90th percentile range. LOW: low graded fibrosis group; HIGH: high graded fibrosis group

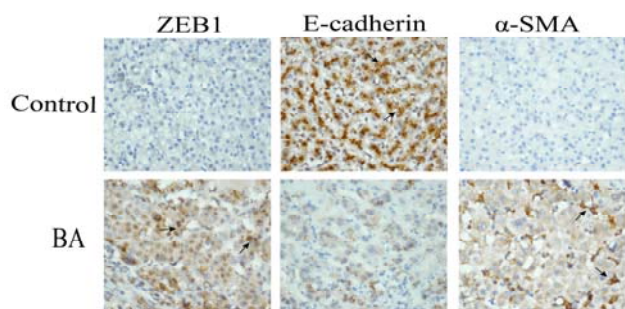


Fig. 4 Expression of ZEB1, E-cadherin and  $\alpha$ -SMA in BA liver tissues vs controls. ZEB1 was expressed predominantly in the cell nucleus and markedly increased in the BA group compared to the control group (arrow). For E-cadherin staining, the control group exhibited more positivity compared to the BA group (arrow). The BA group showed strong  $\alpha$ -SMA immunoreactivity (arrow) and had a much higher expression of  $\alpha$ -SMA compared to the control group ( $\times 400$  magnification)

#### IV. DISCUSSION

MiRNAs are small noncoding RNAs consisting of about 22 nucleotides that negatively regulate the expression of protein-coding genes. Many reports have shown that miR-200a might have a crucial role in organ fibrosis [7], [8], [17]. A previous study indicated that miR-200a increased in liver fibrosis, and was upregulated in a fibrosis progression dependent manner [18]. It also has been demonstrated that miR-200a was prominently upregulated in liver tissues from BA patients, and the upregulation of miR-200a could enhance bile duct proliferation through the IL-6/STAT3 pathway [19]. There were significant histological evidences of EMT in the BA

livers [20]. Deng et al. have demonstrated that biliary epithelial cells from patients with BA showed elevated expression of  $\alpha$ -SMA and S100A4, with a marked transition to a fibroblast-like morphology, and the biliary epithelial cells undergoing EMT might lead to significant bile duct proliferation and directly contribute to fibrogenesis in BA according to the significant histologic evidence [21]. Harada et al. have found that biliary epithelial cells in BA exhibited a lack of epithelial markers and an abnormal expression of vimentin, suggesting that the EMT may result in the occurrence and development of sclerosing cholangiopathy in BA [22]. MiR-200a might repress EMT by directly targeting the mRNA of the E-cadherin transcriptional repressors ZEB1/ZEB2, which were simultaneously the accelerators of EMT [23]. EMT could contribute to the production of more myofibroblasts, which excreted too much extra cellular matrix and furtherly caused organ fibrosis [24], [25]. However, the functional mechanisms of miR-200a in the fibrosis process of BA remain unclear.

In this project, expression of miR-200a was remarkably upregulated in plasma from BA patients compared to the controls. Meanwhile, miR-200a was significantly elevated in liver tissues from BA patients compared to the controls, which was associated with increased liver fibrosis, suggesting that miR-200a specifically increases in BA and miR-200a possibly participated in regulating BA liver fibrosis. These results prompted us to assess the role of miR-200a in the fibrogenesis of BA. It was well known that miR-200a could depress the expression of ZEB1 post-transcriptionally via the 3'-untranslated region (3'-UTR), thereby reducing the repression

at E-cadherin and depressing the process of EMT [23]. In this study, even though miR-200a was markedly increased in BA, the expression of ZEB1 was also upregulated. Simultaneously, expression of E-cadherin was downregulated in BA while expression of  $\alpha$ -SMA was increased. It has been shown that hepatic stellate cells could transdifferentiate into myofibroblasts, which was accompanied with  $\alpha$ -SMA expression [26]. These findings suggest EMT remarkably appears in BA livers and the persistence of EMT may play an important part in BA liver fibrosis; EMT contributes to inducing generation of myofibroblasts and myofibroblasts dramatically emerging in BA, which promote the fibrosis process. However, the upregulation of miR-200a did not

depress the expression of ZEB1. It was indicated that ZEB1 could also repress miR-200a through binding to ZEB-type E-box elements, and there was a double-negative feedback loop controlling the ZEB1 and miR-200a expressions which regulate the cellular phenotype [27], suggesting that the interaction between ZEB1 and miR-200a probably has an important role in BA liver fibrosis. ZEB1 could decrease the expression of E-cadherin and accelerate the process of EMT, and the upregulation of miR-200a might aim to effectively repress the expression of ZEB1 and the process of EMT, which could eventually retard the fibrosis process. It might be a self-regulation mechanism of the human body.

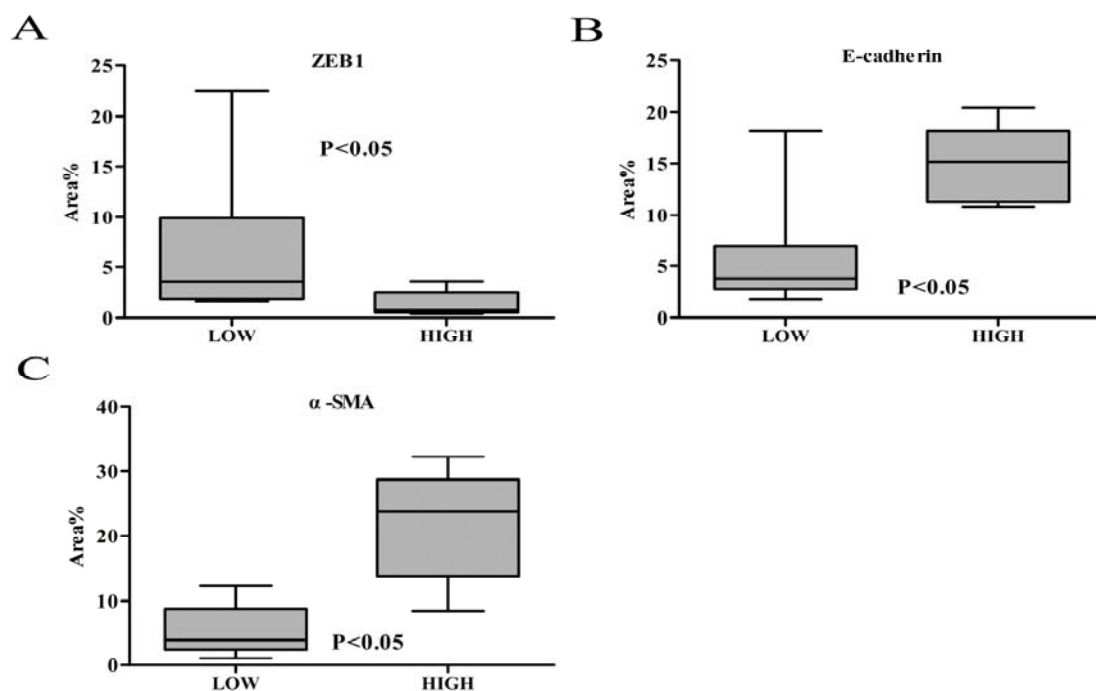


Fig. 5 Comparison of ZEB1, E-cadherin and  $\alpha$ -SMA between the low graded fibrosis group and the high graded fibrosis group in BA. The results were expressed as a proportion of the antibody-positive area. E-cadherin and  $\alpha$ -SMA markedly increased in the high graded fibrosis group compared to the low graded fibrosis group, whereas ZEB1 significantly decreased in the high graded fibrosis group compared to the low graded fibrosis group. The box represents the interquartile range, line across the box median, and the whiskers 90th percentile range. LOW: low graded fibrosis group; HIGH: high graded fibrosis group

Interestingly, we found the expression of miR-200a, E-cadherin and  $\alpha$ -SMA were upregulated with the progression of liver fibrosis in BA, while ZEB1 was downregulated. These findings suggest that the upregulation of miR-200a reduces the expression of ZEB1 and represses EMT, whereas  $\alpha$ -SMA remains elevated. Dong et al. reported that  $\alpha$ -SMA was overexpressed in biliary epithelial cells and in periductular collagen fibers of BA patients, and elevated  $\alpha$ -SMA expression was found to relate to histological fibrosis scores of the livers from BA infants [28]. Moreover, BA Patients who underwent liver transplantation by the age of two years had prominently higher expression of  $\alpha$ -SMA at PE, the expression of  $\alpha$ -SMA was related to patient age at PE, and low the expression of  $\alpha$ -SMA at the time of PE was associated with improved bile flow and native liver survival [29].

Zeisberg et al. found that hepatocytes could gain a fibroblast-like phenotype through EMT in vitro experiments and demonstrated that hepatocytes markedly transdifferentiate into hepatocyte-derived fibroblasts via EMT in the mouse model of CCl4-induced liver fibrosis [30]. It has been well documented that not only hepatic stellate cells but also biliary epithelial cells could undergo EMT and ultimately transdifferentiate into myofibroblasts, which expressed  $\alpha$ -SMA and produced extra cellular matrix, led to liver fibrosis [31], [32]. Too many myofibroblasts induced by EMT might be the reason  $\alpha$ -SMA increased in the high graded fibrosis group. Here, we revealed the characteristics of EMT and miR-200a during the fibrogenesis process of BA, and our data suggest that EMT has an important role in the fibrotic process, the interaction

between miR-200a and ZEB1 may involve in BA liver fibrosis through EMT.

In summary, we found that miR-200a prominently increases in BA, which is associated with the progression of liver fibrosis. The interaction between miR-200a and ZEB1 may regulate EMT and ultimately influence liver fibrosis in BA. Further study on the pathogenesis of BA liver fibrosis will benefit the prognosis of BA patients.

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