Genetics and Molecular Research

Effect of miR-29c and miR-129-5p on epithelial-mesenchymal transition in experimental biliary atresia mouse models

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Genet. Mol. Res. 15 (3): gmr.15037753
Received September 29, 2015
Accepted May 24, 2016
Published September 2, 2016
DOI http://dx.doi.org/10.4238/gmr.15037753

ABSTRACT. Biliary atresia (BA) is a destructive bile duct disease occurring in newborn children within a few weeks after birth. In this study, the effect of miR-29c and miR-129-5p on epithelial-mesenchymal transition (EMT) in experimental BA was explored by constructing BA mouse models via Rhesus rotavirus vaccine infection. miR-29c and miR-129-5p expression was analyzed by real-time quantitative polymerase chain reaction. EMT was established by induction with transforming growth factor (TGF)-β1. miR-29c and miR-129-5p were overexpressed and inhibited, respectively, by Lipofectamine

transfection. EMT-related protein (formin-like 2, FMNL2; E-cadherin; vimentin; and cytokeratin-19, CK-19) expression was analyzed by western blot and immunofluorescent assay. The results indicated that miR-29c and miR-129-5p were downregulated and upregulated in BA mice. TGF-β1 induction caused a time-dependent decrease and increase in miR-29c and miR-129-5p, respectively. Additionally, TGF-β1 induced an increase in FMNL2 and vimentin expression and a decrease in E-cadherin and CK-19 expression ($P < 0.05$). Overexpression or suppression of miRNA-29c or miR-129-5p, respectively, induced the inhibition of FMNL2 and vimentin, and promotion of E-cadherin and CK-19 expression, in the test groups compared to the non-intervention group ($P < 0.05$). However, the FMNL2, vimentin, E-cadherin, and CK-19 expression did not differ between the control and non-intervention groups ($P > 0.05$). Thus, miR-29c upregulation or miR-129-5p downregulation effectively prevented EMT in BA by regulating the expression of EMT pathway-related proteins. Therefore, miR-29c and miR-129-5p could be utilized as therapeutic targets for BA in the future.

Key words: miR-29c; miR-129-5p; Epithelial-mesenchymal transition; Biliary atresia

INTRODUCTION

Biliary atresia (BA) is a disease where in the intrahepatic bile duct, hepatic duct, or the choledoch undergoes atresia or agenesia in a newborn upon birth, or within a few weeks after birth. The major pathological characteristics of BA include the infiltration of a large number of intra- and extra-hepatic inflammatory cells, sustained injury, stenosis, atresia, and progressive liver fibrosis of the bile duct (Barnes et al., 2009), indicating biliary obstruction in the infant (Lampela and Pakarinen, 2013). Fibrosis is a complicated process that occurs for the repair of tissue damage and to protect the host from harm. However, fibrosis can damage organ structure and function via the deposition of a large amount of matrix, when its development does not depend on an initial stimulus. Epithelial-mesenchymal transition (EMT) also plays an important role in organ fibrosis. EMT describes the transformation of the morphology of an epithelial cell to that of a fibroblast or interstitial cell, granting a migratory capacity to the cells. EMT plays an important role in embryonic development and histogeny and is involved in various pathological processes, including wound healing, renal fibrosis, tumorigenesis, and metastasis (Mani et al., 2008; Kalluri and Weinberg, 2009; Zeisberg and Neilson, 2009). Recent studies have focused on the role of EMT in liver fibrosis and its applicability to the development of targeted drugs against liver fibrosis. For example, Rygiel et al. (2008, 2010) reported that biliary epithelial cells are primarily responsible for liver fibrosis. The process of fibrosis is more complicated in the liver than in other organs because of the rapid regenerative capacity of liver cells. EMT has been reported to participate in the process of liver damage, repair, and fibrosis. However, a previous study reported that this process could be reversed or weakened by inducing a change in the production of relevant cytokines and growth factors (Xue et al., 2013). E-cadherin is a member of the transmembrane glycoprotein family that forms a complex with catenins, which in turn links back to the actin cytoskeleton to maintain

the stability of cell adhesion and cell polarity. The downregulation of E-cadherin, an epithelial marker of EMT, is the most important molecular event in the process of EMT (Ansieau et al., 2010). Cytokeratin 19 (CK-19) is a cytokine that is specifically produced by cells in the bile duct and directly reflects the change in the cellular morphology and quantity of bile duct epithelial cells, which in turn plays a major role in EMT (Zheng et al., 2015). Vimentin, the primary member of the intermediate filament protein family, is expressed in normal mesenchymal cells, and plays a major role in maintaining the cell integrity and resisting external trauma (Satelli and Li, 2011). Vimentin is widely regarded as the standard EMT marker (Zhao et al., 2014). Formin-like-2 (FMNL2), a member of the formin family, is an actin nucleation factor that affects cell polarity, cytokinesis, pseudopodia formation, and cell adhesion and movement (Faix and Grosse, 2006).

Although the pathogenesis of BA remains unclear, studies have shown a correlation between BA and virus-induced specific inflammation in the host. Petersen et al. (1997) first reported that an intraperitoneal injection of rotavirus to newborn mice induced the development of biliary atresia in some mice; this method has since been used to induce animal models of biliary atresia. MicroRNA are a class of short (18-23 nucleotide), non-coding, RNA molecules that act as negative regulators of target mRNA stability and translation. The importance of these molecules in normal and diseased livers has been demonstrated; however, their potential role in the pathogenesis of biliary atresia has not been addressed. The microRNA-29 (miR-29) family is a newly discovered small molecule RNA family that is closely related to fibrosis and plays a vital role in the regulation of fibrosis in multiple organs, including the heart, liver, and lung, and in systemic sclerosis (Ge et al., 2011; He et al., 2013; Xing et al., 2014). miR-129-5p has also been shown to modulate EMT (Xiao et al., 2015). However, the functions of miR-29c and miR-129-5p in the EMT of biliary epithelial cells with BA have not been reported. In this study, a BA model was developed by rotavirus infection and the effect of miR-29c and miR-129-5p on EMT in BA has been discussed to clarify the pathogenesis of BA and develop new strategies for prevention.

MATERIAL AND METHODS

Preparation and identification of mouse model of BA

The Rhesus rotavirus vaccine MMU18006 [RRV, 1.0 x 10^3 plaque-forming units (PFU)], conserved in the Jinan University of Medical Sciences, was cultured in MA-104 monkey kidney cells obtained from ATCC (Manassas, VA, USA). The virus was harvested when cells presented evident pathological changes. The virus titer was measured by the plaque formation method. Virus titers >1.0 x 10^6 PFU were used in animal experiments. Healthy newborn BALB/c mice were selected 12 to 18 h after birth. Twenty mice were randomly divided into the experimental (intraperitoneal injection of 30-40 μL RRV suspension) and control groups (intraperitoneal injection of 30-40 μL physiological saline). Fourteen days after the RRV injection, mice presenting jaundice and clay-colored stool were eviscerated to confirm the development of extrahepatic biliary atresia. Liver specimens were stained with hematoxylin and eosin (H&E) to further confirm the success of the experiment. None of the mice (in either the experimental or control group) were excluded because of death.

This study was approved by the Ethics Committee of the Jinan University of Medical Sciences, Shenzhen, China.

Detection of the expression of miR-29c and miR-129-5p

Total RNA was extracted from mouse tails and subjected to reverse transcription using the TaqMan MicroRNA Reverse Transcription kit (Tiangen, Beijing, China) according to the manufacturer instructions. Reverse transcription was performed in a 15-μL reaction system comprising 5 μL total RNA, 0.15 μL 100 mM dNTP (with dTTP), 3 μL miRNA primer, 1 μL MultiScribe™ reverse transcriptase (50 U/μL), 1.5 μL 10X buffer, 0.19 μL RNA enzyme inhibitor (20 U/μL), and 4.16 μL nuclease-eliminating ddH_2O. The generated cDNA was then amplified by real-time fluorescent quantitative PCR (RT-qPCR) using a standard kit (Invitrogen, Carlsbad, CA, USA), with *U6* small nuclear RNA as the internal standard. The 20-μL PCR mixture was composed of 1.33 μL cDNA template, 7.67 μL nuclease-eliminating ddH_2O, 10 μL TaqMan 2X Universal PCR Master mix, and 1 μL TaqMan microRNA assay (20X). The tests to detect each miRNA indicator were repeated for five times. The data obtained were analyzed by the $2^{-\Delta\Delta Ct}$ method.

Establishment of EMT model by TGF-β1 induction

Bile duct epithelial cells were separated and extracted from BA mice and inoculated in Dulbecco's modified Eagle's medium (DMEM)/Hams F12 (1:1) (Gibco, Carlsbad, CA, USA) supplemented with 10 ng/mL erythrocyte growth factor (EGF) (Gibco). This was incubated at 5% CO_2 and 37°C to obtain a primary culture. Cell growth and morphology were observed under an inverted microscope. Cells were grown to a confluence of 80% and subjected to digestion, and DMEM was added to terminate growth; the cell count was then determined using a hemocytometer. The cells were then inoculated at a concentration of 1 x 10^5 cells/mL in each well of a six-well plate. Intervention trials were commenced at a cell confluency of 70-80%. The changes in miR-29c and miR-129-5p expression after induction with 5 ng/mL TGF-β1 for 0, 12, 24, 48, and 72 h were detected by qRT-PCR. The change in expression of FMNL2, E-cadherin, CK-19, and vimentin with the increase in TGF-β1 stimulation time was analyzed by western blot, using specific monoclonal antibodies (against these proteins) purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). In this method, total protein was extracted from each group of cells and subjected to gel electrophoresis on an 8% polyacrylamide gel (Tiangen). The separated proteins were transferred to a cellulose acetate membrane and nonspecific sites were blocked with 5% skim milk for 2 h. The membrane was then incubated with the above mentioned antibodies at 4°C overnight. The membrane was subsequently incubated with a goat-anti-mouse IgG secondary antibody (Santa Cruz Biotechnology) 1 h after a washing step. The membrane was developed using a chemiluminescent solution (Tiangen) after another washing step. The protein bands were obtained by exposing in a dark room and observed by using the digital camera.

Cell transfection

Bile duct epithelial cells with BA induced by incubation with TGF-β1 for 48 h were separately analyzed for miRNA-29c and miRNA-129-5p (Sangon, Shanghai, China) expression.

miRNA-29c expression was analyzed by treating the bile duct epithelial cells as follows: the non-intervention group comprised cell stimulated by 5 ng/mL TGF-β1 for 48 h; the miRNA-29c mimic group comprised cells treated with 50 nM miRNA-29c mimic for 24

h and subsequently with 5 ng/mL TGF-β1 for 24 h; the miRNA-29c mimic negative control group comprised cells treated with 50 nM miRNA-29c mimic negative control for 24 h and subsequently with 5 ng/mL TGF-β1 for 24 h.

miRNA-129-5p expression was analyzed in bile duct epithelial cells stimulated with 5 ng/mL TGF-β1 for 48 h (non-intervention group), treated with 50 nM miRNA-129-5p inhibitor for 24 h and subsequently with 5 ng/mL TGF-β1 for 24 h (miRNA-129-5p inhibitor group), and with 50 nM miRNA-129-5p inhibitor as negative control for 24 h and subsequently with 5 ng/mL TGF-β1 for 24 h (miRNA-129-5p inhibitor-negative control group).

The bile duct epithelial cells were transfected with the miRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer instructions: the cells with BA were digested with pancreatin, centrifuged at 4°C, precipitated, and re-suspended in 1 mL DMEM to prepare a cell suspension. The cells (5 x 10^6) were incubated on a six-well plate for 24 h. Recombinant DNA and liposomes were dissolved in serum-free medium without antibiotics and mixed for 5 min; the mixture was incubated at 20°C to form DNA/liposome compounds. This medium was added to the cells on the six-well plate and incubated at 37°C, 95% humidity, and 5% CO_2 for 6 h. The medium was then changed again to complete DMEM supplemented with 10% fetal bovine serum (Gibco) and the plate was incubated for an additional 48 h.

Detection of protein expression by immunofluorescence assay

Coverslips of the six-well plate were dipped in PBS (Tiangen) ten times. The excess PBS was blotted out, and the coverslips were replaced on the six-well plate and fixed with 2% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 10-15 min. The coverslips were again washed in PBS (ten times) and subsequently placed on a clean glass slide. The slides were then incubated with monoclonal antibodies (mentioned in a previous section) at 4°C overnight. Subsequently, the coverslips were washed with PBS (twenty times) and replaced on the glass slide; the slides were then incubated with the fluorescein isothiocyanate (FITC)-labeled secondary antibody at room temperature for 1 h, washed with PBS (twenty times), and subsequently sealed. The expression of FMNL2, vimentin, E-cadherin, and CK-19 was detected using a laser-scanning confocal microscope (LSCM) (Beckman-Coulter, USA).

Statistical analysis

The data obtained were analyzed using the SPSS 17.0 software (IBM, Armonk, NY, USA). The differences among groups were compared by one-way analysis of variance (ANOVA) and the *t*-test. Differences with P values of <0.05 were considered to be statistically significant.

RESULTS

BA mouse model

The mice in the control group showed no obvious changes in the H&E results at 14 days after vaccination with saline (Figure 1a). Mice vaccinated with RRV had a dark yellow liver after 14 days, with a part of the liver showing bright yellow necrosis. The liver capsule showed obvious tightening, a grainy surface, and strong texture. The gallbladder was atrophied

or inflated while the extrahepatic biliary tract showed funicular stenosis or atresia. The results of H&E staining showed infiltration of a large number of inflammatory cells in the intrahepatic portal, accompanied by obvious intrahepatic cholestasis as well as spotty or large patchy necrosis in the tissues at the liver margins (Figure 1b). qRT-PCR analysis of miRNA expression in liver tissues obtained from mice with BA and the controls showed that miR-29c was downregulated and miR-129-5p was upregulated in the BA model compared to normal mice (Figure 2).

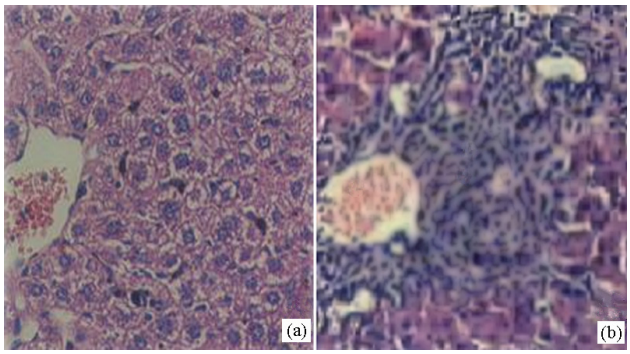

Figure 1. Validation of biliary atresia mouse model by hematoxylin eosin staining (100X). **a.** Control group; **b.** experimental group (we observed the infiltration of a large number of inflammatory cells in the liver portal area, indicating that the BA model was successfully established).

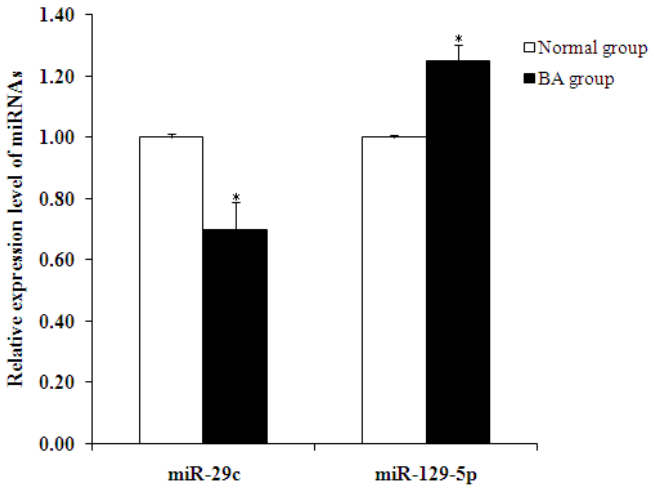

Figure 2. Relative expression of microRNA (miRNA) in each tissue. *P < 0.05.

TGF-β1 induces EMT in bile duct epithelial cells in BA mice

Bile duct epithelial cells obtained from BA mice were cultured and subsequently treated with 5 ng/mL TGF-β1, for varying time periods. The changes in miR-29c and miR-129-5p expression were quantified by qRT-PCR. The results of this analysis (Figure 3A) showed a gradual decrease and increase in the expression of miR-29c and miR-129-5p, respectively, on prolonged

treatment with TGF-β1 ($P < 0.05$). Additionally, we observed an increase in the expression of FMNL2 and vimentin proteins and a decrease in the E-cadherin and CK-19 expression with time in bile duct epithelial cells obtained from BA mice ($P < 0.05$; Figure 3B and C).

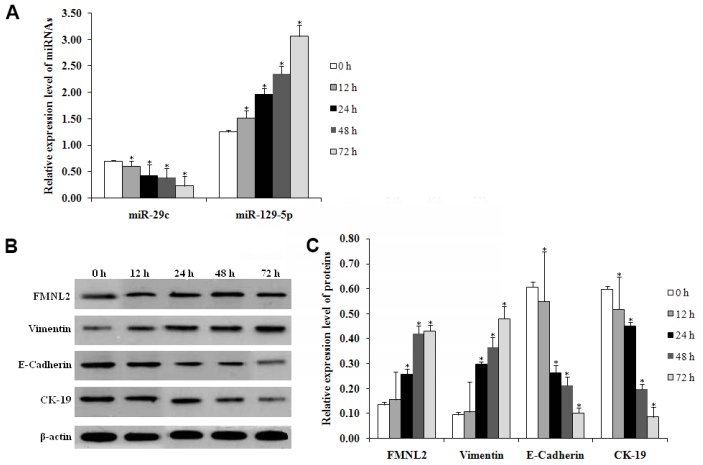

Figure 3. Change in miRNA and protein expression with time after treatment with 5 ng/mL transforming growth factor (TGF)-β1. **A.** Relative expression levels of miR-29c and miR-129-5p; **B.** western blot detection; **C.** relative expression levels of proteins. *$P < 0.05$.

Role of miR-29c and miR-129-5p in EMT of BA

Bile duct epithelial cells from BA mice treated with 5 ng/mL TGF-β1, for 48 h, showed an increase and decrease in the expression of miRNA-29c and miR-129-5p, respectively. We observed a decrease in the expression of FMNL2 and vimentin and an increase in that of E-cadherin and CK-19 in the miRNA-29c mimic (Figure 4A) and miR-129-5p inhibitor (Figure 4B) groups, compared to the non-intervention group ($P < 0.05$). However, the expression of FMNL2, vimentin, E-cadherin, and CK-19 did not differ significantly between the miRNA-29c mimic-negative control and miR-129-5p inhibitor-negative control groups and the non-intervention group ($P > 0.05$).

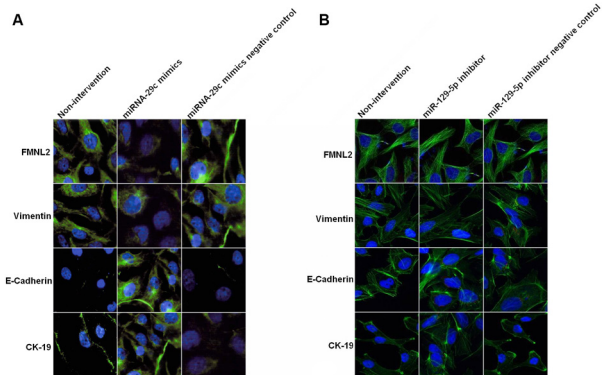

Figure 4. Detection of changes in protein expression by immunofluorescence assay. **A.** Overexpression of miR-29c; **B.** inhibition of miR-129-5p expression.

DISCUSSION

EMT could play a major role in the pathological mechanism of sclerosing cholangitis in biliary atresia, wherein bile duct epithelial cells gradually lose their epithelial characteristics and adopt the features of mesenchymal cells (Schulze et al., 2007; Harada et al., 2009; Deng et al., 2011). Zeisberg et al. (2007) reported that EMT plays an important role in the development of fibrosis in intrahepatic bile ducts, implying a similar role in the development of biliary fibrosis in BA; this theory was validated by Díaz et al. (2008).

Recent studies have identified a close association between miRNA-29 and the EMT mechanism (Ge et al., 2011; He et al., 2013). Some reports have reported that miR-29c expression is downregulated during the development of fibrosis in the liver, heart, lung, and skin (Díaz et al., 2008; van Rooij et al., 2008; Maurer et al., 2010; Ogawa et al., 2010; Cushing et al., 2011; Li et al., 2011). miRNA-29 expression was also shown to be downregulated during EMT in endometrial sarcoma, suggesting a negative regulatory role of miRNA-29 in EMT. In fact, overexpression of miRNA-29 may prevent the occurrence of fibrosis by inhibiting EMT (Hand et al., 2012). This is consistent with our conclusions that miRNA-29c overexpression induced a decrease in FMNL2 and vimentin expression and an increase in E-cadherin and CK-19. This indicated that miRNA-29c overexpression could reduce the degree of fibrosis induced by TGF-β1. A previous study reported that cardiac fibroblasts treated with TGF-β showed downregulated miR-29b expression, suggesting the regulatory role of the TGF-β signaling pathway in miR-29 expression (Yang et al., 2015). TGF-β negatively regulates miR-29 expression; downregulated miR-29 reduces the inhibitory effect of miR-29 on the expression of TGF-β and other pro-fibrogenic genes to amplify the fibrosis signal induced by TGF-β, eventually causing fibrosis. Therefore, miR-29 overexpression may effectively inhibit the fiberization induced by the TGF-β/Smad pathway (Luna et al., 2011). miR-29 directly suppresses the expression of a number of extracellular matrix components and regulates multiple signaling pathways associated with fibrosis. Therefore, miR-29 could be a novel target for the treatment of fibrosis. However, analysis of the miR-29 target genes revealed that miR-29 may also have a positive feedback effect on fibrosis.

The results of this study also showed that the function of miR-129-5p was exactly opposite to that of miR-29c in BA. miR-129-5p inhibition reduced the expression of FMNL2 and vimentin, upregulated E-cadherin and CK-19 expression, and suppressed TGF-β1-induced EMT. A previous study has shown that the TGF-β1/miR-129-5p/SIP-1 or SOX4 pathway plays a significant role in EMT and fibrosis in peritoneal dialysis (Xiao et al., 2015).

Despite the valuable findings obtained in this study, it is subject to some limitations. First, the relationship between miR-129-5p and EMT or fibrosis has not been fully investigated and needs further elaboration. Second, the number of mice used in both the experimental and control groups were too small; future studies must use large number of animals.

In conclusion, EMT plays a major role in the development of fibrosis in BA. Blocking the EMT pathway using an appropriate therapeutic target could inhibit the occurrence and development of liver fibrosis (Nieto, 2009). Therefore, these results provide a theoretical and experimental basis for further research into the treatment of biliary atresia.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by grants from the Medical Scientific Research Foundation of Guangdong Province (#B2014360), the Technical Research and Development Project of Shenzhen (#201402043), and the Technical Research and Development Project of Shenzhen (#JCYJ20150403101028164 and #JCYJ20150403102020231).

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