Published in final edited form as:

Biochem Pharmacol. 2016 October 1; 117: 78–87. doi:10.1016/j.bcp.2016.08.014.

Transcriptome association analysis identifies miR-375 as a major determinant of variable acetaminophen glucuronidation by human liver

Ioannis Papageorgiou¹, Marina Freytsis², and Michael H. Court³

Ioannis Papageorgiou: johnp@vetmed.wsu.edu; Marina Freytsis: Marina.Freytsis@tufts.edu; Michael H. Court: michael.court@vetmed.wsu.edu

¹Program in Individualized Medicine (PrIme), Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Washington State University, Pullman, Washington, USA

²Sackler School of Graduate Biomedical Sciences, Tufts University, Boston, Massachusetts, USA

³Program in Individualized Medicine (PrIme), Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Washington State University, Pullman, Washington, USA

Abstract

Acetaminophen is the leading cause of acute liver failure (ALF) in many countries including the United States. Hepatic glucuronidation by UDP-glucuronosyltransferase (UGT) 1A subfamily enzymes is the major route of acetaminophen elimination. Reduced glucuronidation may predispose some individuals to acetaminophen-induced ALF, but mechanisms underlying reduced glucuronidation are poorly understood. We hypothesized that specific microRNAs (miRNAs) may reduce UGT1A activity by direct effects on the *UGT1A* 3'-UTR shared by all UGT1A enzyme transcripts, or by indirect effects on transcription factors regulating *UGT1A* expression. We performed an unbiased miRNA whole transcriptome association analysis using a bank of human livers with known acetaminophen glucuronidation activities. Of 754 miRNAs evaluated, 9 miRNAs were identified that were significantly overexpressed ($p < 0.05$; > 2 -fold) in livers with low acetaminophen glucuronidation activities compared with those with high activities. miR-375 showed the highest difference (> 10 -fold), and was chosen for further mechanistic validation. We demonstrated using in silico analysis and luciferase reporter assays that miR-375 has a unique functional binding site in the 3'-UTR of the aryl hydrocarbon receptor (AhR) gene. Furthermore overexpression of miR-375 in LS180 cells demonstrated significant repression of endogenous AhR protein (by 40%) and mRNA (by 10%), as well as enzyme activity and/or mRNA of AhR regulated enzymes including UGT1A1, UGT1A6, and CYP1A2, without affecting UGT2B7, which is not regulated by AhR. Thus miR-375 is identified as a novel repressor of UGT1A-mediated hepatic acetaminophen glucuronidation through reduced AhR expression, which could predispose some individuals to increased risk for acetaminophen-induced ALF.

Address correspondence to: Ioannis Papageorgiou, Program in Individualized Medicine (PrIme), Department of Veterinary Clinical Sciences, Washington State University College of Veterinary Medicine, 100 Grimes Way, Pullman, WA 99164, Telephone: 509-335-0704, Fax: 509-335-0880, johnp@vetmed.wsu.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Graphical Abstract

Keywords

acetaminophen; UDP-glucuronosyltransferase 1A; aryl hydrocarbon receptor; miRNA; acetaminophen-induced hepatotoxicity; miRNA transcriptome association analysis

1. Introduction

Acetaminophen is one of the most widely used nonprescription antipyretic and analgesic drugs worldwide. Although considered very safe when used at recommended dosages, excessive dosing of acetaminophen can lead to serious liver injury. An ongoing study by the Acute Liver Failure Study Group has shown that acetaminophen-related acute liver failure (ALF) accounts for nearly 50% of all ALF cases in the United States [1]. The mechanisms underlying acetaminophen-induced hepatotoxicity have been studied extensively [2]. At therapeutic doses, acetaminophen is cleared primarily by conjugative metabolism, including glucuronidation by the UDP-glucuronosyltransferase (UGT) enzymes and sulfation by the sulfotransferase (SULT) enzymes [3, 4]. A small proportion of acetaminophen also undergoes oxidation by cytochrome P450 (CYP) enzymes to the hepatotoxic reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) that is normally rapidly detoxified by glutathione conjugation. However, at toxic doses, the sulfation pathway is saturated such that glucuronidation becomes the predominant rate limiting acetaminophen excretion pathway. There is also substantially increased N-acetyl-p-benzoquinone imine formation, and once available glutathione are depleted, acute liver injury ensues. Various animal and human studies support the importance of drug glucuronidation in minimizing the hepatotoxicity of acetaminophen following supratherapeutic doses. The most direct evidence is from studies of the Gunn rat, a mutant strain of Wistar rat that does not express any of the UGT1A subfamily enzymes as a consequence of a premature stop codon mutation located in an exon shared by all *UGT1A* gene transcripts [5]. Administration of a toxic dose of acetaminophen to mutant Gunn rats resulted in 110-fold greater plasma alanine aminotransferase concentrations (a measure of severity of hepatotoxicity) when compared rats without the mutation [6, 7]. Furthermore, acetaminophen glucuronide formation was inversely correlated with plasma alanine aminotransferase concentrations and glutathione derive metabolites. Mirroring the Gunn rat mutation, we have recently identified a genetic polymorphism (rs8330 C/T) located in the shared 3'-untranslated region of the human *UGT1A* gene that appears to influence the risk for acetaminophen hepatotoxicity in overdose patients through affecting acetaminophen glucuronidation [8, 9]. Specifically the *UGT1A* rs8330 C allele was associated with reduced normal splicing of *UGT1A* exon 4 to exon 5 and decreased acetaminophen glucuronidation in human liver microsomes. Furthermore the rs8330 C allele

was more frequently found in acetaminophen overdose patients that had developed acute liver failure [8].

Other *cis*- or *trans*-acting elements involved in the regulation of human *UGT1A* expression could predispose some individuals to a higher risk for acetaminophen-induced ALF. In recent years, microRNAs (miRNAs) have emerged as a crucial factor involved in *cis*-regulation of gene expression. miRNAs are a class of small non-coding RNAs that have been shown to control gene expression via imperfect base pairing to the 3'-UTR of target genes [12]. Accumulating data demonstrate that miRNAs modulate the expression of drug disposition genes, including CYPs, UGTs, and transporters [13]. For example, a recent study demonstrated that miR-491-3p binds directly to the *UGT1A* 3'-UTR, and that hepatic *UGT1A3* and *UGT1A6* mRNA concentrations show a statistically significant negative correlation with the miR-491-3p relative transcript amounts in human liver samples [14]. However, effects on hepatic glucuronidation activity were not reported. miRNAs can also indirectly control the expression of hepatic drug-metabolizing enzymes and transporters via regulation of transcription factors. For example, miR-148a has been shown to regulate human pregnane X receptor expression, which regulates CYP3A4 expression [15].

Studies aimed at identifying miRNAs that regulate drug metabolizing enzyme expression so far have used an approach that generally entails an initial bioinformatics search to predict putative miRNA binding sites in the target genes, followed by confirmation of binding site functionality through various mechanism-based studies and correlation analysis [16]. Unfortunately this approach frequently demonstrates a relatively high false negative and false positive discovery rate reflecting the imperfect binding of miRNAs to their targets [17]. Furthermore it is biased towards miRNAs that are predicted to bind directly to the 3'-UTR of the gene of interest, or to genes in the currently known regulatory pathway, and may miss miRNAs binding outside of the 3'-UTR or to novel regulatory genes. Consequently, it is likely that the full complement of miRNAs regulating drug metabolism pathway genes including *UGT1A*, either directly (via the *UGT1A* 3'-UTR shared by all UGT1A enzymes) or indirectly (through genes that regulate *UGT1A*) have not yet been discovered.

The objective of the current study was to use an unbiased approach (i.e. whole miRNA transcriptome association analysis) to identify miRNAs that either directly or indirectly regulate UGT1A-mediated glucuronidation in human liver. The ultimate goal of this work is to explain individual variability in susceptibility to adverse reactions to drugs metabolized by UGT1A enzymes, including acetaminophen.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Uridine diphosphate glucuronic acid (UDPGA), diazepam, and alamethicin were obtained from Sigma-Aldrich (St. Louis, MO). Ezetimibe and ezetimibe-D4 were purchased from Toronto Research Chemical (Toronto, ON, Canada). Morphine sulfate, serotonin, and midazolam were obtained from Cerilliant Corporation (Round rock, TX). Formic acid, methanol, and acetonitrile (ACS grade) were purchased from Avantor Performance Materials, Inc. (Center Valley, PA). Acetic acid and ammonium formate (ACS grade) were

obtained from Fisher Scientific (Grand Island, NY). For Western blot experiments the primary rabbit anti-AhR (sc-5579) and anti-beta-actin (sc-7210) antibodies, and the secondary goat anti-rabbit IgG-HRP (sc-2004) antibody were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX).

The pMIR-REPORT plasmid was obtained from Life Technologies (Grand Island, NY) and the Renilla luciferase vector pRL-CMV was purchased from Promega (Madison, WI). The most common variant of the *AhR* 3'-UTR (GenBank: L19872.1) cloned in pBlueScript (pBSK-AhR 3'-UTR) was kindly provided by Dr. Christopher A. Bradfield (McArdle Laboratory, University of Wisconsin-Madison) [18]. All synthesized DNA oligos were obtained from Integrated DNA Technologies, Inc. (Coralville, IA). Precursor miRNAs, mature miRNA mimics, and the respective negative controls (pre-miR miRNA Precursor Negative Control 1 and 2, miRNA mimics negative control 1 and 2), were purchased from Life Technologies (Grand Island, NY). The transfection reagents Lipofectamine 2000, and Lipofectamine RNAiMAX were obtained from Life Technologies and the transfection reagent DharmaFECT Duo was purchased from Dharmacon (Lafayette, CO).

2.2. Cell lines and culture conditions

HEK293 cells were cultured in DMEM (Life Technologies) supplemented with 10% FBS (Life Technologies). LS180 cells were maintained in DMEM (Life Technologies) supplemented with 10% FBS, 1 mM pyruvic acid (Life Technologies), and 0.1 mM MEM non-essential amino acids (Life Technologies). All cells were grown in a humidified chamber at 37°C with 5% CO₂.

2.3. miRNA transcriptome association analysis

Eighteen liver samples were selected from our human liver bank (N = 54) that demonstrated either the 9 lowest (0.4 - 2.4 nmoles / min / mg protein; N = 9; 5 males, 4 females; 1 black, 8 white) or 9 highest (3.3 – 6.8 nmoles / min / mg protein; 7 males, 2 females; all white) acetaminophen glucuronidation activities at 40 mM acetaminophen concentration. This substrate concentration was chosen since previous studies indicated that multiple UGT1A isoforms (including UGT1A1, UGT1A6 and UGT1A9) should contribute to the observed hepatic glucuronidation activity [4]. Complete details of the assay, results and the source of the liver bank tissues have been published recently [8, 19]. These de-identified liver samples were obtained from publicly available sources, including the National Disease Research Interchange (Philadelphia, PA) and the Liver Tissue Procurement and Distribution Service (Minneapolis, MN). The use of these tissues for this study was approved by the Washington State University Institutional Review Board.

Total RNA was extracted from individual liver samples using Trizol (Life Technologies). Equal amounts of total RNA were then pooled (3 different livers per pool) to create 3 pooled samples from high acetaminophen glucuronidation activity livers and 3 pooled samples from low acetaminophen glucuronidation activity livers. miRNA abundances were then determined in each of these 6 pooled samples using TaqMan Array Human MicroRNA A and B Cards v3.0 microfluidic cards with Megaplex RT and PreAmp Primers (Life Technologies) run on an ABI PRISM 7900HT quantitative real-time PCR instrument

(Applied Biosystems). These arrays included 754 unique assays providing comprehensive coverage of the human miRNAs listed in version 14 of the Sanger miRBase (<http://www.mirbase.org/>). Relative miRNA concentrations were derived by normalizing to the average of four replicates of the mammalian U6 reference transcript included on each card using the C_t method. Relative miRNA concentrations for the low acetaminophen glucuronidation activity livers were compared to the high acetaminophen glucuronidation activity livers using an unpaired t-test. The results with these pooled samples were confirmed by quantifying selected miRNAs in the 18 individual RNA samples (described below).

2.4. Construction of reporter plasmids

The *AhR* 3'-UTR [18] was PCR amplified from plasmid pBSK-AhR 3'-UTR using primers AhR F and AhR R (Table 1) and cloned at *HindIII* sites of vector pMIR-REPORT downstream of the firefly luciferase gene generating pMIR-AhR. The pMIR-UGT1A firefly luciferase plasmid containing the entire *UGT1A* 3'-UTR was constructed as described previously [8]. Plasmid pMIR-AhR and 5'-end phosphorylated primers 375 F and 375 R (Table 1) were employed for the generation of plasmid pMIR-AhR 375 which carries a deletion of the putative miR-375 binding sequence in the *AhR* 3'-UTR. The KAPA HiFi Polymerase (Kapa Biosystems, Inc., Wilmington, MA) was used for all the PCR reactions employed in the generation of the reporter plasmids. To generate all triplicate constructs (UGT1A 3x F/R, HNF1A 3x F/R, HNF1B 3x F/R, Nrf2 3x F/R, and AhR 3x F/R), a 5'-end phosphorylated oligonucleotide containing the triplicated miR-375 binding sequence in the 3'-UTR of the respective gene was annealed with its complementary sequence (Table 1) and cloned in pMIR-REPORT vector at *HindIII* sites. Nucleotide sequences of all plasmids used in this study were confirmed by DNA sequencing analysis performed at the Washington State University Molecular Biology and Genomics Core.

2.5. Luciferase reporter assays

HEK293T cells were plated in 96-well optical plates at 10^4 cells/well in Opti-MEM Reduced Serum Medium without antibiotics (Life Technologies). After overnight culture, cells were co-transfected in quadruplicate with a pMIR-REPORT vector carrying the triplicated miR-375 putative binding sequences of UGT1A (UGT1A 3x F/R), HNF1A (HNF1A 3x F/R), HNF1B (HNF1B 3x F/R), Nrf2 (Nrf2 3x F/R) or AhR (AhR 3x F/R) (10 ng/well), the pRL-CMV reporter vector (5 ng/well) and pre-miR for miR-375 or pre-miR negative controls 1 and 2 at 30 nM using Lipofectamine 2000 (0.25 μ L/well). 48 hrs after transfection, cells were harvested and firefly and Renilla luciferase activities were measured with a SpectraMax i3 microplate reader (Molecular Devices, Sunnyvale, CA) using the Dual-Glo Luciferase Assay System (Promega).

For transfection assays with the pMIR-UGT1A, pMIR-AhR or pMIR-AhR 375 firefly luciferase expression vectors HEK293 cells were transformed with the firefly luciferase construct (1.5 ng/well), the pRL-CMV Renilla luciferase control vector (0.5 ng/well) and the miRNA mimic or one of the mimic negative controls at 30 nM final concentration using DharmaFECT Duo (0.05 μ L/well). Transfection assays were performed in 384-well flat clear bottom white optical plates (Corning, NY) in quadruplicate. Briefly for each well the

transfection reagent was diluted in Opti-MEM, incubated 20 min at room temperature, miRNA mimics added and then incubated for 30 min at room temperature. HEK293 cells were harvested at 80–90% confluency, diluted in Opti-MEM, mixed with the transfection reagent-plasmid/miRNA complexes and plated to 5×10^3 cells/well. 24 hrs after transfection, cells were harvested and firefly and Renilla luciferase activities were measured with SpectraMax i3 microplate reader (Molecular Devices, Sunnyvale, CA) using the Dual-Glo Luciferase Assay System (Promega).

For all the transfections performed the firefly luciferase activity was normalized to the Renilla activity. The normalized values of cells transfected with negative controls (pre-miR negative controls 1 and 2 or miRNA mimics negative controls 1 and 2) and non-transfected cells were averaged. Subsequently the luciferase values of cells transfected with the pre-miR-375 or the mature miR-375 were normalized to these averaged values and presented relative to the luciferase values of cells transfected with the empty pMIR-REPORT vector (set at a value of 100%).

2.6. LS180 cell transfection and RNA extraction

LS180 cells (passage number 18–35) were transfected with mature miRNA mimics (miR-375 or the scrambled negative controls NC1, NC2) at 30 nM in duplicate. Briefly for each well, 3 μ L (12-well format) or 5 μ L (6-well format) of the RNAiMAX transfection reagent was diluted in 500 μ L of Opti-MEM Reduced Serum Medium without antibiotics, incubated 10 min at room temperature, followed by the addition of miRNA mimics and subsequently incubated for 20 min at room temperature. LS180 cells were harvested at 80–90% of confluency and diluted in Opti-MEM so that we finally had 1.5×10^6 cells/well (6-well format) or 8×10^5 cells/well (12-well format). Cells were mixed with the miRNA mimic-transfection reagent complexes, plated, and incubated at 37°C, 5% CO₂. 48 hrs after transfection, total RNA was extracted with the use of Trizol according to the manufacturer's instructions.

2.7. Quantitative real-time PCR (qPCR)

cDNAs were synthesized from total RNA with the use of the TaqMan Reverse Transcription Reagents (Life Technologies). TaqMan Gene Expression Master Mix and Taqman primer-probe mixes were from Life Technologies for the detection of human mRNA sequences for *UGT1A1* (Hs02511055_s1), *UGT1A6* (Hs01592477_m1), *UGT2B7* (Hs00426592_m1), *AhR* (Hs00169233_m1), *CYP1A2* (Hs00167927_m1), and *GAPDH* (Hs02758991_g1). To determine the relative concentrations of mature miRNAs in human liver samples and LS180 cells, the miRNA enriched fraction was reverse transcribed with miRNA-specific primers using MultiScribe Reverse Transcriptase (Life Technologies). Taqman miRNA assays and TaqMan Universal Master Mix II without UNG (Life Technologies) were used to measure the relative expression of miR-375 (#000564), miR-152-3p (#000475), and miR-23b (#002126). All assays were performed in triplicate. Gene expression was compared to an endogenous internal control (GAPDH for mRNA; the geometric mean of miR-152-3p and miR-23b concentrations for miRNA as described previously [20]) using the C_t method. To determine relative mRNA concentrations, the C_t values of non-transfected cells and cells transfected with the miR mimics negative controls (NC1, NC2) were averaged

(Negative control). These values were subsequently used to calculate fold change in relative expression of target genes (C_t method) in cells transfected with miR-375.

2.8. Glucuronidation assays

Glucuronidation of the probe drugs ezetimibe and morphine were used to measure UGT1A1 and UGT2B7 activity, respectively [21, 22]. LS180 cells were plated in 12-well plates (8 x 10⁵ cells/well) and transfected with mature miRNA mimics (miR-375 or the negative controls NC1, NC2) at 30 nM concentration. 48 hrs after transfection cell culture medium was aspirated, cells were washed twice with pre-warmed phosphate buffered saline, and then incubated (37°C, 5% CO₂) with Opti-MEM containing either ezetimibe (100 µM) for 1 hr or morphine (5 mM) for 4 hrs. The supernatant was collected to measure ezetimibe glucuronide formation rate (UGT1A1-mediated glucuronidation activity) and morphine-3-glucuronide formation rate (UGT2B7-mediated glucuronidation activity). Ezetimibe glucuronidation reactions were stopped by adding an equal volume of ice-cold acetonitrile/acetic acid solution (95:5) containing the internal standard ezetimibe-D4 (0.5 µM final concentration) and centrifuged (15,000 g, 10 min) before drying down in a speedvac and reconstituting in 40 µL of 0.1% formic acid in water/acetonitrile (85:15). Morphine glucuronidation assays were terminated by the addition of an equal volume of ice-cold acetonitrile/acetic acid solution (95:5) containing the internal standard diazepam (12.5 µM final concentration), centrifuged (15,000 g, 10 min) before drying down in a speedvac and reconstituting in 40 µL of 2 mM ammonium formate/methanol (9:1). All reactions were performed in duplicate.

Glucuronidation of serotonin was used as a selective indicator of UGT1A6 activity [23]. LS180 cells were plated in 6-well plates and transfected with miR-375 or the mimic negative controls at 30nM. 48hrs after transfection cell culture medium was aspirated; cells were washed twice with pre-warmed phosphate buffered saline, scraped from the plate and crude cell extracts were prepared as described previously [24]. Protein concentration was determined with the BCA assay kit (Life Technologies) and measured using SpectraMax i3 microplate reader. Serotonin glucuronidation assays were performed as described previously [23] using 100 µM serotonin and an incubation period of 60 min at 37°C. The reactions were stopped by adding an equal volume of ice-cold acetonitrile/acetic acid solution (95:5) containing the internal standard diazepam (12.5 µM final concentration), centrifuged (15,000 g, 10min) before drying down in a speedvac and reconstituted in 40 µL of 0.1% aqueous formic acid/acetonitrile (95:5).

All glucuronidation reactions were performed in duplicate. Measurement of glucuronide metabolites was performed using LC-MS/MS technology. The LC-MS/MS system consists of an Agilent 110 Series LC (Agilent Technologies, Santa Clara, CA) interfaced with API 4000 spectrometer (AB Sciex LLC, Framingham, MA) using electrospray ionization in the positive mode. HPLC separation was performed using a Synergi™ 4µm Fusion-RP 80Å (100x2 mm; Phenomenex, Torrance, CA) and data was quantified with the Analyst 1.6 software (AB Sciex LLC). The normalized activity values of non-transfected cells and cells transfected with the mimic negative controls were averaged (Negative control). Activity values of cells transfected with miR-375 were presented relative to the negative control. The

concentration of metabolites was normalized to the total number of cells per well (ezetimibe, morphine) or to protein concentration per assay (serotonin).

Gradient elution for ezetimibe and serotonin glucuronides was performed using a mobile phase system consisting of (A) 0.1% formic acid in water, (B) acetonitrile and flow rates of 0.35 and 0.4 mL/min, respectively. For ezetimibe glucuronide a mobile phase composition of 15% B was ramped to 90% B over 5 min, returned to 15% B over 1 min and maintained at 15% B for 4 min. Positive ion (M/Z+) mass transitions monitored included 584.5→271.3 for ezetimibe glucuronide and 412.5→271 for ezetimibe-D4. The gradient conditions for serotonin glucuronide were 10% B for 3 min, increased to 95% B over 0.05 min, held at 95% B for 4.95 min, returned to 10% B over 0.05 min and maintained at 10% B for 3.95 min. Positive ion (M/Z+) mass transitions monitored included 353→177 for serotonin glucuronide and 285→257 for diazepam. HPLC separation for morphine-3-glucuronide was achieved using a mobile phase system consisting of (A) 2 mM ammonium formate, (B) methanol and a flow rate of 0.3 mL/min. The gradient conditions were 10% B for 4 min, increased to 90% B over 0.05 min, held at 90% B for 3.95 min, returned to 10% B over 0.05 min and held at 10% B for 3.95 min. Positive ion (M/Z+) mass transitions monitored included 462→286 for morphine 3-glucuronide and 285→257 for diazepam.

2.9. Western blot analysis

Relative concentrations of AhR protein were determined in LS180 cells by semiquantitative immunoblotting. LS180 cells were plated and transfected in 6-well plates. 48 hrs after transfection cells were harvested and crude cell extracts were prepared as described previously [24]. Protein concentration was determined with the BCA assay kit (Life Technologies) and measured using SpectraMax i3 microplate reader (Molecular Devices). Whole cell lysates (20–30 µg) were adjusted to equal amounts of total protein, separated using Mini-PROTEAN TGX stain-free precast gels (Biorad) and transferred to Trans-Blot Turbo Mini PVDF membranes (Biorad) using the Trans-Blot Turbo Transfer System (Biorad). The membranes were blocked in 2% milk in TBS with Tween-20 (TBS-T) for 1hr, probed with rabbit anti-AhR or rabbit anti-beta-actin antibodies (1:200 dilution for each) overnight at 4°C and the corresponding secondary goat anti-rabbit IgG-HRP antibody (1:600). Protein bands were visualized using Clarity Western ECL Blotting Substrate (Biorad) and the band densities were quantified with ChemiDoc MP Imaging System (Biorad). The band densities of non-transfected cells and cells transfected with the two scrambled miRNA mimics negative controls (NC1, NC2) were averaged (Negative control). Band densities of cells transfected with the miR-375 mimic were presented relative to the Negative control values (set as a value of a 100%). Beta-actin served as a loading control.

2.10. Statistical analysis

Statistical analysis was performed with using SigmaPlot Version 11 (Systat Software, Inc., San Jose, CA) and included a two-tailed Student's *t* test and correlation analysis by Spearman's rank method. A *p* value of less than 0.05 was considered statistically significant.

3. RESULTS

3.1. Hepatic UGT1A activity is inversely correlated with miR-375

A miRNA transcriptome association analysis was conducted using RNA from banked human livers showing the highest (N=9) versus the lowest (N=9) acetaminophen glucuronidation (a measure of UGT1A activity) out of 54 phenotyped livers. As shown in Figure 1A, 9 miRNAs were identified in the initial screen with 2-fold higher expression and an unadjusted p value of less than 0.05 in the group of samples with low UGT1A activity compared to the group of high UGT1A activity individuals. Of these miRNAs, miR-375 showed the highest fold difference (10.6-fold, $p = 0.045$) between low and high UGT1A glucuronidation activity livers (Figure 1A). Next we determined miR-375 expression by quantitative RT-PCR in the same 18 liver samples. Confirming the whole miRNA transcriptome results, a significant inverse correlation was observed between miR-375 transcript concentrations and UGT1A activity ($R_s = -0.835$, $p < 0.001$). Moreover the expression of miR-375 was significantly higher ($p < 0.05$) in individual livers with low UGT1A activity as compared to individual livers with high UGT1A activity (Figure 1B). Consequently, we decided to evaluate the potential role of miR-375 in the regulation of hepatic UGT1A expression and function.

3.2. Functional miR-375 binding sites were not identified in the UGT1A-3'UTR or shared exons 2 - 5

First we sought to determine whether miR-375 could interact directly with the 3'-UTR and/or exons 2 - 5 shared by all UGT1A subfamily members. The lack of any miR-375 binding sites in the *UGT1A* 3'-UTR was validated using a plasmid carrying the full length *UGT1A* 3'-UTR (pMIR-UGT1A). As shown in Figure 2A the luciferase values of HEK293 cells transfected with this plasmid were not different compared to the average luciferase values of cells transfected with the mimic negative controls and cells transfected with the empty pMIR-REPORT vector (negative control). Putative binding sites of miR-375 in the *UGT1A* gene locus were identified by a bioinformatics approach that included the well validated programs miRanda (<http://www.microrna.org/microrna/home.do>) and MicroCosm (<http://www.ebi.ac.uk/enright-srv/microcosm/cgi-bin/targets/v5/search.pl>) [25–27]. Although MicroCosm failed to identify any miR-375 sites, miRanda identified a single putative miR-375 site located in exon 3 at a position -577 to -556 bp upstream of the *UGT1A* stop codon (Figure 2C). Results from other groups have demonstrated that miRNA response elements (MREs) can be located within the protein coding sequence of target genes [28]. The functionality of this site was evaluated using serially triplicated miRNA response element (3x MRE) constructs that were cloned in the forward (F) or reverse (R) orientation downstream of the firefly luciferase gene in the pMIR-REPORT vector (plasmid UGT1A 3x F/R). These constructs were transiently transfected into HEK293T cells with pre-miR-375 or the pre-miR negative controls together with the pRL-CMV vector (serving as a transfection control). As shown in Figure 2B the luciferase values of cells transfected with the forward and reverse constructs were not different ($p > 0.05$) compared to the average luciferase values of pre-miR negative control transfected cells and cells transfected with the empty pMIR-REPORT vector (negative control).

3.3. A functional miR-375 binding site is located in the AhR 3'-UTR

Next we investigated whether miR-375 could target the 3'-UTR of well characterized transcription factors known to regulate UGT1A expression, namely AhR, HNF1A, HNF1B, and Nrf2. Using miRanda we identified one putative miR-375 binding site in the 3'-UTRs of *AhR* located at position 1249–1270 bp downstream of the *AhR* stop codon (Figure 3C). Using MicroCosm we identified three putative miR-375 binding sites in the 3'-UTRs of *HNF1A* (75–96 bp downstream of the stop codon), *HNF1B* (307–330 bp downstream of the stop codon), and *Nrf2* (364–385 bp downstream of the stop codon) (Figure 3C).

To examine whether *AhR*, *HNF1A*, *HNF1B*, and *Nrf2* are indeed direct targets of miR-375, 3x MREs of each gene were then cloned in the forward or the reverse orientation downstream of the firefly luciferase gene in the pMIR-REPORT vector (plasmids AhR 3x F/R, HNF1A F/R, HNF1B F/R, and Nrf2 F/R). Only cells transfected with the AhR 3x F construct showed a statistically significant ($p = 0.0094$) decrease in luciferase activity (~40%) when compared to the average luciferase values of pre-miR negative control transfected cells and cells transfected with the empty pMIR-REPORT vector (Negative control) (Figure 3A).

To further confirm that AhR is a direct target of miR-375, we cloned the *AhR* 3'-UTR containing the predicted miR-375 MRE directly downstream of the firefly luciferase gene in the pMIR-REPORT vector (pMIR-AhR). We also generated a construct with a deletion of the predicted miR-375 MRE (pMIR-AhR 375). Cells transfected with the *AhR* 3'-UTR showed a statistically significant decrease (by 30%, $p = 0.033$) in luciferase activity compared with luciferase activities of the miRNA negative control transfected cells and cells transfected with the empty pMIR-REPORT vector (Figure 4). However, this reduction was abrogated in the pMIR-AhR 375 construct that lacked the miR-375 MRE (Figure 4).

3.4. Hepatic *AhR* mRNA expression is inversely correlated with miR-375 expression in human liver

To further assess the functional effect of hepatic miR-375 on *AhR* mRNA expression, we measured the relative amount of miR-375 transcripts and *AhR* mRNA by qPCR in the same 18 liver samples of the original discovery set. A significant inverse correlation was observed between miR-375 and *AhR* transcript concentrations ($R_s = -0.469$, $p = 0.049$).

3.5. miR-375 overexpression negatively regulates AhR mRNA and protein concentrations in LS180 cells

We next examined the effect of overexpression of a mature miR-375 mimic on endogenous AhR mRNA and protein concentrations in LS180 cells. We chose this human intestinal cell line since in preliminary studies LS180 cells demonstrated readily measurable AhR protein and mRNA with much higher UGT1A1 and UGT1A6 glucuronidation activities than the commonly used hepatic cell lines (HepG2 and Huh7) (data not shown). miR-375 transcript concentrations were also 32 to 256-fold higher in LS180 cells compared with human liver samples. Despite the intestinal origin of this cell line preliminary data indicated that the relative mRNA amounts of *AhR* and its downstream targets were comparable with the respective expression levels in human liver samples. This indicated that LS180 cells were a

good model to study the miR-375-dependent changes in AhR and UGT1A relative mRNA and protein amounts. LS180 cells were transiently transfected with mature miR-375 mimic or miRNA mimic negative controls (NC1, NC2) at 30 nM and cell homogenates and total RNA was collected. As can be seen in Figure 5A the AhR protein concentrations in cells transfected with miR-375 were significantly lower (by 40%, $p = 0.0014$) compared with the control, while beta-actin protein concentrations were unaffected. qPCR analysis showed that *AhR* mRNA transcripts were significantly decreased (by 10%, $p = 0.028$) in cells transfected with miR-375 (Figure 5B).

3.6. miR-375 overexpression downregulates the AhR target genes UGT1A1, UGT1A6, and CYP1A2, but not UGT2B7 in LS180 cells

Finally, we investigated whether the miR-375-dependent suppression of AhR could decrease the mRNA expression and enzyme activity of some known AhR target genes within the UGT1A subfamily (*UGT1A1* [29] and *UGT1A6* [30]) and in the CYP family (CYP1A2) without affecting a UGT gene (UGT2B7) that is not regulated by AhR. As shown in Figure 6A ezetimibe glucuronidation activity (UGT1A1 marker [21]) was reduced by 20% ($p = 0.028$) in LS180 cells transfected with miR-375 compared to the negative control. Moreover *UGT1A1* mRNA transcripts were reduced by 20% ($p < 0.001$), (Figure 6A). LS180 cells transfected with miR-375 showed a markedly reduced serotonin glucuronide formation (by 50%, $p < 0.001$) as shown in Figure 6B. *UGT1A6* mRNA expression showed a 30% ($p < 0.001$) in cells transfected with miR-375 (Figure 6B). In addition CYP1A2 mRNA expression was reduced by 50% ($p < 0.001$) in cells transfected with miR-375 (Figure 6C). Finally there was no difference in morphine glucuronidation (UGT2B7 marker) or *UGT2B7* mRNA expression in LS180 cells transfected with miR-375 ($p > 0.05$) (Figure 6D).

4. DISCUSSION

The most important novel finding of this study was the discovery of miR-375 as a major negative regulator of the UGT1A enzymes responsible for glucuronidation of acetaminophen in human liver (including UGT1A1 and UGT1A6) through post-transcriptional repression of *AhR* gene expression. Consequently, differences in miR-375 expression explain part of the high variability in UGT1A glucuronidation activities we have observed in our human liver bank samples [19]. These findings are also likely to extend to other drug handling genes regulated by AhR other than *UGT1A* in the liver such as CYP1A2 (as demonstrated here), and other oxidative enzymes, conjugative enzymes, and transporters, as well as AhR regulated proteins involved in cell proliferation/apoptosis, differentiation and inflammation [30].

A previous study suggested that UGT1A gene expression may also be directly regulated by miR-491-3p [14]. Although a negative correlation was found between hepatic *UGT1A3* and *UGT1A6* mRNA concentrations and miR-491-3p expression, effects on hepatic UGT1A enzyme activity were not evaluated. miR-491-3p expression was measured by real-time PCR as part of the Taqman array panel we used for the transcriptome association analysis. However miR-491-3p concentrations were consistently below the quantitation threshold ($C_t = 40$ cycles) despite performing a preamplification step (data not shown).

miR-375 plays a key role in many biological processes including pancreatic islet development, glucose homeostasis, and tumorigenesis among others [31, 32]. Recent studies have indicated the potential utility of measuring the concentrations of miRNAs in the plasma and/or urine as biomarkers of various pathological states including cancer, diabetes, as well as acetaminophen-induced hepatotoxicity [33]. Interestingly serum and urine concentrations of miR-375 were observed to be elevated in children hospitalized after acetaminophen overdose compared with children receiving therapeutic doses of acetaminophen, or no acetaminophen [33]. In addition, miR-375 concentrations in the serum showed a significant positive correlation with serum acetaminophen protein adduct concentrations, suggesting a potential role of miR-375 as a biomarker of the severity of acetaminophen-induced liver toxicity. However, since samples were not collected from these children prior to acetaminophen exposure, the observed elevation in miR-375 concentrations may have preceded exposure. Consequently, at present it is unclear whether the elevation of miR-375 was a direct consequence of liver injury (such as by release from damaged hepatocytes into the circulation), or was a reflection of pre-existent elevation of miR-375 expression in certain individuals that had predisposed them to greater injury through lower UGT1A glucuronidation, as suggested by the results of this study. Future studies are needed to clarify this.

We observed over 10-fold variation in miR-375 concentrations in our liver samples. Unfortunately, relatively little is known about the regulation of miRNA expression, including miR-375. Interestingly, increased circulating amounts of miR-375 have been observed in patients with type-2 diabetes mellitus in association with hypomethylation of the miR-375 promoter [34]. Results from a recent study demonstrated drug-induced changes in the relative expression amounts of miRNAs in various human cell lines [35]. Future studies are required to examine the association between acetaminophen administration and miRNome profile changes in individuals.

The results of our work is likely to have significant impact beyond acetaminophen hepatotoxicity since UGT1A mediated glucuronidation is essential for detoxification and clearance of a large variety of drugs, some of which have a narrow therapeutic index. Notable examples include irinotecan that is used to treat colon cancer and shows treatment-limiting toxicity in individuals with UGT1A1 polymorphisms; and mycophenolic acid, an immunosuppressant that is associated with transplant rejection in individuals with UGT1A9 polymorphisms [19]. Consequently, miR-375 could be of value in identifying individuals requiring dose adjustments to prevent toxicity, in conjunction with UGT genotyping.

The mouse *Ahr* gene was also identified as a potential target of miR-375 in the first study to evaluate the utility of the bioinformatics program PicTar to predict miRNA targets [36]. However preliminary studies failed to show an effect of miR-375 transfection on AhR protein by immunoblotting in mouse neuroblastoma (N2A) cell. This discrepancy with our finding could be explained by species (mouse versus human) and cell type (neuronal versus intestinal) differences. For example, miR-22 has been demonstrated to regulate mitochondrial targeted 2C-type serine/threonine protein phosphatase (PP2Cm) in human but not in mouse cells [37].

There were several limitations to our study that should be noted. We found statistically significant but relatively small decreases in AhR protein concentrations (less than 2-fold; Figure 5A) and *AhR* relative mRNA expression (10%, Figure 5B) in miR-375 transfected cells. A possible explanation for this modest effect is that miR-375 expression in LS180 cells is relatively high compared with human liver samples (by 32 to 256-fold), thus making it more difficult to further inhibit *AhR* expression. Although LS180 cells recapitulate many aspects of UGT expression and regulation in human liver, additional studies using primary human hepatocytes will be required to verify and further explore the association between miR-375 overexpression and reduced UGT1A glucuronidation through *AhR* and possibly other gene regulatory pathways. Furthermore, studies will be needed to determine whether our findings can be extrapolated to glucuronidation in vivo.

Our unbiased exploratory experimental approach also identified eight more miRNAs associated with low UGT1A activity, as well as a larger group of miRNAs associated with higher UGT1A activity. Ongoing research in our lab is evaluating the contribution of these miRNAs in the variability of UGT1A expression in human liver and their direct or indirect involvement in the control of UGT1A expression.

Acknowledgments

This work was supported by the National Institutes of Health through National Institute of General Medical Sciences Grant NIH-R01-GM102130 (to M.H.C.) and National Institute of Diabetes and Digestive and Kidney Diseases Grant N01-DK-7-0004 (to the Liver Tissue Cell Distribution System, University of Minnesota and University of Pittsburgh). Dr Court was also supported by the William R. Jones endowment to Washington State University College of Veterinary Medicine.

Abbreviations

ALF	acute liver failure
NAPQI	N-acetyl-p-benzoquinone imine
UGT	UDP-glucuronosyltransferase
SULT	sulfotransferase
CYP	cytochrome P450
3'-UTR	3'-untranslated region
SNP	single nucleotide polymorphism
miRNA	microRNA
UDPGA	uridine diphosphate glucuronic acid
qPCR	quantitative real-time PCR
AhR	aryl hydrocarbon receptor
MRE	miRNA response element
HNF1A	hepatocyte nuclear factor 1-alpha

HNF1B	hepatocyte nuclear factor 1-beta
Nrf2	nuclear factor erythroid 2-related factor 2
NC1 and 2	miRNA mimic negative control 1 and 2
Ez-Glu	ezetimibe glucuronide
5HT-Glu	serotonin glucuronide
M3-Glu	morphine-3 glucuronide

References

1. Lee WM. Acute liver failure. *Semin Respir Crit Care Med*. 2012; 33:36–45. [PubMed: 22447259]
2. Larson AM. Acetaminophen hepatotoxicity. *Clin Liver Dis*. 2007; 11:525–548. vi. [PubMed: 17723918]
3. Prescott LF. Kinetics and metabolism of paracetamol and phenacetin. *Br J Clin Pharmacol*. 1980; 10(Suppl 2):291S–298S. [PubMed: 7002186]
4. Court MH, Duan SX, von Moltke LL, Greenblatt DJ, Patten CJ, Miners JO, Mackenzie PI. Interindividual variability in acetaminophen glucuronidation by human liver microsomes: identification of relevant acetaminophen UDP-glucuronosyltransferase isoforms. *J Pharmacol Exp Ther*. 2001; 299:998–1006. [PubMed: 11714888]
5. Chowdhury JR, Kondapalli R, Chowdhury NR. Gunn rat: a model for inherited deficiency of bilirubin glucuronidation. *Adv Vet Sci Comp Med*. 1993; 37:149–173. [PubMed: 8273513]
6. de Morais SM, Wells PG. Deficiency in bilirubin UDP-glucuronyl transferase as a genetic determinant of acetaminophen toxicity. *J Pharmacol Exp Ther*. 1988; 247:323–331. [PubMed: 3139868]
7. de Morais SM, Wells PG. Enhanced acetaminophen toxicity in rats with bilirubin glucuronyl transferase deficiency. *Hepatology*. 1989; 10:163–167. [PubMed: 2501210]
8. Court MH, Freytsis M, Wang X, Peter I, Guillemette C, Hazarika S, Duan SX, Greenblatt DJ, Lee WM. The UDP-glucuronosyltransferase (UGT) 1A polymorphism c.2042C>G (rs8330) is associated with increased human liver acetaminophen glucuronidation, increased UGT1A exon 5a/5b splice variant mRNA ratio, and decreased risk of unintentional acetaminophen-induced acute liver failure. *J Pharmacol Exp Ther*. 2013; 345:297–307. [PubMed: 23408116]
9. Court MH, Peter I, Hazarika S, Vasiadi M, Greenblatt DJ, Lee WM. Candidate gene polymorphisms in patients with acetaminophen-induced acute liver failure. *Drug Metab Dispos*. 2014; 42:28–32. [PubMed: 24104197]
10. Laine JE, Auriola S, Pasanen M, Juvonen RO. Acetaminophen bioactivation by human cytochrome P450 enzymes and animal microsomes. *Xenobiotica*. 2009; 39:11–21. [PubMed: 19219744]
11. Yamamoto A, Liu MY, Kurogi K, Sakakibara Y, Saeki Y, Suiko M, Liu MC. Sulphation of acetaminophen by the human cytosolic sulfotransferases: a systematic analysis. *J Biochem*. 2015; 158:497–504. [PubMed: 26067475]
12. Chekulaeva M, Filipowicz W. Mechanisms of miRNA-mediated post-transcriptional regulation in animal cells. *Curr Opin Cell Biol*. 2009; 21:452–460. [PubMed: 19450959]
13. Yu AM, Tian Y, Tu MJ, Ho PY, Jilek JL. MicroRNA Pharmacoeugenetics: Posttranscriptional Regulation Mechanisms behind Variable Drug Disposition and Strategy to Develop More Effective Therapy. *Drug Metab Dispos*. 2016; 44:308–319. [PubMed: 26566807]
14. Dluzen DF, Sun D, Salzberg AC, Jones N, Bushey RT, Robertson GP, Lazarus P. Regulation of UDP-glucuronosyltransferase 1A1 expression and activity by microRNA 491–3p. *J Pharmacol Exp Ther*. 2014; 348:465–477. [PubMed: 24399855]
15. Takagi S, Nakajima M, Mohri T, Yokoi T. Post-transcriptional regulation of human pregnane X receptor by micro-RNA affects the expression of cytochrome P450 3A4. *J Biol Chem*. 2008; 283:9674–9680. [PubMed: 18268015]

16. Akhtar MM, Micolucci L, Islam MS, Olivieri F, Procopio AD. Bioinformatic tools for microRNA dissection. *Nucleic Acids Res.* 2016; 44:24–44. [PubMed: 26578605]
17. Yue D, Liu H, Huang Y. Survey of Computational Algorithms for MicroRNA Target Prediction. *Curr Genomics.* 2009; 10:478–492. [PubMed: 20436875]
18. Dolwick KM, Schmidt JV, Carver LA, Swanson HI, Bradfield CA. Cloning and expression of a human Ah receptor cDNA. *Mol Pharmacol.* 1993; 44:911–917. [PubMed: 8246913]
19. Court MH. Interindividual variability in hepatic drug glucuronidation: studies into the role of age, sex, enzyme inducers, and genetic polymorphism using the human liver bank as a model system. *Drug Metab Rev.* 2010; 42:209–224. [PubMed: 19821798]
20. Lamba V, Ghodke-Puranik Y, Guan W, Lamba JK. Identification of suitable reference genes for hepatic microRNA quantitation. *BMC Res Notes.* 2014; 7:129. [PubMed: 24606728]
21. Ghosal A, Hapangama N, Yuan Y, Achanfuo-Yeboah J, Iannucci R, Chowdhury S, Alton K, Patrick JE, Zbaida S. Identification of human UDP-glucuronosyltransferase enzyme(s) responsible for the glucuronidation of ezetimibe (Zetia). *Drug Metab Dispos.* 2004; 32:314–320. [PubMed: 14977865]
22. Court MH, Krishnaswamy S, Hao Q, Duan SX, Patten CJ, Von Moltke LL, Greenblatt DJ. Evaluation of 3'-azido-3'-deoxythymidine, morphine, and codeine as probe substrates for UDP-glucuronosyltransferase 2B7 (UGT2B7) in human liver microsomes: specificity and influence of the UGT2B7*2 polymorphism. *Drug Metab Dispos.* 2003; 31:1125–1133. [PubMed: 12920168]
23. Krishnaswamy S, Duan SX, Von Moltke LL, Greenblatt DJ, Court MH. Validation of serotonin (5-hydroxytryptamine) as an in vitro substrate probe for human UDP-glucuronosyltransferase (UGT) 1A6. *Drug Metab Dispos.* 2003; 31:133–139. [PubMed: 12485962]
24. Court MH, Hazarika S, Krishnaswamy S, Finel M, Williams JA. Novel polymorphic human UDP-glucuronosyltransferase 2A3: cloning, functional characterization of enzyme variants, comparative tissue expression, and gene induction. *Mol Pharmacol.* 2008; 74:744–754. [PubMed: 18523138]
25. Betel D, Wilson M, Gabow A, Marks DS, Sander C. The microRNA.org resource: targets and expression. *Nucleic Acids Res.* 2008; 36:D149–153. [PubMed: 18158296]
26. Enright AJ, John B, Gaul U, Tuschl T, Sander C, Marks DS. MicroRNA targets in *Drosophila*. *Genome Biol.* 2003; 5:R1. [PubMed: 14709173]
27. John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS. Human MicroRNA targets. *PLoS Biol.* 2004; 2:e363. [PubMed: 15502875]
28. Tay Y, Zhang J, Thomson AM, Lim B, Rigoutsos I. MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation. *Nature.* 2008; 455:1124–1128. [PubMed: 18806776]
29. Togawa H, Shinkai S, Mizutani T. Induction of human UGT1A1 by bilirubin through AhR dependent pathway. *Drug Metab Lett.* 2008; 2:231–237. [PubMed: 19356098]
30. Bock KW, Bock-Hennig BS. UDP-glucuronosyltransferases (UGTs): from purification of Ah-receptor-inducible UGT1A6 to coordinate regulation of subsets of CYPs, UGTs, and ABC transporters by nuclear receptors. *Drug Metab Rev.* 2010; 42:6–13. [PubMed: 19831498]
31. Poy MN, Hausser J, Trajkovski M, Braun M, Collins S, Rorsman P, Zavolan M, Stoffel M. miR-375 maintains normal pancreatic alpha- and beta-cell mass. *Proc Natl Acad Sci U S A.* 2009; 106:5813–5818. [PubMed: 19289822]
32. Yan JW, Lin JS, He XX. The emerging role of miR-375 in cancer. *Int J Cancer.* 2014; 135:1011–1018. [PubMed: 24166096]
33. Yang X, Salminen WF, Shi Q, Greenhaw J, Gill PS, Bhattacharyya S, Beger RD, Mendrick DL, Mattes WB, James LP. Potential of extracellular microRNAs as biomarkers of acetaminophen toxicity in children. *Toxicol Appl Pharmacol.* 2015; 284:180–187. [PubMed: 25708609]
34. Sun K, Chang X, Yin L, Li J, Zhou T, Zhang C, Chen X. Expression and DNA methylation status of microRNA-375 in patients with type 2 diabetes mellitus. *Mol Med Rep.* 2014; 9:967–972. [PubMed: 24366165]
35. Rodrigues AC, Li X, Radecki L, Pan YZ, Winter JC, Huang M, Yu AM. MicroRNA expression is differentially altered by xenobiotic drugs in different human cell lines. *Biopharm Drug Dispos.* 2011; 32:355–367. [PubMed: 21796641]

36. Krek A, Grun D, Poy MN, Wolf R, Rosenberg L, Epstein EJ, MacMenamin P, da Piedade I, Gunsalus KC, Stoffel M, Rajewsky N. Combinatorial microRNA target predictions. *Nat Genet.* 2005; 37:495–500. [PubMed: 15806104]
37. Pan BF, Gao C, Ren SX, Wang YB, Sun HP, Zhou MY. Regulation of PP2Cm expression by miRNA-204/211 and miRNA-22 in mouse and human cells. *Acta Pharmacol Sin.* 2015; 36:1480–1486. [PubMed: 26592513]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1.

Association of hepatic UGT1A-mediated acetaminophen glucuronidation activity with miR-375 concentrations. A) Volcano plot showing the results of miRNA transcriptome association analysis used to identify miRNAs expressed more highly in liver samples with low acetaminophen glucuronidation activity (a UGT1A marker) compared with those with high rates of acetaminophen glucuronidation activity. Data for each miRNA assayed (out of 754 total human miRNAs assayed from Sanger mirBase v14) are shown as individual circles on the plot. The x-axis shows the $\log_2$ ratio of mean miRNA concentrations measured in livers with high glucuronidation activities divided by mean miRNA concentrations in livers with low glucuronidation activities (3 pools of 3 livers in each group). The y-axis shows the $-\log_{10} p$ value of a t-test comparing these mean values for each miRNA, indicating the strength of the association. Filled circles identify 9 candidate miRNAs with 2-fold difference and $p < 0.05$. miR-375 (indicated with an arrow) was chosen for further analysis. B) miR-375 transcripts were quantified in liver samples of individuals with high ($N = 9$) and

low ($N = 9$) UGT1A activity via qPCR. Data are expressed relative to the sample with the highest miR-375 concentration (set to 1). * $p < 0.05$.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 2. miR-375 does not have a binding site in the *UGT1A* 3'-UTR. Luciferase activities of HEK293 cells transfected with the pMIR-REPORT vector carrying the full length *UGT1A* 3'-UTR (A) or the triplicated putative miRNA response elements of *UGT1A* (B). The activity of the reporter constructs was first normalized to the activity of the pRL-CMV vector and then presented relative to the average values of cells transfected with the empty pMIR vector or the empty vector and the pre-miR negative controls (Negative control, set at a value of 100%). Data are shown as mean $\pm$ S.D. from three independent experiments performed in quadruplicate. C) Exon 3 of *UGT1A* contains putative miR-375 binding sites. Complementarity of miR-375 to the predicted target sequences of this gene is indicated. Nucleotide positions in the mRNA are indicated relative to the last nucleotide of the stop codon.

Figure 3.

Identification of a miR-375 binding site in the *AhR* 3'-UTR. Luciferase activities of cells transfected with the pMIR-REPORT vector carrying the triplicated putative miRNA response elements of *HNF1A*, *HNF1B*, *Nrf2*, or *AhR* cloned in forward (A) or reverse (B) orientation, and co-transfected with either pre-miR-375 or pre-miR negative control. The activity of the reporter constructs was first normalized to the activity of the pRL-CMV vector and then presented relative to the average values of cells transfected with the empty pMIR vector or the empty vector and the pre-miR negative controls (Negative control, set at a value of 100%). Data are shown as mean $\pm$ S.D. from three independent experiments performed in quadruplicate. ** $p < 0.01$. C) The 3'-UTRs of *HNF1A*, *HNF1B*, *Nrf2*, and *AhR* contain putative miR-375 binding sites. Complementarity of miR-375 to the predicted target sequences of these genes is indicated. Nucleotide positions in the mRNA are indicated relative to the last nucleotide of the stop codon.

Figure 4.

Effect of miR-375 on AhR 3'-UTR-mediated luciferase activity. Luciferase reporter assays using HEK293T cells co-transfected with plasmids pMIR-AhR 3'-UTR or pMIR-AhR

375, the pRL-CMV vector and miR-375 or the miR mimics negative controls at 30 nM. The activity of the reporter constructs was first normalized to the activity of the pRL-CMV vector and then presented relative to the average values of cells transfected with the empty pMIR vector or the empty vector and the pre-miR negative controls (Negative control, set at a value of 100%). Data shown are mean $\pm$ S.D. from three independent experiments performed in quadruplicate. * $p < 0.05$.

Figure 5.

Effect of miR-375 overexpression on AhR mRNA and protein in LS180 cells. miR-375 mimic or the negative controls (NC1, NC2) were transfected into LS180 cells at 30 nM concentration. After 48 hrs the cells were harvested and total RNA and protein was isolated. A). Representative Western blot of AhR and beta-actin protein in whole cell lysates. B). Data from Western blots showing AhR protein concentrations normalized to beta-actin concentrations. Values are the mean $\pm$ S.D. from three independent experiments. ** $p < 0.01$. C). *AhR* mRNA concentrations were determined by qPCR and normalized to *GAPDH* mRNA concentrations and expressed relative to the averaged C_t *AhR* values of cells transfected with the miRNA mimics negative controls and non-transfected cells (Negative control). The values are the mean $\pm$ S.D. from three independent experiments. *** $p < 0.001$.

Figure 6.

Effect of miR-375 mimic overexpression on UGT1A1, UGT1A6, and UGT2B7 activity and mRNA, and *CYP1A2* mRNA in LS180 cells. miR-375 or the mimic negative controls were transfected into LS180 cells at 30 nM. 48 hrs after transfection enzyme activity measurements and qPCR analysis were performed as described in the Materials and Methods. A) Ezetimibe glucuronide formation and *UGT1A1* mRNA relative to the negative control. B) Serotonin glucuronide formation and *UGT1A6* mRNA relative to the negative control. C) *CYP1A2* mRNA relative to the negative control. D) Morphine-3-glucuronide formation rate and *UGT2B7* mRNA relative to the negative control. The values are the mean $\pm$ S.D. from three independent experiments. * $p < 0.05$, *** $p < 0.001$.

Table 1

List of oligonucleotides used in this study as PCR primers, for mutagenesis, or to construct microRNA response elements. Underlined are shown the *HindIII* restriction sites used for cloning in the pMIR-REPORT vector. All oligonucleotides used for the generation of the triplicate and deletion constructs were 5'-end phosphorylated.

Oligo	Sequence (5' – 3')
AhR F	AATTA <u>AAGCTT</u> GTGAATTGAATGGTGCATTG
AhR R	AATTAAGCTTGTTTAACGTGATTTTATTATTCTTAG
375 F	GGTTTAAACAGTTAAGCTTGTGAATTGAATGGTGCA
375 R	GGACTTTTTTAAATGAAAACTGCAAATATCTTACAAATAAT
AhR MRE 3x F	AGCTTGAATGGTGCATTGTATAGATATAATGAACAAAATTAT TTGTAGAATGGTGCATTGTATAGATATAATGAACAAAATTATT TGTAAGAATGGTGCATTGTATAGATATAATGAACAAAATTATTTGTA
AhR MRE 3x R	AGCTTACAAATAATTTTGTTCATTATATCTATACAATGCACCATT CTACAAATAATTTTGTTCATTATATCTATACAATGCACCATTCTA CAAATAATTTTGTTCATTATATCTATACAATGCACCATT
UGT1A MRE 3x F	AGCTTAGCCACACATCAATTTGGTTGTTGCGAACGGACTTTG TTTTGAGCCACACATCAATTTGGTTGTTGCGAACGGACTTTG TTTTGAGCCACACATCAATTTGGTTGTTGCGAACGGACTTTGTTTGA
UGT1A MRE 3x R	AGCTTCAAAACAAAGTCCGTTTCGCAACAACCAAATTGATGTGTGGC TCAAAACAAAGTCCGTTTCGCAACAACCAAATTGATGTGTGGCTCAA ACAAAGTCCGTTTCGCAACAACCAAATTGATGTGTGGCTA
HNF1A MRE 3x F	AGCTTCCTGCCTGGAGGACCTGAGCCTGCCGAGCAACCGTGGCCC TTCCTGCCTGGAGGACCTGAGCCTGCCGAGCAACCGTGGCCCTCC TGCTTGGAGGACCTGAGCCTGCCGAGCAACCGTGGCCCTTA
HNF1A MRE 3x R	AGCTTAAGGGCCACGGTTGCTCGGCAGGCTCAGGTCCTCCAGGCA GGAAGGGCCACGGTTGCTCGGCAGGCTCAGGTCCTCCAGGCAGGA AGGGCCACGGTTGCTCGGCAGGCTCAGGTCCT
HNF1B MRE 3x F	AGCTTGACTGTCTATGTTGTGATCCTTCATCGAACAAACTGATGCG AAGTACTGTCTATGTTGTGATCCTTCATCGAACAAACTGATGCGAAG TACTGTCTATGTTGTGATCCTTCATCGAACAAACTGATGCGA
HNF1B MRE 3x R	AGCTTCGCATCAGTTTGTTCGATGAAGGATCACAACATAGACAGTACT TCGCATCAGTTTGTTCGATGAAGGATCACAACATAGACAGTACTTCGC ATCAGTTTGTTCGATGAAGGATCACAAC
Nrf2 MRE 3x F	AGCTTATGTACTTTTTTATAAATACTGTATGGACAAAAAATGGCATTATG TACTTTTTTATAAATACTGTATGGACAAAAAATGGCATTATGTACTTTTTT ATAAATACTGTATGGACAAAAAATGGCATT
Nrf2 MRE 3x R	AGCTTAATGCCATTTTTTGTCCATACAGTATTTATAAAAAAGTACATAATTT GCCATTTTTTGTCCATACAGTATTTATAAAAAAGTACATAATGCCATTTT TGTCATACAGTATTTATAAAAAAGT