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MicroRNA-21/PTEN/Akt axis in the fibrogenesis of biliary atresia☆,☆☆

Wenjun Shen [1], Gong Chen *, Rui Dong [1], Rui Zhao [1], Shan Zheng [1]

Department of Pediatric Surgery, Children's Hospital of Fudan University, Shanghai 201102, China

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ABSTRACT

Background: MicroRNAs (miRNAs) are short, noncoding RNA molecules that act as post-transcriptional negative regulators of target mRNAs. Increasing evidence suggests that miRNAs are involved in liver fibrotic processes. Biliary atresia (BA) is characterized by rapid and progressive liver fibrosis. Therefore, we investigated the role of miRNA-21in the pathogenesis of BA.

Methods: We collected liver samples from patients with BA or liver trauma to examine the role of miRNA-21. We examined RNA expression of *miRNA-21*, *phosphatase and tensin homolog deleted on chromosome ten* (PTEN), and α-smooth muscle actin (α-SMA) in liver tissue using real-time fluorescence quantitative PCR. Western blot analyses and immunohistochemical staining were performed to evaluate protein expression of PTEN, α-SMA, and phosphorylated AKT in liver.

Results: We found that miRNA-21was upregulated in liver samples from BA patients, whereas PTEN negatively correlated with suppression of the 3′-untranslated region (3′-UTR). Activation of the downstream AKT pathway provoked liver fibrosis by enhancing α-SMA levels.

Conclusions: The miRNA-21/PTEN/AKT axis promotes the fibrosis process in BA, which might be a potential therapeutic target to improve the prognosis of patients with BA.

Biliary atresia (BA), an inflammatory sclerosing cholangiopathy, is the leading cause of cholestasis in infants younger than 3 months of age. Pathologic features of BA include micro-inflammation and intra- and extra-hepatic hepatic bile duct fibrosis. Children suffer from progressive liver fibrosis and cirrhosis without treatment, and do not typically survive longer than 2 years [1]. Although the Kasai procedure significantly improves the prognosis of BA patients, most still require liver transplantation during their lifetimes [2]. Despite the efforts of researchers worldwide, the cause of BA remains unknown. BA is characterized by rapid and progressive liver fibrosis, which often presents clinically after surgery. Understanding liver fibrosis in BA should benefit patient prognosis [3].

MicroRNAs (miRNAs) represent a class of endogenous non-coding short RNA molecules (~22 nucleotides in length), which post-transcriptionally regulate target mRNAs [4]. It is estimated that there are nearly 1000 miRNAs in mammalian cells, and that greater than 30% of all genes are regulated by miRNAs. Recent studies indicate that several miRNAs regulate extracellular matrix protein deposition and inflammatory cell behavior [5], both of which are involved in liver fibrotic processes [6]. Recent studies have suggested that miRNA-21 may have a crucial role in fibrotic processes of many organs [7–9]. A miRNA profiling study reported up-regulated miRNA-21 in experimental BA models [10], whereas hepatic stellate cell (HSC) activation was mediated via the miR-21/PTEN/AKT axis [11]. We studied miRNA-21alterations in BA patients and the association with phosphatase and tensin homolog deleted on chromosome ten (PTEN) using PCR and western blot analyses. We used immunohistochemistry (IHC) to evaluate hepatic lobule localization of PTEN, p-AKT, and α-smooth muscle actin (α-SMA). Our findings report regulation of AKT signaling in BA, and suggest that the miRNA-21/PTEN/AKT axis is a molecular target that can be used therapeutically to treat patients with BA.

1. Materials and methods

1.1. Liver tissue and pathology

Liver tissue specimens were obtained from 12 patients with BA. As controls, we obtained normal liver tissues from four patients who

Abbreviations: miRNAs, microRNAs; miR-21, MicroRNA-21; BA, biliary atresia; PTEN, phosphatase and tensin homolog deleted on chromosome ten; a-SMA, a-smooth muscle actin; IHC, immunohistochemistry; HSC, hepatic stellate cell; MEM, minimum essential medium; H&E, hematoxylin and eosin; OD, optical density; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; TBS, Tris-buffered saline; DAB, 3,3'-diaminobenzidine tetrahyochloride; UTR, untranslated region;EMT, epithelial to mesenchymal transition; IKK, IκB kinase; NF-κB, nuclear factor- κB.

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* Corresponding author at: Department of Pediatric Surgery, Children's Hospital of Fudan University, 399 Wanyuan Road minhang district Shanghai China. Tel.: +86 21 64 931 117; fax: +86 21 64 931 901.

E-mail addresses: doc_albert@126.com (W. Shen), chengongzlp@hotmail.com (G. Chen), dongrui_1982@126.com (R. Dong), zhao007rui007@sina.com (R. Zhao), szheng@shmu.edu.cn (S. Zheng).

[1] Children's Hospital of Fudan University, 399 Wanyuan Road minhang district Shanghai China (201102).

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underwent surgeries due to liver injuries. Each tissue sample was isolated, fixed in formalin, and embedded in paraffin. Sections were stained with hematoxylin-eosin (H&E) and Masson's trichrome for light microscopy analyses. Liver tissues for PCR and western blot analyses were preserved at −80 °C. The Ethics Committee at the Children's Hospital of Fudan University approved this study.

1.2. RNA extraction and quantitative reverse transcription polymerase chain reaction (RT-PCR)

After warming to room temperature, tissue fractions (~500 mg) were homogenized in 600 μL Trizol reagent (Life Technologies, USA). We typically extracted 2 μg to 9 μg of total RNA, and OD260/280 ratios typically ranged from 1.8 to 2.0, indicating high RNA purity. cDNA synthesis was performed using the First Strand RT kit (Thermo Scientific, #K1621, USA). PCRs were carried out using the 7900HT Fast Real-Time PCR System (Applied Biosystems 7900HT, ABI, USA) and the SYBR® Premix Ex Taq kit (Takara, DRR081A, Japan) to quantify mRNA levels. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, was used as an endogenous control.

To quantify miRNA-21, 1 μL of RNA was reverse transcribed and amplified using the One-step PrimeScript® One Step miRNA cDNA Synthesis kit (Takara, D350A, Japan). U6 small nuclear RNA served as a control. Quantitative RT-PCR (qRT-PCR) was performed using the ABI 7900HT instrument. All PCRs were performed in triplicate. The sequences of the primers are as follows: PTEN: 5′-ACCCCTTCATTGACCTCAACTA-3′ (forward) and 5′-TCTCGCTCCTGGAAGATGGTGA-3′ (reverse); α-SMA: 5′-GACAATGGCTCTGGGCTCTGTAA-3′ (forward) and 5′-CTGTGCTTCGTC ACCCACGTA-3′ (reverse); GAPDH: 5′-GCACCGTCAAGGCTGAGAAC-3′ (forward) and 5′-TGGTGAAGACGCCAGTGGA-3′ (reverse).

1.3. Protein isolation and western blot analyses

Frozen tissue specimens (~50 mg) were homogenized in 500 μL of ice-cold RIPA buffer (Beyotime Institute of Biotechnology, P0013C, China). After incubation for 30 min on ice, specimens were spun for 5 min at 14,000 rpm at 4 °C and supernatants were collected. Protein concentrations were determined using a bicinchoninic acid (BCA) kit (Beyotime Institute of Biotechnology, P0009, China). Each sample (~1.5 mg protein) was denatured for 10 min at 90 °C in the presence of Coomassie brilliant blue.

Proteins were subjected to 12% SDS-PAGE and subsequently transferred to nitrocellulose membranes. After washing for 20 min in TBS (Tris-buffered saline, pH 7.6), membranes were blocked in TBS containing 5% (w/v) nonfat dry milk at room temperature. Blots were incubated overnight at 4 °C with following primary antibodies: rabbit anti-human PTEN, rabbit anti-human α-SMA, rabbit anti-human p-AKT, and rabbit anti-human AKT (all from Abcam, USA). All antibodies were diluted 1:1000 in 5% (w/v) nonfat dry milk. After three washes (5 min each) in TBS–Tween-20 (TBST), membranes were incubated for 1 h at room temperature with goat anti-rabbit IgG secondary antibodies combined with horseradish peroxidase (1:1000; Santa Cruz Biotechnology, USA). After three washes (5 min each) in TBST, membranes were visualized with a chemiluminescent substrate (Immobilon Western HRP, Millipore, USA) and imaged using a fusion imaging system.

1.4. Immunohistochemical analyses

Liver sections were first deparaffinized with xylene and hydrated using a 95% ethanol solution. After antigen heat retrieval for 10 min in citrate buffer (pH 6.0), sections were returned to room temperature and washed twice (for 2 min each) in PBS. Sections were incubated for 2 h at room temperature with the primary antibodies (rabbit anti-human PTEN and rabbit anti-human α-SMA, 1:100 dilution, Abcam). Next, sections were washed twice (for 2 min each) in PBS, stained with 3,3'-diaminobenzidine tetrahyochloride (DAB), and counterstained with hematoxylin. After two more washes in PBS, sections were dehydrated, cleared, and mounted in XAM (BDH, Poole, UK).

Sections were visualized using an Olympus microscope. Medical image analysis software was used to determine the positive staining area of PTEN and α-SMA.

1.5. Statistical analysis

Results are expressed as mean values ± SD. The analysis of t-test and was used to search for statistical differences between two groups by SPSS 12. A P value less than 0.05 was considered statistically significant.

2. Results

2.1. Histological evaluations

We observed changes in pathology due to BA as evidenced by inflammation and fibrosis in the hepatic portal area using H&E staining. The BA group had more severe bile canalicular hyperplasia and widespread inflammation. In the BA group, we also observed debris from necrosis, which was accompanied by increased liver fibrosis, disappearance of the lobular structure, and formation of numerous pseudo-lobules. Liver specimens were stained with Masson's trichrome stain, and fibrosis was significantly increased in BA samples compared with control samples (Fig. 1).

2.2. Expression of miRNA-21 and mRNA in BA patients

We quantitatively evaluated the expression of miRNA and mRNA in samples. We found significantly higher miRNA-21expression in the BA group compared to the control group (2.48 ± 0.65 *vs.* 0.98 ± 0.41, $p < 0.05$). In contrast, PTEN expression was decreased in the BA group

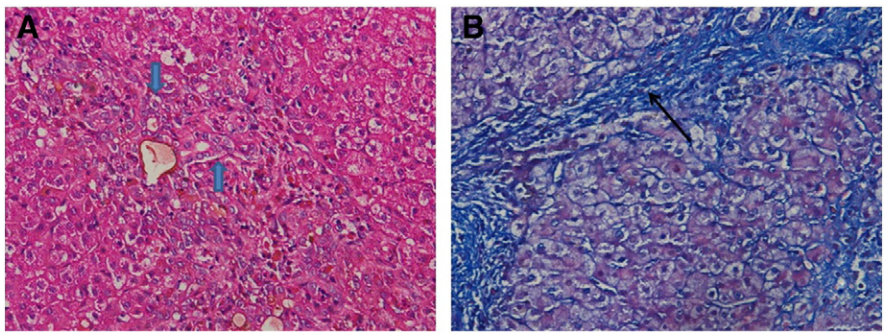

Fig. 1. Histological liver sections from BA patients. (A) Hepatic cells exhibit diffuse ballooning degeneration, with bile canalicular hyperplasia (blue arrowhead) and widespread inflammation (H&E staining; original magnification, 200×). (B) Fibroplasia (arrow) and collagen deposition were noted in the portal tract and septum area (Masson's trichrome stain; original magnification, 200×).