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Role of miR-511 in the Regulation of OATP1B1 Expression by Free Fatty Acid

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Abstract

MicroRNAs (miRNAs) are a family of non-coding RNA that are able to adjust the expression of many proteins, including ATP-binding cassette transporter and organic cation transporter. We sought to evaluate the effect of miR-511 on the regulation of OATP1B1 expression by free fatty acids. When using free fatty acids to stimulate Chang liver cells, we found that the expression of miR-511 increased significantly while the expression of OATP1B1 decreased. We also proved that SLCO1B1 is the target gene of miR-511 with a bioinformatics analysis and using the dual luciferase reporter assay. Furthermore, the expressions of SLCO1B1 and OATP1B1 decreased if transfecting Chang liver cells with miR-511, but did not increase when transfecting the inhibitors of miR-511 into steatosis cells. Our study indicates that miR-511 may play an important role in the regulation of OATP1B1 expression by free fatty acids.

Key Words: miR-511, OATP1B1, SCLO1B1, Expression, Free fatty acid

INTRODUCTION

Drugs are mainly metabolized in the liver and absorbed through transporters after being released into the blood. Drug transporters include solute carrier transporters (SLC) and ATP-binding cassette transporters (ABC). OATP1B1, also named OATP2, OATP-C and LST-1, belongs to solute carrier transporters, and its coding gene is SLCO1B1; it is mainly expressed on the sinusoidal membrane of human hepatocytes. SLCO1B1 mRNA has also been detected in other tissues, including small intestinal enterocytes (Abe *et al*., 1999; Glaeser *et al*., 2007). Substrates of OATP1B1 include endogenous organic anions and structurally diverse drugs, such as HMG-CoA reductase inhibitors and rifampicin (Kalliokoski and Niemi, 2009). Previous studies have shown that inflammatory cytokines (TNF-α, IL-6), hepatocyte growth factor, progressive familial intrahepatic cholestasis and free fatty acids (FFA) can decrease the expression of SLCO1B1 mRNA and OATP1B1 protein (Geier *et al*., 2005; Keitel *et al*., 2005; Le Vee *et al*., 2009a; Le Vee *et al*., 2009b).

MicroRNAs (miRNAs/miRs) are small, non-coding RNAs with the functions of negatively regulating gene expression through binding to a 3′-untranslated region (3′-UTR) of mRNA. A number of miRNAs are significantly up- or down-regulated by FFA (Zhu *et al*., 2013; Lin *et al*., 2014). Many studies have demonstrated that miRNAs regulate the expression of ATP-binding cassette transporter (Adlakha *et al*., 2013; de Aguiar Vallim *et al*., 2013), but the relationship of miRNA and solute carrier transporters, especially OATP, is unclear. Lim *et al*. reported that miR-1764 and miR-1700 bind directly to SLC13A2 and SLC35B4 transcripts, respectively, to regulate their expressions (Lim *et al*., 2012).

Our study indicated that miR-511 significantly decreased and OATP1B1 increased when the Chang liver cell was treated with FFA. We also proved that SLCO1B1 was the target gene of miR-511 by bioinformatics analysis and the research of the dual luciferase reporter gene. Furthermore, the expressions of SLCO1B1 mRNA and OATP1B1 protein decreased if Chang liver cells were transfected with miR-511, but the inhibitors of miR-511 did not increase the expressions of SLCO1B1 mRNA and OATP1B1 protein in steatosis cells.

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MATERIALS AND METHODS

Materials

Palmitic acid (PA) and oleic acid (OA) in sodium salts and fatty acid free bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA), RPMI 1640 medium and fetal bovine serum (FBS) from GIBCO (Shanghai, China) and Sijiqing Bio-Tech (Hangzhou, China), respectively. MTT was purchased from Sigma-Aldrich (St. Louis, MO, USA) and Lipofectamine 2000 from Invitrogen (USA). Oligonucleotide primers for SLCO1B1 expression were synthesized by Sangon Biotech (Shanghai, China). All miRNA mimics, mimic negative control, inhibitors, inhibitor negative controls and primers for miRNA PCR were purchased from Ribobio (Guangzhou, China). Rabbit anti-human OATP1B1 polyclonal antibody was purchased from Abcam (ab15442, Abcam, Hong Kong) and mouse anti-human GAPDH antibody was from Zhongshan Inc. All other chemicals and solvents were of the highest commercial grade.

Treating the cells with FFA

Chang liver cells (obtained from the Institute of Clinical Pharmacology, Central South University) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C/5% CO_2. Steatosis was induced, as previously described (Feldstein *et al*., 2004; Ricchi *et al*., 2009). PA and OA in sodium salts were co-dissolved in 10% fatty acid free BSA prepared in ddH_2O. A stock solution of 10 mM FFA (PA 3.3 mM + OA 6.6 mM)/10% BSA was prepared and stored at -20°C until later use. Chang liver cells were cultured in 0.5, 1 and 2 mM FFA containing 1% BSA for steatosis, while those cultured in plain medium RPMI 1640 were supplemented with 1% BSA as a control. After incubating for 24 h, cellular viability was tested using an MTT assay and the fluorimetric fat content was determined using oil red O staining. Then the ratio of triglyceride (TG) accumulation to total protein amount (TG/protein, mg/g) was evaluated according to the kit instructions (JianCheng Bio-Tech, China). Expressions of miR-511 and SLCO1B1 mRNA were detected by real-time PCR and the protein was detected using Western blot.

Prediction of miRNA targets and luciferase reporter assay

Bioinformatics miRNA databases were used to identify SLCO1B1-related miRNAs, including miR-Base (http://www.mirbase.org/), and target scan human (http://www.targetscan.org/). The segment of OATP1B1 mRNA 3′-UTR from 2181 to 2800 nt (620 bp; accession no. NM_006446.4) was cloned into the pmiR-RB-REPORT™ vector (Ribobio, Guangzhou, China) via *Xho*I (CTCGAG) and *Not*I (GCGGCCGC) restriction sites (Fermentas). The renilla luciferase gene with SLCO1B1 mRNA 3′-UTR acted as the report luciferase, while the firefly luciferase gene in the same plasmid acted as an internal control.

The Chang liver cells were seeded into 24-well plates. Then, 0.1 μg firefly luciferase and SLCO1B1 3'-UTR reporter vector named pmiR-RB-Report™, along with mimics, mimic negative control, inhibitors, or inhibitor negative controls of miR-511, were transfected into Chang liver cells with Lipofectamine 2000, according to the manufacturer's protocol. After a 24-hour incubation, the luciferase activities were measured with a luminometer (Tecan Infinite 200 Pro, Switzerland) using the Dual-Luciferase Reporter Assay System (Promega, Valencia, CA, USA). Renilla luciferase (Rluc) activity was normalized using firefly luciferase (Fluc) activity.

Transfection of mimics and inhibitors

All of the transfection was performed by Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. All the miRNA mimics, the mimic's negative control, the inhibitors (antisense oligonucleotides sequence of miR-511 mimics), and the inhibitor negative controls (antisense oligonucleotides sequence of the mimic negative control) were purchased from the Ribobio Co. The sequences are as follows: miR-511 mimics, 5′-GUGUCUUUUGCUCUGCAGUCA-3′; mimic negative control (cel-miR-239b-5p), 5′-UUUGUACUACACAAAAGUACUG-3′. A general miRNA containing fluorescence provided by the Ribobio Co. was used to estimate the efficiency of transfection. The expression of miR-511 was detected using real-time PCR.

Real-time PCR for miR-511 and *SLCO1B1* mRNA

Small RNA was extracted with an miRNA kit (Omega, Japan) and was reverse transcribed using ReverTra Ace qPCR RT Kit (TOYOBO, Japan) with the primers for specified miRNA. The miRNA quantification using Bulge-loop™ miRNA qRT-PCR Primer Set (one RT primer and a pair of qPCR primers for the set) specific for miR-511 was designed by RiboBio (Guangzhou, China). Real-time PCR was performed using the SYBR Green PCR Master Mix (TOYOBO, Japan) according to the following conditions: 95°C for 15 s followed by 40 cycles of amplification at 95°C for 15s and 60°C for 30s. The levels of mature miR-511 were normalized using U6.

MiR-511 mimics of 40 nM, 80 nM or 120 nM and the mimic negative control of 80 nM were transfected into Chang liver cells with Lipofectamine 2000 in 24-well plates. Furthermore, miR-511 inhibitors (100 nM) and their negative control were transiently transfected into the in vitro model of NAFLD that was incubated by 1 mM FFA after 24 h in Chang liver cells. After 24 h, total RNAs were extracted using the TRIzol method (Life Technologies, CA, USA), and the concentration and quality of RNA were measured using an Eppendorf Biophotometer. Total mRNAs were reverse transcribed into cDNAs using the ReverTra Ace qPCR RT Kit (TOYOBO, Japan). Real-time PCR was performed using the SYBR Green PCR master Mix (ABI, USA) according to the following conditions: 95°C for 10 min followed by 40 cycles of amplification of 95°C for 10 s and 59°C for 30s. GAPDH (forward primer: 5'-AGAAGGCTGGGGCTCATTTG-3'; reverse primer: 5'-AGGGGCCATCCACAGTCTTC-3') was used as an internal control for normalizing the expression of SLCO1B1 (F: 5'-AGCCCATTTGGGTGAA-3'; R: 5'-CGTTGTATCAATCAGAGCC-3').

Western blot analysis

Cells were harvested at 24 h post-transfection in six-well plates, and whole cell lysates were prepared with Radio Immunoprecipitation Assay lysis buffer (Beyotime, China) supplemented with complete protease inhibitor phenylmethanesulfonyl fluoride (PMSF; Beyotime, China). Protein concentrations were determined using the BCA Protein Assay Kit (Beyotime, China). Whole-cell proteins (40 μg) were separated on 10% SDS-polyacrylamide gels (PAGE) and were electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After incubation with

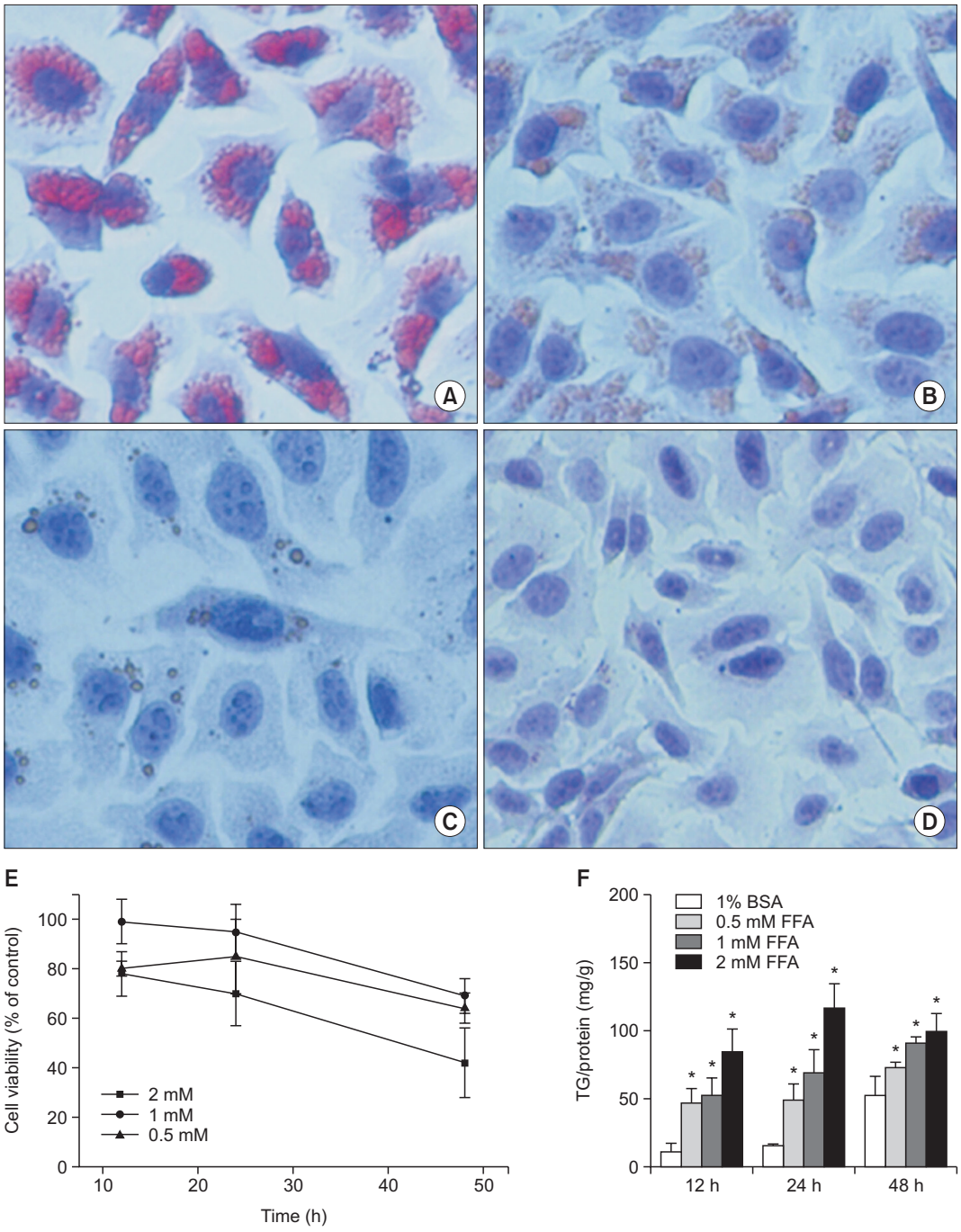

Fig. 1. The lipid accumulation. (A, B, C, D) Representative microscopy photograph. Chang liver cells were exposed to 2 mM, 1 mM, 0.5 mM FFA and 1% BSA for 24 h. The control cells were treated only with 1% BSA. (E) Cellular viability after incubating with different FFAs for various durations. Chang liver cells were incubated with 2 mM, 1 mM, and 0.5 mM FFA for 12 h, 24 h and 48 h respectively. The control cells were only treated with 1% BSA. Single points included the means of at least three independent experiments. (F) Effects of different fatty acids on triglyceride accumulation. The ratio of triglyceride accumulation to the total protein amount (TG/protein, mg/g) was evaluated as the concentration of TG in cell lysate after radio immunoprecipitation. The column represented the mean ± SD of three independent experiments. **p*<0.05 versus control.

selective rabbit anti-human OATP1B1 polyclonal antibody (ab15442, Abcam, Hong Kong), mouse anti-human GAPDH antibody (Zhongshan Inc., China) and the corresponding second antibody of horseradish peroxidase goat anti-rabbit IgG (Zhongshan Inc., China), rabbit anti-mouse IgG (Zhongshan Inc., China), chemoluminescence images were acquired using GE Healthcare ImageQuant 350, and the band densities were quantified using GeneTools (SynGene).

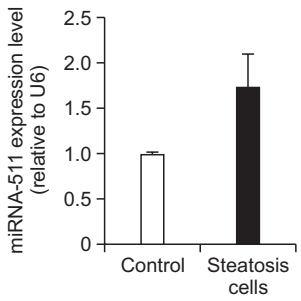

Fig. 2. Expression levels of miR-511 in the steatosis cells. The mature miR-511 levels in steatosis cells were determined by real time Q-PCR. The control cell was treated only with 1% BSA. The values were the levels of mature miR-511 normalized with the level of U6 snRNA. The values were mean ± SD for three independent experiments.

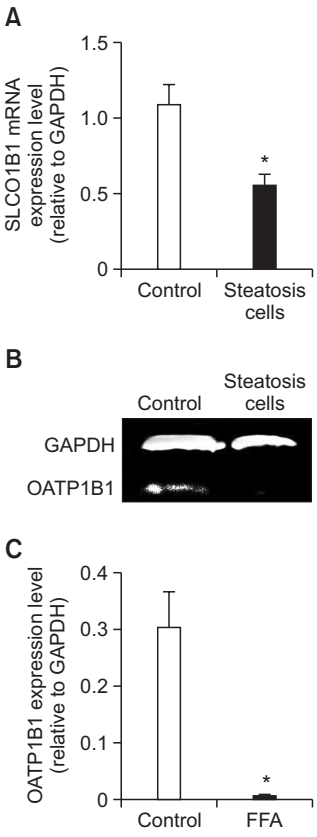

Fig. 3. SLCO1B1 mRNA and protein expression in the steatosis cells. (A) The SLCO1B1 mRNA level was determined by real time Q-PCR and normalized with the GAPDH mRNA level. (B) The OATP1B1 protein level was determined using Western blot analysis and normalized with the GAPDH protein level. (C) The gray values of OATP1B1 were measured using Image J software and normalized with GAPDH. The control was treated only with 1% BSA, while steatosis cells were exposed to 1mM FFA for 24 h. Each column represented the mean ± SD of three independent experiments. *p<0.05 versus control.

Statistical analysis

All values were expressed as the mean ± SD. Comparisons of variables between groups were performed using an unpaired, two-tailed student's t-test. A p-value <0.05 was considered statistically significant.

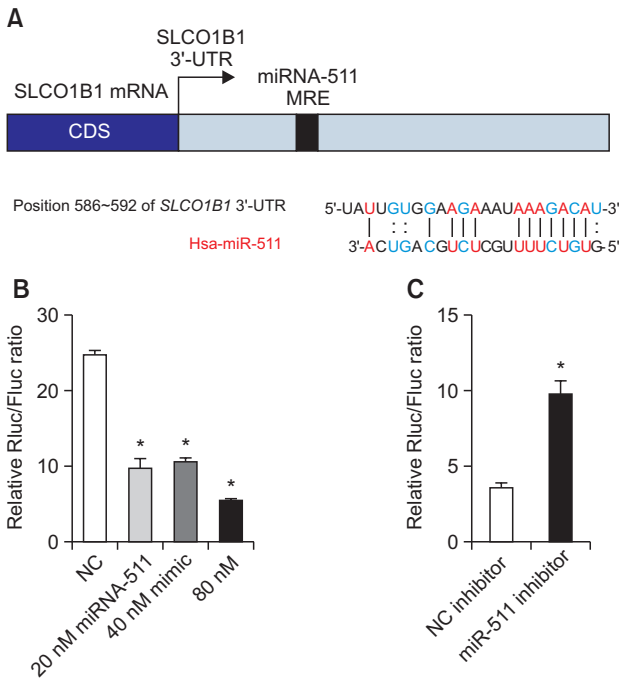

Fig. 4. Rescue validation for miR-511. (A) The miR-511 MRE site (miRNA recognition element) within human SLCO1B1 3′-UTR predicted by TargetScan was conserved. (B) The luciferase expression of the SLCO1B1 3′-UTR vector was significantly decreased by differential concentrations (20nM, 40 nM and 80 nM) of miR-511. The values were the mean ± SD for three independent experiments (*p<0.05 versus control). (C) The luciferase expression of the SLCO1B1 3′-UTR vector was significantly increased by inhibitors of miR-511 (200 nM).

RESULTS

FFA increased the miR-511 and decreased the SLCO1B1 mRNA and protein

After the Chang liver cells were exposed to successively graded concentrations of FFA (2 mM, 1 mM, 0.5 mM) for 24 h, oil red O staining was performed and the results shown in Fig. 1 A~D indicating the lipid accumulation was FFA concentration-dependent. The cellular viability was tested using the methyl thiazolyl tetrazolium (MTT) method and the ratio of triglyceride (TG) accumulation to the total protein amount (TG/protein, mg/g) was assessed to confirm lipid accumulation. As shown in Fig. 1E, 1F, the cellular viability and TG/protein ratio exhibited a time-dependent and FFA concentration-dependent phenomenon. For overall considerations of the cellular viability and the lipid accumulation, the optimal incubation time was 24 hours and the optimal incubation concentration of FFA was 1 mM.

The expression levels of mature miR-511 in steatosis cells were determined using a real time RT-PCR analysis. Small RNAs was extracted using a miRNA kit, and reverse transcription was performed using a PrimeScript RT Reagent kit with the primers for specified miRNA. The U6 snRNA levels were used for normalization. As seen in Fig. 2, the mature miR-511

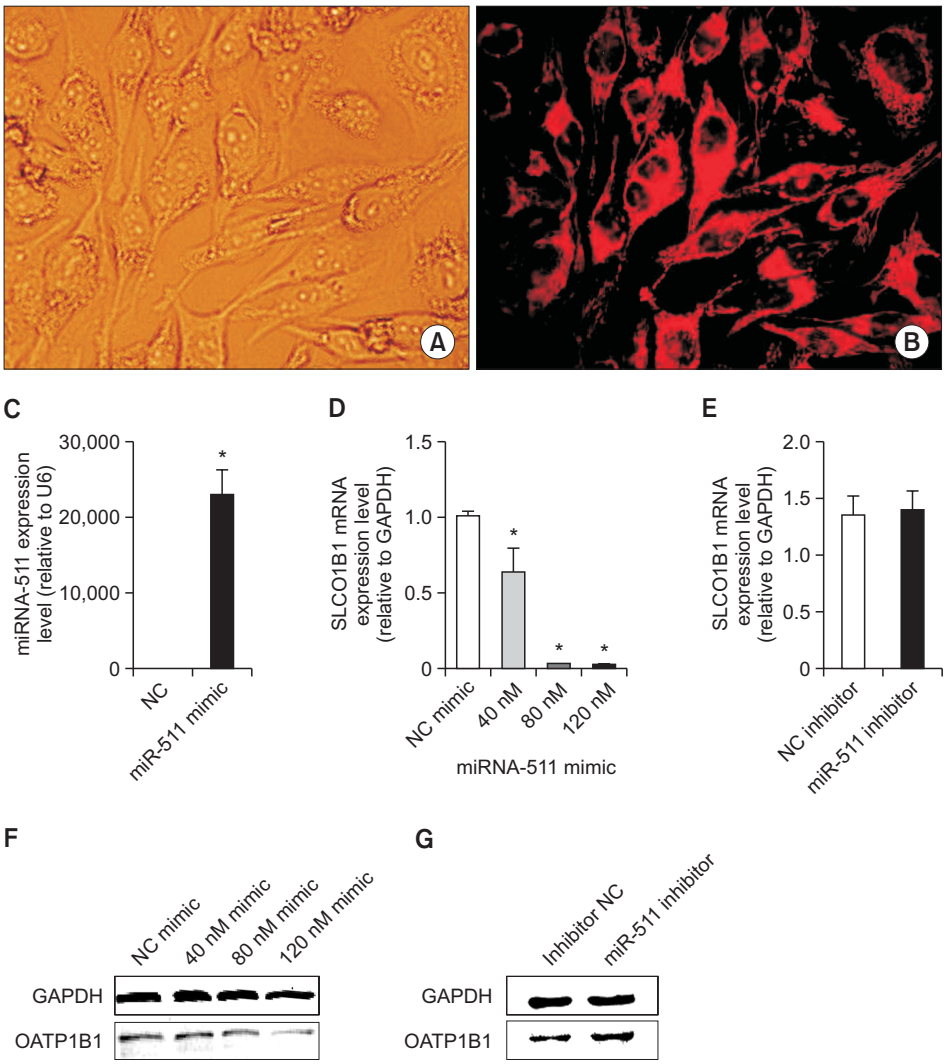

Fig. 5. Effects of miR-511 mimics or inhibitors on SLCO1B1 mRNA and protein levels. (A, B) The cellular morphology did not change after being transfected by a general miRNA (40 nM) and the red fluorescence showed that miRNA can be transfected into the cells sufficiently. (C) The level of miR-511 was determined after 80 nM miR-511 mimics or mimic negative controls (NC, 80 nM) were transfected into Chang liver cells. The miR-511 mimics with different concentrations (40 nM, 80 nM & 120 nM) or mimic negative controls (NC, 80 nM) were transfected into Chang liver cells, and miR-511 inhibitors (200 nM) or inhibitor negative controls (NC, 200 nM) were transfected into steatosis cells. (D, F) The SLCO1B1 mRNA was determined using real time Q-PCR and normalized with GAPDH mRNA. (E, G) The OATP1B1 protein level was determined by Western blot analysis and normalized with the protein level of GAPDH. The values were the mean ± SD for at least three independent experiments (*p<0.05 versus control).

levels were higher (1.74 times) in the steatosis cells than in the control cells.

The SLCO1B1 mRNA level was first examined using real time RT-PCR and then OATP1B1 protein expression was detected in the steatosis cells. As shown in Fig. 3A, a statistically significant decrease was observed in the steatosis cells versus the Chang liver cells (p=0.004). The gray values were measured using Image J software and OATP1B1 was normalized by GAPDH. Likewise, the OATP1B1 protein level decreased (Fig. 3B, 3C). Furthermore, the result of immunocytochemistry also verified that FFA decreased the expression of OATP1B1 (data not shown).

SLCO1B1 mRNA is the target of miR-511

Regarding the question of whether SLCO1B1 could be directly regulated by the mature miR-511, luciferase assays were performed using Chang liver cells. MicroInspector and TargetScan algorithms were employed initially to screen antisense matches of SLCO1B1 3′-UTR against human miRNAs (Fig. 4A). Then, dual-luciferase assays were performed by constructing SLCO1B1 3′-UTR vectors to test the effect of miR-511. miR-511 mimics/inhibitors and SLCO1B1 3′-UTR vectors were co-transfected. For the inhibitor experiment, miR-511 inhibitors and the vector were co-transfected. The results of co-transfecting showed that miR-511 interacted with SLCO1B1 3′-UTR for its role in post-transcriptional regulation (Fig. 4B, 4C).

Effects of miR-511 on OATP1B1 expression

Regarding the effects of miR-511 on the SLCO1B1 mRNA and protein, the changes were detected by adding mimics or inhibitors of miR-511. A general miRNA containing fluorescence could be sufficiently transfected into the cells and red fluorescence could be seen in cells transfected with miRNA (Fig. 5A, B). As shown in Fig. 5C, the amount of miR-511 significantly increased compared with the control when adding mimics (80 nM) (p<0.05). By transfecting miR-511 mimics with different concentrations (40, 80 & 120 nM), the SLCO1B1 mRNA and protein levels significantly decreased compared with the control (p<0.05) (Fig. 5D, 5F). However, by transfecting miR-511 inhibitors at a concentration of 200 nM into steatosis cells with rising levels of mature miR-511, the levels of SLCO1B1 mRNA and protein did not change (Fig. 5E, 5G).

DISCUSSION

In this study, Chang liver cells were exposed to incremental doses of FFAs for various time periods and under treatment for the same time, the cellular viability was in the following order of the FFA concentration: 2 mM <0.5 mM <1 mM. This might be explained by the fact that a low concentration of FFAs somewhat served as nutrients for promoting cell growth while inhibition occurred when at a high concentration of 2 mM. Added to the culture medium, FFA was taken up and used as nutrients to support cell growth. 1 mM FFA provided more nutrients to cells, so the cellular viability of 1mM was greater than 0.5 mM. However, because the excessive accumulation of FFA (2 mM) was harmful to cells, the cellular viability in 1 mM FFA was also greater than in 2 mM. Further accumulation of TGs was proportional to the concentration of FFA in the culture medium. This indicated that different FFA compositions induced a time-dependent increase of fat over-accumulation. Cell survival and TG accumulation was in accordance with other studies (Gómez-Lechón *et al*., 2007; Ricchi *et al*., 2009), and 1 mM FFA incubation for 24 h was selected for further studies.

OATP1B1 is an important drug transporter for human, and its expression is affected by inflammatory cytokines, hepatocyte growth factor, intrahepatic cholestasis and FFA (Geier *et al*., 2005; Keitel *et al*., 2005; Le Ve *et al*., 2009a; Le Vee *et al*., 2009b). We had proven that FFA could decrease SLCO1B1 mRNA and protein using qPCR, Western blot and ICC, and miR-511 was increased. But Gao reported that the Chang liver cell line was contaminated by Hela cells, while Hela cells did not express OATP1B1 (Gao *et al*., 2011), so if Chang liver cells express OATP1B1, they can meet the requirements of our experiments. In our study, we found that Chang liver cells express OATP1B1 definitely, which was expressed differently from the Hela cells. We proved that the OATP1B1 expression in Chang liver cells was higher than in the HepG2 cell (another liver cell line), which expressed OATP1B1. In addition, we obtained good results by using the specific monoclonal OATP1B1 antibody from Abcam (ab15442, Abcam, Hong Kong) in our Western blot and immunocytochemistry experiments, and the SLCO1B1 mRNA was well amplified by qRT-PCR (Ct value was 25-30), so Chang liver cells met the requirements for our studies.

There has not been a study about the relationship of miRNA and OATP1B1 to date, so we tried to discover it. In our study, although the inhibitors of miR-511 did not increase the expression of OATP1B1 in the steatosis cells, miR-511 obviously decreased SLCO1B1 mRNA and protein expression. Furthermore, results of the luciferase reporter assay and in silico identification of putative miRNA binding sites indicated that SLCO1B1 is the target gene of miR-511. The expression of SLCO1B1 mRNA was not rescued by miR-511 inhibitor treatment, while the miR-511 inhibitor worked well in the luciferase reporter assay. We speculated that expression of SLCO1B1 mRNA might be more complex than is the luciferase reporter assay, after all, only SLCO1B1 mRNA 3′-UTR from 2181 to 2800 nt was cloned into the pmiR-RB-REPORT™ vector. As mentioned above, OATP1B1 was affected by many factors, which might have led to the negative result of inhibitors of miR-511. While miR-511 mimics had an excellent function of negative regulation, miR-511 was likely to be a regulatory factor of OATP1B1.

Based on our study, we concluded that miR-511 might play an important role in the regulation of OATP1B1 expression by free fatty acids. We will continue our research and hope it may arouse the interests of other researchers.

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