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Post-transcriptional regulation by miR-137 underlies the low abundance of CAR and low rate of bilirubin clearance in neonatal mice

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ABSTRACT

Aim: Jaundice, potentially fatal encephalopathy, is common in approximately two-thirds of all well term infants. It is largely due to low expression of constitutive androstane receptor (CAR) in newborns; however, the mechanisms for this low expression were poorly understood.
Materials and methods: Expression of miR-137 and CAR was compared between neonatal and adult mice and between healthy and a mouse model of obstructive jaundice (OJ) using real-time RT-PCR and Western blot methods. Rate of bilirubin clearance was measured. DNA methylation of miR-137 was analyzed.
Key findings: Inverse expressions of miR-137 and CAR were consistently observed between newborn and adult mice, with a significantly higher miR-137 level and lower CAR protein and mRNA levels in neonatal liver than in adult liver. Similar reciprocal relationship was found existing between adult OJ mice and healthy control animals with a higher miR-137 level and lower CAR protein and mRNA levels in OJ than in healthy mice. Forced expression of miR-137 in primary hepatocytes repressed CAR protein levels, which was prevented by the inhibitor of miR-137. Knockdown of endogenous miR-137 by its inhibitor increased the rate of bilirubin clearance in OJ mice. Finally, we found that miR-137 was epigenetically over-activated due to hypomethylation in neonatal mice and in adult OJ mice, relative to adult healthy animals.
Significance: Our findings indicate that miR-137 is a repressor of CAR and thus a critical determinant of bilirubin clearance and may be considered a molecular target for the treatment of neonatal hyperbilirubinemia.

Introduction

Hyperbilirubinemia is common in the newborn period, and while the vast majority of babies are unaffected, significant neurological impairment remains a risk associated with extremely high levels of bilirubin (Woodgate and Jardine, 2011; Lauer and Spector, 2011). The number of babies affected by severe neonatal hyperbilirubinemia is expected to be increasing. Bilirubin deposition in the skin causes jaundice and most jaundice in newborn infants is a result of increased red cell breakdown and decreased bilirubin excretion.

Bilirubin is the yellow breakdown product and one of the oxidative end products of normal heme catabolism primarily from breakdown of hemoglobin, which is produced daily at an amount of 250–400 mg in adult humans (McDonagh et al., 1980). Bilirubin is normally excreted into the bile for clearance from the body through the liver. Functional deficit of bilirubin clearance results in the accumulation of this yellow pigment in blood or hyperbilirubinemia and jaundice. hyperbilirubinemia in newborns can lead to accumulation of bilirubin in certain brain regions (particularly the basal nuclei) with consequent irreversible damage to these areas manifesting as various neurological deficits, seizures, abnormal reflexes and eye movements. Thus, maintaining normal bilirubin clearance is a critical process in preventing neonatal hyperbilirubinemia and the associated neurotoxicity, and a better understanding of the mechanisms for bilirubin clearance is of paramount importance.

Recent reports have suggested that the expression of bilirubin-detoxifying enzymes and transporters is under the transcriptional control of orphan nuclear receptors pregnane X receptor (PXR) and constitutive androstane receptor (CAR) (Huang et al., 2003; Saini et al., 2005; Laurenzana et al., 2012). CAR (encoded by NR1I3) is a key regulator of bilirubin clearance in the liver, as it regulates the expression of genes involved in xenobiotic metabolism as well as hormone, energy, and lipid homeostasis. Unexpectedly, CAR expression is low in neonatal mice and humans, which could clearly have pathologic consequences in some cases, such as neonatal hyperbilirubinemia and the associated neurotoxicity (Huang et al., 2003, 2004; Saini et al., 2005; Laurenzana

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et al., 2012). However, the mechanisms for low expression of CAR in infants remained unknown.

We thought to shed light on this issue to elucidate the regulatory mechanisms underlying the low level of CAR expression. It has recently been emerging that microRNAs or miRNAs, a new class of non-protein-coding RNAs, play a pivotal role in fine-tune regulation of protein-coding genes at the post-transcriptional level through repressing translation and destabilizing target mRNAs. Our pilot study using computational analysis predicted CAR as a putative target for miR-137, as the coding sequence and the 3′ untranslated region (3′UTR) of NR1I3 contains a cluster of seed site for this miRNA. We therefore carried out a series of experiments aiming to characterize the expression of miR-137 and CAR in the liver of neonatal and adult mice, the rate of bilirubin clearance, and miR-137 targeting of CAR. We also exploited the epigenetic mechanisms for the expression alterations of miR-137. Our findings indicate that miR-137 is a repressor of CAR and thus a critical determinant of bilirubin clearance and may be considered a molecular target for the treatment of neonatal hyperbilirubinemia.

Methods and materials

Animals

Adult BALB/c mice of both genders weighing 30–35 g and pregnant mice were obtained from the animal facilities of the Chongqing Medical University. Neonatal mice of 3 days old were used for our experiments. The experimental protocols involving animal uses conformed to the guidelines of and approved by the Animal Care and Use Committee of the Chongqing Medical University.

Preparation of obstructive jaundice (OJ) models and associated therapeutic regimen

Twenty adult mice were randomly divided into two groups: control and OJ model groups. Mouse OJ model was developed according to the methods described by Kirkland et al. (2010). After mice were anesthetized by 2.5% sodium pentobarbital (i.p., 2 mg/L), the abdomens were then shaved and prepared in sterile fashion. Next, their abdominal cavity was opened to expose and dissociate the common bile duct along the hepatoduodenal ligament. For animals in the model group, the proximal end of common bile duct was double-ligated with surgical threads and the common bile duct was cut off. Finally, a layered suture (6-0 silk suture) of the abdominal wall was performed to close the abdominal cavity. All steps excluding ligation of the bile duct were performed for sham operations.

Synthesis and administration of miRNA and anti-miRNA antisense inhibitor

miR-137 (5′-UUAUUGCUUAAGAAUACGCGUAG-3′), the antisense oligonucleotides inhibitor anti-miR-137 (5′-CUACGCGUAUUCUUAAGCAAUAA-3′), and negative control inhibitor anti-miR-137 (5′-CUACGCGUAUUCUUAAGuccgcA-3′) were synthesized by Integrated DNA Technologies Inc (IDT). Five nucleotides or deoxynucleotides at both ends of the antisense molecules were Locked Nucleic Acid (LNA) modified with the ribose ring being constrained by a methylene bridge between the 2′-*O*- and the 4′-C atoms to enhance cellular stability and target affinity (Elmén et al., 2008). Seven days after bile duct ligation, miR-137 mimic, anti-miR-137 inhibitor or the negative control inhibitor was delivered into mice via tail vein injection at a dosage of 50 mg/kg/100 μL once a day for continuous three days.

Transfection procedures

Primary liver cells were transfected with miR-137 (10 nM), anti-miR-137 (4 nM), or negative control inhibitor (anti-miR-137 at 4 nM) with lipofectamine 2000 (Invitrogen), according to manufacturer's instructions. Forty-eight hours after transfection, cells were collected for RNA or nuclear extract and subsequent analyses.

Bilirubin clearance

Mice were maintained in a pathogen-free animal facility under a standard 12-h light/dark cycle, and were fed standard rodent chow and water *ad libitum* or given water containing 0.1 mg/mL phenylhydrazine in aluminum foil-wrapped bottles. After 30 days, blood was drawn and serum was prepared by centrifugation at 1200 ×*g* for 10 min. Seven days after pretreatment with miR-137 or anti-miR-137 inhibitor, mice were injected via the tail vein with a single dose of bilirubin (10 mg/kg) dissolved in an isotonic solution containing 0.5 g of Na_2CO_3 and 0.52 g of NaCl per 100 mL. After 1 h, blood samples were collected, and bilirubin levels were determined by using total Bilirubin Assay Kits (Biocompare) according to the manufacturer's instructions.

Isolation and culture of primary hepatocytes

Primary mouse hepatocytes were isolated from adult mice using a two-step collagenase perfusion protocol described in detail by Klaunig et al. (1981). Hepatocytes harvested at time '0' were pelleted and then lysed in lysis buffer A (10 mM Tris–HCl (pH7.4), 150 mM NaCl, 1% NP-40, 1 mM EDTA, and proteinase inhibitor cocktail (cOmplete, Roche, 20× stock solution prepared as 1 tablet/2 mL of water)) and the lysates were frozen at −80 °C for subsequent immunoblotting analysis. The mouse hepatocytes were then seeded onto collagen gels in the wells of 6-well plates at a density of 1×10^5 cells/cm^2 in 1.3 mL William's E medium (1.3 mm medium depth) supplemented with 10% FBS, 1 mg/mL aprotinin, 1× antibiotic/antimycotic (Gibco), 10 mM HEPES, 0.1 μM dexamethasome, 10 μg/mL ITS (Roche), and 2 mM glutamax (Sigma-Aldrich). The collagen gels (1.6 mg/mL of collagen I in PBS with 2 g/L glucose and 3.7 g/L sodium bicarbonate) were prepared as described previously (Martinez et al., 2010). Cells were maintained in serum-free medium with 3% Matrigel.

Drug treatment

To achieve DNA demythylation, a total of 2×10^5 primary liver cells/mL were treated with 0.5 μM 5-aza-2′-deoxycytidine (5-aza, Sigma, St. Louis, MO) for 3 days prior to subsequent measurements, during which medium was replaced daily. Control cells were not drug treated.

Quantitative real-time RT-PCR analysis

After experimental treatment, total RNA samples were isolated from cultured mouse liver and cultured primary liver cells using Trizol reagent (Invitrogen, USA) according to manufacturer's protocol. RNA (0.5 μg) was then reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) to obtain first-strand cDNA. Levels of miR-137 and NR1I3 mRNA were determined using SYBR Green I incorporation methods on ABI 7500 fast Real Time PCR system (Applied Biosystems, USA), with U6 or GAPDH as an internal control. Data are presented relative to adult data.

Western blot analysis

Nuclear extract was prepared from mouse liver samples of varying groups and from cultured primary liver cells as well. Fifty micrograms of each sample was fractionated on 10% polyacrylamide gel and immunoblotted with a CAR antibody (rabbit polyclonal; Abcam). The same blot was stripped and then immunoblotted with actin monoclonal antibody (1:1000, Santa Cruz Biotechnology) as a control. The density of immunoblot bands was quantified using QuantityOne software and