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Elevated hepatic MDR3/ABCB4 is directly mediated by MiR-378a-5p in human obstructive cholestasis

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Abstract. – OBJECTIVE: The function of MDR3 is important in bile acid transport. The miRNAs can suppress the expression of gene through combining mRNA of target gene. The regulation about MDR3 mediated by FXR or PPARα in cholestasis is clear, but the mechanism through miRNA is hardly reported. We aimed to find out the miRNA, which could suppress MDR3 expression and the significance of this connection in cholestasis.

PATIENTS AND METHODS: We measured hsa-miR-378a-5p expression level in liver tissues from 20 patients with cholestasis and 15 patients without cholestasis by quantitative PCR. We also tested the level of clinical features of the same group. HepG2 cell lines were performed experiments to discover the connection between hsa-miR-378a-5p and MDR3, including transient transfection, RNA and protein extraction, qPCR, Western blotting and luciferase reporter assay.

RESULTS: A significant decrease of miR-378a-5p was observed in obstructive cholestasis patient liver tissues compared to control group. We also find that the miR-378a-5p expression is correlated to several clinical features, which are important biomarkers in cholestatic liver injury. Then we predicted that MDR3 may be the target gene of miR-378a-5p through miRanda v3.3a. We programed the transient transfection of mimics and inhibitor on HepG2 cell lines, and detected the mRNA and protein expression of MRP2, MRP3 and MDR3. The results suggested that miR-378a-5p could negatively regulate MDR3 expression in both mRNA and protein expression level, and this regulation is specific. We didn't find same regulation in MRP2 and MRP3. Dual luciferase assays proved this regulation is mediated by a direct binding between miR-378a-5p and CDS of MDR3.

CONCLUSIONS: We found that hsa-miR-378a-5p expression was down-regulated in cholestatic liver tissues, compared to control liver tissues. Transient transfection and luciferase reporter assay in HepG2 cell lines results suggest that hsa-miR-378a-5p can directly combine MDR3 mRNA and suppress MDR3 protein expression. The down-regulated hsa-miR-378a-5p may cause a protective alteration through up-regulating MDR3 expression in cholestasis.

Key Words:
Cholestasis, miRNA, MDR3(ABCB4), hsa-miR-378a-5p.

Abbreviations

ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP, alkaline phosphatase; GGT = gamma-glutamyl transferase; TBIL = total bilirubin; DBIL = direct (conjugated) bilirubin; IBIL = indirect (unconjugated) bilirubin; BA = bile acids. * $p < 0.001$.

Introduction

Cholestasis is a kind of hepatobiliary disease characterized by abnormity of synthesis and transport of bile acid[1,2]. Toxicity constituents, such as bile acid, will injury hepatocytes and bile duct cells, resulting in inflammation, fibrosis, then cirrhosis and even carcinoma[1]. To resist the cholestasis, a decrease of bile acid synthesis *de novo* and adaptive alteration of bile acid transporters expression have been found in human liver tissue[3]. The function of bile acid transporter is important in cholestasis. Wagner and Trauner[4] show that the bile acid uptake transporters (e.g. OATPs, NTCP) expression are down-regulated, meanwhile the expression of bile acid efflux transporters (e.g. OSTα/β, MRP3) are up-regulated. Canalicular ATP-binding cassette (ABC) transporters family, which are expressed at basolateral and apical membrane of hepatocyte, play a key role in bile formation and secretion[1,5,6]. There are many subfamilies in ABC transporter family

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with different function, exporting constituents like BA, bili-gluc, cholesterol[1,6-9]. Dysfunctions of these transporters lead to an accumulation of bile acid, and subsequent liver injury[1,6]. Chai et al[9] suggested that kinds of representative ABC transporters expression are up-regulated obviously in cholestasis, such as MRP3, MRP4 and MDR3. MDR3 (ABCB4), locating at apical membrane, is a 170-kDa member of the B subfamily (ABCB) of ABC transporters, mainly including two nucleotide-binding and two 6-helical transmembrane domains. MDR3 is a floppase translocating phospholipids from inner canalicular membrane to outer[1,6,7]. Phospholipids can be excreted to bile, and form mixed micelles with bile acid, which protect the bile duct epithelium from the detergent properties of bile acids[10-12]. Dysfunction of MDR3 caused by mutations results in deficiency of phospholipids in bile, then the cholangiocyte apical membrane will be exposed to detergent toxicity of bile acid, and the subsequent progress will be liver injury[5,11,12]. It was reported that MDR3 mRNA and protein expression level are up regulated in PFIC3 patient liver tissues compared to control group[9]. MiRNAs are small endogenous non-coding RNAs that could post-transcriptionally regulate gene expression by formatting RISC preferentially targeting the 3'-UTR of specific mRNAs[13,14]. This miRNA: mRNA interaction may cause translational repression or enhanced mRNA degradation[15]. Also, there are studies suggesting that miRNA can combine the CDS region even 5'-UTR, and if microRNA binds the CDS region of target gene mRNA, the protein expression of target gene could also be repressed[16,17]. In the past 2 decades, the researches of miRNAs suggest that miRNAs are deeply related to digestive system diseases[18-24]. MiRNAs also play a key role in liver cholestasis in many aspects[25]. Cholestasis changes the miRNAs which induce cell death, including necroptosis and apoptosis[26]. Recently scholars[27,28] suggest that inflammatory regulation and immune activation induced by miRNAs is crucial in cholestasis. Furthermore, miRNAs are also reported to be related to cholangiocyte proliferation[29]. Up-regulated hsa-miR-378a-5p may regulate SULT2A1 in PSC, but in different subtypes of cholestatic diseases, the expression level of miR-378a-5p will be different[30]. Thus, we have investigated the alteration of miR-378a-5p in obstructive cholestasis, and its connection with MDR3, to find out miR-378a-5p clinical significance.

Patients and Methods

Patients and Liver Sample Collection

Patients were recruited from the Institute of Hepatobiliary Surgery, Southwest Hospital. Corresponding written informed consent was obtained from all patients prior to the study. Cholestatic liver samples (n=20) were obtained from patients suspected to have a pancreatic or periampullary malignancy. All patients had typical symptoms of obstructive cholestasis and jaundice caused by periampullary tumor growth. Control liver tissues were obtained from other patients who accept resection of liver metastases without cholestasis (n=15). The biochemical characteristics of the patients were described in Table I. We carried out this study conforming to the Declaration of Helsinki (2008) of the World Medical Association. The study protocol was reviewed and approved by the Institutional Ethics Review Board at the Southwest Hospital Chongqing, China.

Target Gene Prediction

We used miRanda v3.3a to predict the target gene of miR-378a-5p, based on microRNA Target Scanning Algorithm, as reported by Enright et al[31].

Cell Culture

Human hepatoma HepG2 cells were obtained from ATCC (Manassas, VA, USA). HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS), glutamax-1, and penicillin/streptomycin (Gibco, Rockville, MD, USA) in a humidified incubator at 37°C with 5% CO_2.

Cell Transfection

We obtained the miR-378a-5p mimics, miR-378a-5p inhibitor, miR-378a-5p mimics-negative control, miR-378a-5p inhibitor-negative control from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). We seeded HepG2 cells in 6-well plate, each well was seeded 5×10^8 HepG2. We transiently transfected these cells with a miR-378a-5p inhibitor *vs.* the negative control or a miR-378a-5p mimic *vs.* the negative control. We prepared a pre-mixture of 200 ml DMEM (Gibco, Rockville, MD, USA), 10 ml transfected reagent (Roche, Mannheim, Germany) and 5 nmol mimics or inhibitor or negative control for each well. These cells were harvested after 24 h transfection for RNA extraction, and 36 h transfection for protein extraction.

RNA Extraction and qPCR Real-Time Quantitative RT-PCR (qPCR)

We extracted the total RNA of transient transfected cells with Promega total RNA extraction kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. TaKaRa reverse transcription kit (TaKaRa Biotechnology, Otsu, Shiga, Japan) was used for reverse transcription. The specific RT primers and qPCR primers of miR-378a-5p and U6 were also obtained from Guangzhou RiboBio Co., Ltd. (Guangzhou, China) (Table II). The probes of MDR3, MRP2, MRP3 were obtained by ABI (Table III). We used the GAPDH as the relative control for the analysis of mRNA levels. The miR-378a-5p expression level was standardized to U6.

Table I. Clinical features of patients.

Clinical features	Cholestasis (N=20)	Control (N=15)
Samples (Male/Female)	13/7	12/3
Age (years)	53 ±13	49 ±11
ALT (IU/L)	210.3 ± 39.22*	35.41 ± 8.440
AST (IU/L)	153.6 ± 21.22*	33.83 ± 7.377
ALP (IU/L)	563.5 ± 82.67*	91.33 ± 6.223
GGT (IU/L)	847.7 ± 119.7*	35.33 ± 7.341
TBIL (μmol/L)	189.8 ± 98.45*	18.45 ± 4.141
DBIL (μmol/L)	118.4 ± 60.66*	5.544 ± 2.263
IBIL (μmol/L)	100.8 ± 50.72*	12.91 ± 1.979
BA (μmol/L)	137.7 ± 24.37*	5.525 ± 0.812

Values are means ± SD.

Western Blotting Analysis

Total protein was harvested after 36 h transient transfection. Proteins were extracted from cells using RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA) containing protease and phosphatase inhibitors (Roche, Basel, Switzerland). Next, the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked with 5% dry milk in TBST and incubated with a specific primary antibody overnight at 4°C. Incubation was carried out with horseradish peroxidase (HRP) conjugated secondary IgG(H+L) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA USA). The blots were processed with Immobilon™ Western chemiluminescent HRP substrate (Millipore, Billerica, MA, USA) and analyzed by gel imaging analysis system (BioRad, Hercules, CA, USA). GAPDH was used as a control. The dilutions of primary antibodies were as follows: MDR3 1:1000, MRP2 1:2000, MRP3 1:2000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), GAPDH 1:5000 (Proteintech Group, Chicago, IL, USA).

Luciferase Assays

According to the prediction from miRanda v3.3a, the predicted binding sequence of MDR3 for miR-378a-5p was cloned into the pmirGLO dual Luciferase reporter plasmid, which was designed by TsingKe (Chengdu, China). HepG2 cells were seeded at 48-well plate and co-transfected with miR-378a-5p mimics and pmirGLO constructs for the reporter assay. Each group was divided into triplicate in 48-well plates. The luciferase activity was detected by Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) after 48 h of transfection. Firefly luciferase activity was normalized against Renilla luciferase activity.

Statistical Analysis

All data were analyzed using the independent-samples Student's *t*-test (two-tailed) and multiple comparison between the groups was performed by SNK method. All data were expressed as the mean ± standard deviation (SD), using GraphPad Prism 6.01 (GraphPad Software Inc., La Jolla, CA, USA). A value of $p<0.05$ was considered statistically significant.

Results

Significant Down-Regulation of miR-378a-5p Expression in Cholestatic Liver Tissues and the Correlations between miR-378a-5p and Clinical Features

In obstructive cholestatic liver tissues, we found that miR-378a-5p expression level was down-regulated. We chose 20 patients with obstructive

Table II. Sense and antisense primers used for quantitative Real-time PCR (Sybr green).

Gene	Forward Primer (5′Ð3′)	Reverse primer (5′Ð3′)
MiR-378a-5p	ggctctcctgactccaggt	gtgcgtgtcgtggagtcggcacacagg
U6	gcttcggcagcacatatactaaaat	cgcttcacgaatttgcgtgtcat

Table III. Product information for Taqman pre-designed primer-probe reagent from Applied Biosystems.

Gene nomenclature	Product no.
MRP2	Hs00166123_m1
MRP3	Hs00166123_m1
MDR3	Hs00240956_m1

cholestatic liver disease and 15 patients without obstructive liver disease (Table I). Significant decrease of miR-378a-5p was observed (2-fold $p<0.05$ *vs.* controls; Figure 1A). We also detected representative clinical features. After analyzing the values of those features, we found GGT, ALT, AST, ALP, TBIL, DBIL, IBIL levels were evidently higher in obstructive cholestatic liver tissues than control group (Table I). We performed the correlation analysis between miR-378a-5p expression and these features, and found that TBIL, DBIL, IBIL are negatively correlated to the expression level of miR-378a-5p in patients (n=20) by linear regression analysis (*$p<0.05$ *vs.* controls. Figure 1B-D), but we didn't find significant correlations between miR-378a-5p and other clinical features.

Overexpression of miR-378a-5p Down-Regulated MDR3 mRNA and Protein Expression Level in HepG2 Cells

According to the predictive target gene of miR-378a-5p from miRanda v3.3a, we speculated that the miR-378a-5p may regulate MDR3. In the preparation of the previous period, we used miRanda v3.3a to predict the target gene of miR-378a-5p, including MDR3 (ABCB4). To show the correlation between miR-378a-5p and MDR3, transient

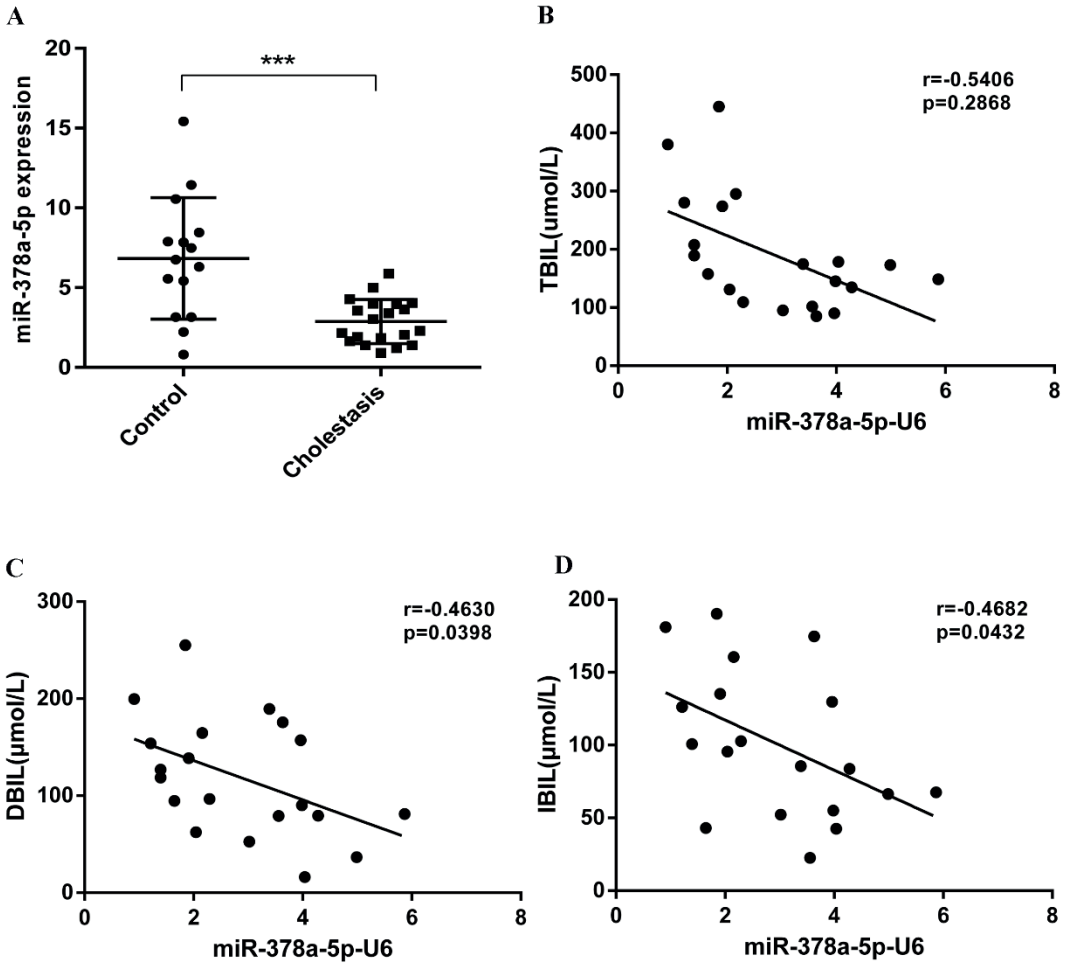

Figure 1. Significant down-regulation of miR-378a-5p expression in cholestatic liver tissues and the correlations between miR-378a-5p and clinical features. ***(A)*** The miR-378a-5p expression level in obstructive cholestatic patients, relative to control group (n=15 for control group, n=20 for obstructive cholestatic patients). *$p<0.05$ *vs.* controls. ***(B-D)*** The correlations between miR-378a-5p and clinical features in obstructive cholestasis patients (n=20) by linear regression analysis. *$p<0.05$ *vs.* controls.

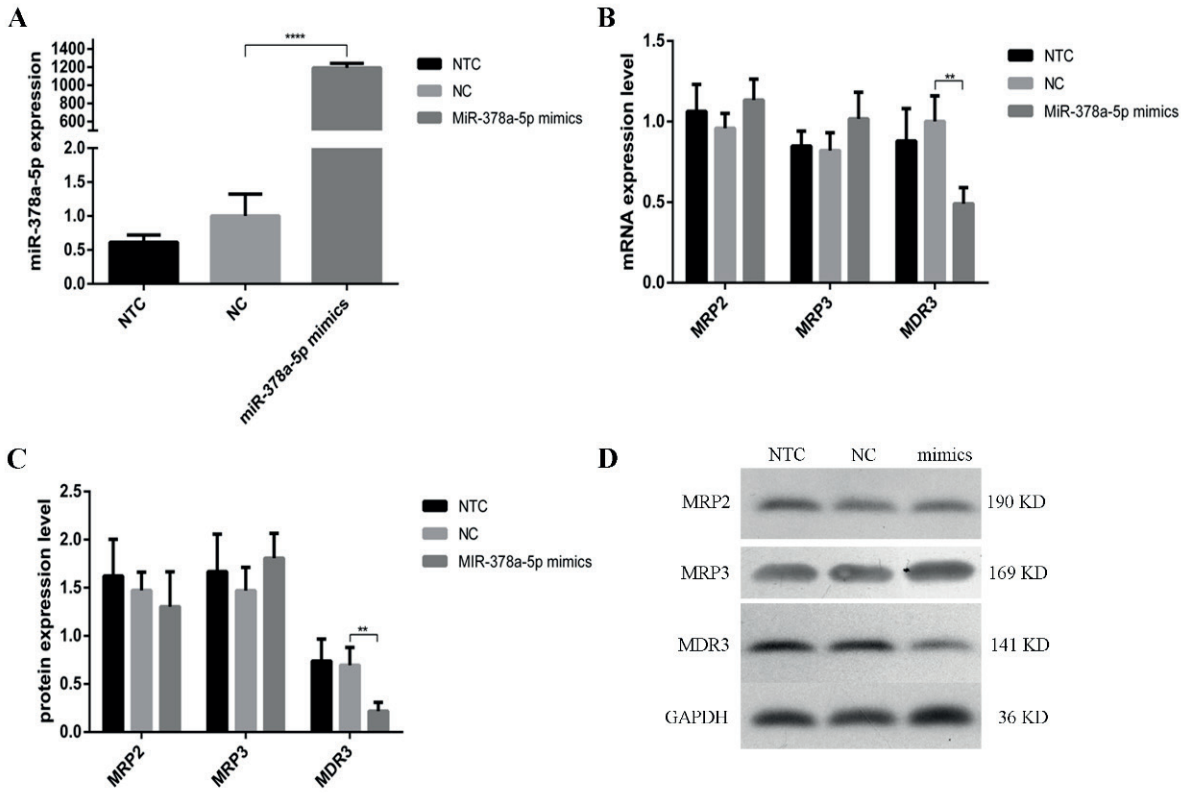

Figure 2. Overexpression of miR-378a-5p down-regulated MDR3 mRNA and protein expression level in HepG2 cells. ***(A)*** The expression level of miR-378a-5p after transient transfection of miR-378a-5p mimic. ***(B)*** The expression level of MDR3, MRP2, MRP3 mRNA after transient transfection of miR-378a-5p mimics. ***(C, D)*** The expression level of MDR3, MRP2, MRP3 protein after transient transfection of miR-378a-5p mimics.

transfection with miR-378a-5p mimics, corresponding negative control was performed. After transient transfection, we found that the miR-378a-5p expression level was obviously increased 1190.44-fold, meaning that miR-378a-5p mimics was transfected successfully (Figure 2A). Then we detected the mRNA expression of MDR3; there was a 51% decrease of MDR3 in miR-378a-5p mimics group compared to negative control group (Figure 2B). We detected the mRNA expression level of MRP2 and MRP3, but there were no significant statistical differences (Figure 2B). We found that the expression of the proteins above was identical to the mRNA expression, and that the protein expression of MDR3 decreased 3.04-fold after transient transfection of miR-378a-5p mimics compared to the negative control group (Figure 2C,2D).

Inhibition of miR-378a-5p Up-Regulate MDR3 Protein Expression Level in HepG2 Cells

To verify our conjecture of the connection between miR-378a-5p and MDR3 in protein expression level, we simulated the change of miR-378a-5p in cholestasis, and we transiently transfected miR-378a-5p inhibitor into HepG2 cells. After transfection, we verified the miR-378a-5p expression, which has a 43.2% decrease (Figure 3A). Next, we detected the mRNA and protein expression level of MDR3. There was a 1.78-fold increase in mRNA; at the same time, MDR3 protein expression level increased 1.7-fold (Figure 3B-D). Similarly, we also carried out the detection of MRP2 and MRP3 mRNA and proteins expression level, but we didn't find significant changes of these proteins in inhibitor group compared to negative control group (Figure 3B-D).

MiR-378a-5p can Bind mRNA CDS Region Directly According to Luciferase Assays

The above experimental results suggest that miR-378a-5p was related to MDR3 expression in both RNA and protein level, but the detailed mechanism is unknown, and the most possible regulation is direct binding. As expected, miRanda v3.3a also predicted the potential binding re-

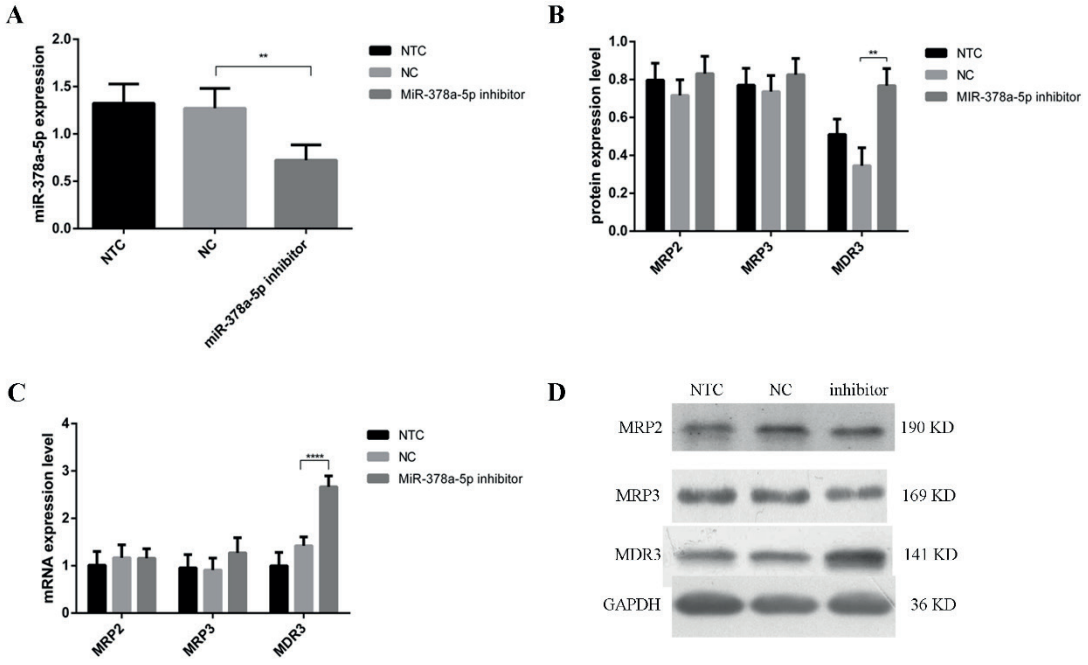

Figure 3. Inhibition of miR-378a-5p up-regulate MDR3 protein expression level in HepG2 cells. ***(A)*** The expression level of miR-378a-5p after transient transfection of miR-378a-5p inhibitor. ***(B)*** The expression level of MDR3, MRP2, MRP3 mRNA after transient transfection of miR-378a-5p inhibitor. ***(C, D)*** The expression level of MDR3, MRP2, MRP3 protein after transient transfection of miR-378a-5p inhibitor.

gion of MDR3 mRNA and miR-378a-5p (Figure 4A) co-transfected with miR-378a-5p mimics into HepG2 cells. The luciferase activities were significantly decreased by 46.5% in the reporter with wild-type binding sites, but not with mutant ($p<0.01$, Figure 4B), suggesting that miR-378a-5p regulated MDR3 expression in a site-specific manner.

Discussion

In our studies, we aimed at confirming the connection between miR-378a-5p and MDR3 in cholestasis. We found a decrease of miR-378a-5p expression in obstructive cholestasis patient liver tissues compared to control group (Figure 1). At the same time, it is observed that some clinical features are correlated to miR-378a-5p in cholestasis patient liver tissues (Figure 2). *In vitro*, by transient transfection of miR-378a-5p mimics, our results showed a decrease of MDR3 mRNA and protein expression. Next, we performed a transient transfection of miR-378a-5p inhibitor to simulate the same alteration of miR-378a-5p in human obstructive cholestasis. We observed a result consistent with detection of MDR3 in human liver tissues, but it is opposite to transient transfection of miR-378a-5p mimics (Figure 3). Luciferase assays also suggested that miR-378a-5p can bind MDR3 mRNA directly (Figure 4). Cholestasis results in complex alteration in liver. Previous studies suggested that miR-378a-5p could impact liver expression of several proteins. Wunsch et al[30] reported that miR-378a-5p may regulate SULT2A1 expression in PSC. Gill et al[32] also identified that CYPs might be regulated by miR-378a-5p in the metabolism and subsequent development of APAP toxicity in liver. Above studies prove miR-378a-5p could influence various aspects in cholestasis. To discover the change of miR-378a-5p in obstructive cholestatic liver, we detected miR-378a-5p in human liver tissues. We divided patients into two groups, patients with obstructive cholestasis and patients without obstructive cholestasis. As expected, miR-378a-5p down-regulated significantly in obstructive cholestasis patients compared to patients without obstructive cholestasis, and its expression is correlated to several clinical features such as TBIL, IBIL and DBIL, which are important in liver injury caused by obstructive cholestasis. We infer miR-378a-5p might play a role in liver injury cau-

sed by cholestasis. To clear the detailed function of miR-378a-5p in cholestasis, we used the miRanda v3.3a to predict the target gene of miR-378a-5p, and the MDR3 is one of those genes. MiR-378a-5p might bind MDR3 mRNA directly, and the binding region locates in CDS region of MDR3, so the miR-378a-5p could influence MDR3 expression in obstructive cholestasis. As we know, MDR3 is important in cholestasis, its function is translocating phospholipids into bile and forming mixed micelles. The mixed micelles could protect the bile duct epithelium from detergent properties of bile acids. Previous studies of Chai et al[8,9] pointed out that MDR3 expression is up-regulated in patients obstructive cholestatic liver disease compared to control patient[32]. The trend of MDR3 expression is in accordance with our prediction. We also observed results according to our assumption about interaction between miR-378a-5p and MDR3 in both mRNA and protein expression level, after transient transfection of miR-378a-5p mimics and inhibitor in HepG2 cells. Considering the connection between MDR3, MRP2 and MRP3, we detected the 3 genes in both mRNA and protein expression level. Results suggested that miR-378a-5p specifically regulate MDR3. There are two classic regulatory mechanisms of MDR3. One of those is FXR-ABCD4 pathway[33-35], another is PPARα-ABCB4 pathway[36]. Our study firstly found miR-378a-5p could regulate MDR3 directly by binding the CDS region of MDR3 mRNA. It is a new regulation of MDR3, and the new finding will provide a potential drug target for PFIC3 and human obstructive cholestasis. If we could block the binding of miR-

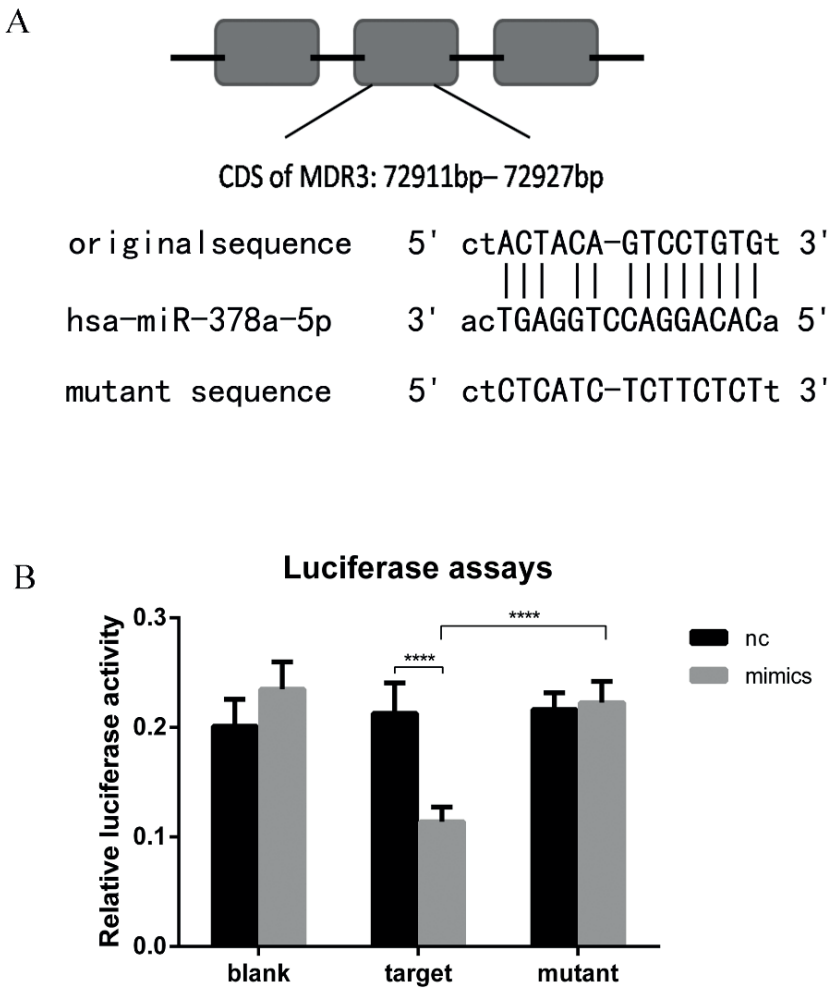

Figure 4. MiR-378a-5p can binding MDR3 mRNA CDS region directly according to luciferase assays. ***(A)*** Schematic diagram of the dual luciferase miRNA target reporter vector, the wild type and mutant MDR3 CDS sequences are shown with the miR-378a-5p sequence. (***B)*** Luciferase assays in HepG2 cells with wild-type or mutant MDR3 CDS vectors and miR-378a-5p mimic or NC.

378a-5p and MDR3 effectively, or down-regulate miR-378a-5p expression, the function of MDR3 will be enhanced, and the protection of phospholipids that keeps the bile duct epithelium from the detergent properties of bile acids will be stronger. In conclusion, a novel insight of miR-378a-5p in cholestasis was identified in the study, and miR-378a-5p might potentially be an effective biomarker for obstructive cholestasis. At the same time, our studies bring a new sight to clinical therapy program in cholestasis. However, further clinical trials are need. Considering the key role of MDR3 in gallstone production, we plan to discover the function of miR-378a-5p in cholelithiasis.

Conclusions

Taken together, the findings on the protective effect of miR-378a-5p in human obstructive cholestasis and HepG2 cells provide a new sight for detecting and treating cholestasis and deserve attention in the clinical practice of precision medicine.

Conflict of interest

The authors declare no conflicts of interest.

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