Published in final edited form as:

Biochem Pharmacol. 2019 November ; 169: 113617. doi:10.1016/j.bcp.2019.08.019.

MicroRNAs hsa-miR-495-3p and hsa-miR-486-5p suppress basal and rifampicin-induced expression of human sulfotransferase 2A1 (SULT2A1) by facilitating mRNA degradation

Dongying Li, Bridgett Knox, Si Chen, Leihong Wu, William H. Tolleson, Zhichao Liu, Dianke Yu¹, Lei Guo, Weida Tong, Baitang Ning*

National Center for Toxicological Research (NCTR), U.S. Food and Drug Administration (FDA), 3900 NCTR Road, Jefferson, AR 72079, USA

Abstract

Drug metabolizing enzymes mediate biotransformation of drugs and play an essential role in drug efficacy and toxicity. Human sulfotransferases are a superfamily of Phase II detoxification enzymes that metabolize a wide spectrum of endogenous compounds and xenobiotics. SULT2A1 is one of the most abundant hepatic sulfotransferases and it catalyzes the sulfate conjugation of many endogenous substrates, such as bile acids and steroids. In the current study, we utilized a systematic approach by combining a series of computational analyses and *in vitro* methods to identify miRNAs that repress SULT2A1 expression post-transcriptionally. Our *in silico* analyses predicted miRNA response elements for hsa-miR-495-3p and hsa-miR-486-5p within the 3'-UTR of SULT2A1 mRNA and the levels of these miRNAs were inversely correlated with that of SULT2A1 mRNA in human liver. Using fluorescence-based RNA electrophoretic mobility shift assays, we found that hsa-miR-495-3p and hsa-miR-486-5p interacted directly with the SULT2A1 3'-UTR. The activity of a luciferase reporter gene construct containing sequences from the SULT2A1 3-UTR was suppressed by hsa-miR-486-5p and hsa-miR-495-3p. Furthermore, gain- and loss-of-function assays demonstrated that hsa-miR-486-5p and hsa-miR-495-3p negatively modulate basal and rifampicin-induced expression of SULT2A1 in HepG2 cells by decreasing mRNA stability.

*Corresponding author: National Center for Toxicological Research, 3900 NCTR Road, HFT100, Jefferson, AR 72079, USA. baitang.ning@fda.hhs.gov (B. Ning).

¹Current Address: School of Public Health, Qingdao University, Qingdao, China.

Author contributions

Participated in study design: Li, Yu, Tong, and Ning.

Conducted experiments: Li, Knox, Wu, and Liu.

Performed data analysis: Li, Chen, Tolleson, and Ning.

Wrote or contributed to the writing of the manuscript: Li, Chen, Tolleson, Guo, and Ning.

Conflict of interest

The authors declare no competing financial interests.

Publisher's Disclaimer: Disclaimer

Publisher's Disclaimer: This article is not an official guidance or policy statement of the U.S. Food and Drug Administration (FDA). No official support or endorsement by the U.S. FDA is intended or should be inferred.

Keywords

MicroRNA; Sulfotransferase (SULT); SULT2A1; miR-495-3p; miR-486-5p; Epigenetics; Drug metabolism

1. Introduction

Drug metabolizing enzymes (DMEs) mediate the biotransformation of drugs and other xenobiotics, thus affecting drug efficacy and toxicity [1]. Human sulfotransferases (SULTs) are a superfamily of Phase II detoxification enzymes that catalyze a broad range of sulfation reactions; endogenous biochemicals and exogenous compounds, including steroid hormones, neurotransmitters, therapeutics, and carcinogens, are conjugated with a sulfate group to form more polar metabolites, which facilitates their elimination [2,3]. The human SULTs are comprised of four families, SULT1, SULT2, SULT4, and SULT6 [4]. The SULT2 family contains two genes (*SULT2A* and *SULT2B*) encoding three functional isoforms, SULT2A1, SULT2B1a, and SULT2B1b. The *SULT2A* and *SULT2B* genes encode homologous proteins with 48% amino acid sequence identity and similar substrate specificities [5]. Human SULT2A1 is expressed primarily in the liver and adrenal glands [4], and it is one of the most abundant hepatic SULTs [6]. SULT2A1 is involved in the transformation of endogenous compounds, such as bile acid and steroids [3], and xenobiotics, such as the breast cancer drug 4-hydro-xytamoxifen [7], the anti-inflammatory drug budesonide [8], and various environmental estrogens [9]. In some cases, reactive electrophilic metabolites are produced by SULT-dependent reactions by which procarcinogens, such as hydroxymethyl polyaromatic hydrocarbons, are activated to generate DNA and protein adducts associated with carcinogenesis and toxicity [10].

The expression of SULT2A1 is an important factor for the homeostasis of steroid hormones and bile acids and for the efficiency of drug metabolism and clearance. High inter- and intra-individual variability in SULT2A1 expression has been observed in human liver samples [11]. Individual dissimilarities in the level of SULT2A1 expression may be due to alterations in DNA sequences, such as copy number variations [11], or due to epigenetic differences, such as altered DNA methylation status [12].

MicroRNAs (miRNAs) are a class of small non-coding RNAs that are involved in many biological and pathological processes by mediating post-transcriptional gene silencing. miRNAs post-transcriptionally regulate the expression of both Phase I enzymes, such as cytochrome P450s (CYPs) [13,14], and Phase II DMEs, including UDP-glucuronosyltransferases (UGTs) [15–17] and SULTs [18,19]. Our previous study [19] demonstrated that miRNAs are involved in suppressing SULT2A1 in liver cells exposed to excessive acetaminophen. Under acetaminophen overdose conditions, miR-877-5p up-regulation caused down-regulation of the nuclear receptor NR1I2 and decreased expression of SULT2A1. In the current study, we investigated systematically the regulatory role of miRNAs in the expression of SULT2A1 using an integrated approach that combined computational predictions with biochemical, molecular, and cellular assays to identify miRNAs that potentially suppress SULT2A1 expression. The results of our study

demonstrate that miR-495-3p and miR-486-5p down-regulate SULT2A1 expression by binding to the 3'-untranslated region (UTR) of SULT2A1 mRNA and promoting SULT2A1 mRNA degradation.

2. Materials and methods

2.1. Cell culture

The HepG2 human hepatocellular carcinoma cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in a complete medium containing Dulbecco's Modified Eagle's Medium (DMEM, ATCC) supplemented with 10% fetal bovine serum (FBS, ATCC), and 1× Antibiotic-Antimycotic (ThermoFisher, Waltham, MA). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. Cells within passages 2–10 were used in experiments.

2.2. *In silico* analyses

In silico prediction of miRNAs targeting the 3'-UTR of SULT2A1 mRNA was performed using the public databases <http://microRNA.org> (<http://www.microrna.org/>) [20] and TargetScan [21] (Release 7.1, <http://www.targetscan.org>). Predictions obtained using the miRanda algorithm from www.microRNA.org comprised conserved miRNAs with a mirSVR score equal to or less than -0.1. Results predicted by the two algorithms were compared and a Venn diagram was generated using <http://bioinformatics.psb.ugent.be/webtools/Venn/>. Integrated DNA Technologies' (IDT, Coralville, IA) OligoAnalyzer Tool and RNAhybrid (<https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid/>) were used to calculate the free energies required for the formation of the miRNA:mRNA duplexes between predicted miRNAs and miRNA recognition elements (MREs) on SULT2A1 mRNA.

2.3. Correlation analysis

The Cancer Genome Atlas Liver Hepatocellular Carcinoma (TCGALHC) RNA-seq and miRNA-seq datasets were downloaded from <http://firebrowse.org/> (Broad Institute, Boston, MA). The expression profiles of SULT2A1 mRNA and 10 miRNAs predicted by both miRanda and TargetScan were extracted from the datasets and subjected to Pearson's correlation analysis, which was performed using GraphPad Prism 5. All expression levels were presented as reads per million RNA mapped.

2.4. Fluorescence-based RNA electrophoretic mobility shift assays (FREMSAs)

The 5' ends of the miRNA oligonucleotides for hsa-miR-495-3p and hsa-miR-486-5p were labeled with cy5.5TM dye, while IRDye800 was added to the 5' end of the 2' O-methyl-modified target mRNA oligonucleotides containing the miR-495-MRE-1, miR-495-MRE-2, and miR-486-MRE. Probe sequences are presented in Table 1. The probes were synthesized by IDT.

FREMSA were performed as in prior reports [19,22]. Briefly, 20 nM fluorophore-tagged synthetic miRNA and/or target mRNA oligonucleotides were mixed with H₂O and EMSA binding buffer (LightShift Chemiluminescent RNA EMSA kit, ThermoFisher) in a 20 µL RNA binding reaction. Cold miRNA or cold negative control (cold-NC) probe was added for

competitive binding with a final concentration of 1 μ M (50-fold). The reaction mixtures were incubated at 25°C for 20 min and then subjected to RNA polyacrylamide gel electrophoresis (PAGE) on a 12% native acrylamide gel at 4°C for 3 h. The gel was imaged with an Odyssey CLx Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) to detect the mobility shifts based on the signals from the fluorophore labels. Bands were shown with virtual colors. Red was for miRNAs, and green was for mRNA, while yellow indicated the duplex formation between miRNAs and their cognate mRNA targets.

2.5. Reporter construct cloning and dual-luciferase reporter assay

A HepG2 cDNA library was generated via total RNA extraction from HepG2 cells followed by reverse transcription. The SULT2A1 3'-UTR sequence was PCR-amplified from the cDNA library using primers containing XhoI and XbaI restriction sites at their 5'-ends flanking the 3'-UTR sequence. Sequences of the primers for SULT2A1 3'-UTR amplification and cloning are listed in Table 1. The nucleotide sequence of SULT2A1 3'-UTR is capitalized and restriction sites are in lowercase and underlined. Additional nucleotides in lowercase flanking the restriction sites were added to facilitate enzymatic digestion of the PCR amplicon. The SULT2A1 3'-UTR sequence was then cloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI) using XhoI and XbaI restriction enzymes (New England Biolabs, Ipswich, MA). Constructs were confirmed by double enzyme digestion and sequencing.

HepG2 cells were seeded in 96-well plates (3×10^4 cells/well) 24 h before transfection. Cells were co-transfected with pmirGLO-SULT2A1-3'-UTR or pmirGLO empty vector (EV) plasmid (100 ng/well) in combination with 10 nM of mimics for hsa-miR-486-5p, hsa-miR-495-3p, or miRNA mimic-NC (Dharmacon, Lafayette, CO) using Lipofectamine 2000 Transfection Reagent (ThermoFisher). Culture medium containing transfection reagent mixture was replaced 6 h after transfection with fresh complete medium. Cells were then cultured for another 42 h before being harvested for luciferase assays using a Dual-Luciferase® Reporter Assay System (Promega, Madison, WI). Three independent experiments were performed, each in triplicate.

2.6. Transfection of miRNA mimic and inhibitors for gain- or loss-of-function assays

HepG2 cells were seeded in 24-well plates (1.75×10^5 cells/well) 24 h before transfection. Lipofectamine 2000 Transfection Reagent (ThermoFisher) was used to transfect cells with miRNA mimics or inhibitors at the final concentrations indicated in the figures. Culture medium containing the transfection reagent mixtures was replaced with fresh complete medium 6 h after transfection. To assess the effect of miRNAs on the basal expression of SULT2A1, transfected cells were cultured for another 42 h before being harvested for RNA or protein extraction.

2.7. RNA extraction and RT-qPCR

RNeasy Mini kits and miRNeasy Mini kits (Qiagen, Valencia, CA) were used to extract total RNA from HepG2 cells for mRNA and miRNA quantification, respectively. QuantiTect Reverse Transcription kits (Qiagen) and TaqMan MicroRNA Reverse Transcription Kits (ThermoFisher) were used for reverse transcription for mRNA and miRNA quantification,

respectively. qPCR was performed using SsoAdvanced Universal SYBR Green Supermix (BioRad, Hercules, CA) and TaqMan Universal Master Mix II, no UNG (ThermoFisher) for mRNA and miRNA quantification. Primer sequences for SULT2A1, SULT2B1a, SULT2B1b, and GAPDH are listed in Table 1. These primers were synthesized by IDT. TaqMan assays (including miRNA-specific reverse transcription primers and probes for miRNA qPCR) for RNU6B (ThermoFisher, Cat# 4427975, Assay ID 001093), hsa-miR-486-5p (ThermoFisher, Cat# 4427975, Assay ID 001278), and hsa-miR-495-3p (ThermoFisher, Cat #4427975, Assay ID 001663) were used for miRNA detection and quantification. The assay for hsa-miR-495-3p (Thermo-Fisher, Cat #4427975, Assay ID 001663), with the commercial name of mmu-miR-495, is appropriate for detection of both hsa-miR-495-3p-3p in humans and mmu-miR-495-3p in mice because miR-495-3p is conserved between human and mice. The mRNA and miRNA levels were measured using the BioRad CFX96 Touch Real-Time PCR Detection System (BioRad). GAPDH was used as a housekeeping gene for normalization of SULT2A1, SULT2B1a, and SULT2B1b expression, while RNU6B was used for normalization of hsa-miR-486-5p and hsa-miR-495-3p expression. The 2^{-C_t} method was used to analyze the quantification data.

2.8. Protein extraction and Western blots

Whole cell lysates were extracted using RIPA Lysis and Extraction Buffer (ThermoFisher). Briefly, HepG2 cells after transfection or chemical treatment were rinsed once with ice-cold phosphate-buffered saline (PBS) and incubated in ice-cold RIPA buffer containing 1× protease/phosphatase inhibitor cocktail (Cell Signaling, Danvers, MA) for 10 min. Samples were then centrifuged at 14,000g for 15 min at 4°C to pellet the cell debris. Lysate supernatants were collected and subjected to SDS-PAGE and Western blotting. Antibodies against SULT2A1 and GAPDH (Abcam, Cambridge, MA) were used to detect SULT2A1 and GAPDH proteins. Odyssey CLx Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) and the Image Studio software were used for imaging and quantification of protein levels.

2.9. SULT2A1 mRNA stability

Twenty-four hours after transfection, HepG2 cells transfected with 10 nM of mimics for hsa-miR-486-5p, hsa-miR-495-3p, or mimic-NC were treated with 5 µg/mL of actinomycin D and harvested after 0, 8, 12, and 24 h. Cells were then subjected to mRNA extraction and RT-qPCR analysis.

2.10. SULT2A1 induction by rifampicin

HepG2 cells transfected with 10 nM of mimics for hsa-miR-486-5p, hsa-miR-495-3p, or miRNA mimic-NC were treated 24 h after transfection with 50 µM rifampicin or 0.05% DMSO, which is the empty vehicle solution for rifampicin, in serum free DMEM. Treated cells were harvested for mRNA extraction and RT-qPCR 24 h after adding DMSO or rifampicin.

2.11. Statistical analyses

Statistical analyses were performed using GraphPad Prism 5. Two-tailed student *t* tests were used to compare two groups of data. One-way analysis of variance with Bonferroni's Multiple Comparison Test was used to compare all pairs of data when there were more than two groups of data. Data are presented as the mean and standard deviation from three independent experiments. **p* < 0.05 was considered statistically significant.

3. Results

3.1. *In silico* identification of miRNA candidates that regulate SULT2A1 expression

We first analyzed the mRNA sequences of SULT2 isoforms to identify potential miRNA recognition elements in the 3'-UTRs of their transcripts. As shown in Fig. 1A, SULT2A1 3'-UTR is 988 bp in length, while the 3'-UTRs of SULT2B1a and SULT2B1b are only 49 bp and 17 bp long, respectively, suggesting that SULT2A1 mRNA is likely to be more susceptible to miRNA targeting. We then used two different miRNA prediction tools, miRanda at www.microrna.org and TargetScan (V7.1) at http://www.targetscan.org/vert_71/, to identify human miRNA candidates, based on seed match and conservation across species, that might modulate SULT2A1 expression. The miRanda algorithm predicted 284 miRNA candidates that putatively target the 3'-UTR of SULT2A1 mRNA, of which 12 were conserved miRNAs with good miRSVR scores (< -0.1), while the TargetScan algorithm predicted 309 miRNA candidates with various levels of conservation. The predictions generated by the two algorithms were compared and 10 out of the 12 conserved miRNA candidates predicted using miRanda were also identified by TargetScan (Fig. 1B). These 10 miRNA candidates selected for further analysis had seed matches of at least 6mer at different loci within the SULT2A1 3'-UTR (Fig. 1C).

To confirm that these miRNA candidates should specifically target SULT2A1, but not SULT2B1, which shares 48% amino acid sequence identity with SULT2A1, we examined the SULT2B1 3'-UTR for miRNA targeting. miRanda prediction generated no candidate miRNAs that target SULT2B1 3'-UTR and TargetScan found only one candidate, which did not overlap with the SULT2A1 prediction from either miRanda or TargetScan. These results indicate that the 10 miRNA candidates should specifically target SULT2A1 but not SULT2B1.

Next, we evaluated the correlation between the expression levels of SULT2A1 mRNA and that of each of the 10 candidates in 49 non-tumor human liver samples from the LIHC dataset of the TCGA database. Among the 10 candidates, the levels of hsa-miR-495-3p and hsa-miR-486-5p showed statistically significant inverse correlations with SULT2A1 mRNA levels, with *r* = -0.4267, *p* = 0.0022 and *r* = -0.3480, *p* = 0.0143, respectively (Fig. 2). The expression correlation between each of the other eight miRNAs and SULT2A1 mRNA had higher *r* and *p* values. These data suggest a potential role of hsa-miR-495-3p and hsa-miR-486-5p in negatively regulating SULT2A1 expression.

3.2. hsa-miR-495-3p and hsa-miR-486-5p directly bind to SULT2A1 3'-UTR for gene silencing

To evaluate whether hsa-miR-495-3p and hsa-miR-486-5p would form stable duplexes with their recognition elements in the SULT2A1 3'-UTR, we used RNAhybrid to calculate the free binding energy between either of the two miRNAs and SULT2A1 mRNA. The results showed that hsa-miR-495-3p could interact with SULT2A1 mRNA at two predicted binding sites, miR-495-MRE-1 and miR-495-MRE-2, with their ΔG s being -18.6 kcal/mol and -19.6 kcal/mol, respectively (Fig. 3A, top). Heterodimer analysis using the IDT OligoAnalyzer Tool showed that the energy required for hybridization between hsa-miR-495-3p and miR-495-MRE-1 (-13.9 kcal/mol) was much lower than that of miR-495-MRE-2 (-6.44 kcal/mol) (Fig. 3A, bottom). Additionally, the binding energy for the interaction between hsa-miR-486-5p and its predicted site (miR-486-MRE) within the SULT2A1 3'-UTR was -10.5 kcal/mol based on analysis using IDT OligoAnalyzer; however, RNAhybrid was unable to detect this interaction (Fig. 3A).

We then conducted FREMSA assays to verify the interactions between hsa-miR-495-3p or hsa-miR-486-5p and their MREs on SULT2A1 3'-UTR. In the top panel of Fig. 3B, the cy5.5-tagged hsa-miR-495-3p (lane 1) is shown in virtual red color, and the IRDye800-tagged miR-495-MRE-1 (lane 2) or miR-495-MRE-2 (lane 3) is shown in virtual green color. Incubation of the probes for hsa-miR-495-3p and miR-495-MRE-1 resulted in a single yellow band (lane 4, white arrow), indicating the merge of the red hsa-miR-495-3p and green miR-495-MRE-1, which resulted from the formation of a duplex between hsa-miR-495-3p and miR-495-MRE-1. In the presence of the cold-hsa-miR-495-3p probe at a 50-fold concentration, the intensity of the yellow band was reduced (lane 6) compared to that in lane 4, while the cold-NC probe at a 50-fold concentration had little effect on the duplex formation between hsa-miR-495-3p and miR-495-MRE-1 (represented by the yellow band, lane 5), suggesting specificity of the interaction between hsa-miR-495-3p and miR-495-MRE-1. However, a yellow band was not detected when the probes for hsa-miR-495-3p and miR-495-MRE-2 were incubated together in the absence (lane 7) or presence (lane 9) of cold-hsa-miR-495-3p, compared to bands when the probes for hsa-miR-495-3p (lane 1) and miR-495-MRE-2 (lane 3) were incubated alone. We observed two bands for the hsa-miR-495-3p probe in the gel (lane 1, hollow arrowheads). In the presence of miR-495-MRE-1, but not miR-495-MRE-2, the two red bands for hsa-miR-495-3p were diminished, suggesting that hsa-miR-495-3p existed in two conformations with different mobility in a native gel and that duplex formation with miR-495-MRE-1 on the SULT2A1 mRNA consumed hsa-miR-495-3p of both conformations and formed a single yellow band in lane 4. The reduction of the yellow band representing the duplex in lane 6 was concomitant with the re-appearance of the two faint red bands for hsa-miR-495-3p (one above and the other under the yellow band), indicating the availability of the "hot" hsa-miR-495-3p probe caused by the presence of 50-fold cold-hsa-miR-495-3p probe that competitively interacted with miR-495-MRE-1.

Although not detected by RNAhybrid, incubation of probes for hsa-miR-486-5p and miR-486-MRE led to a shifted band in yellow (Fig. 3B, bottom panel, lane 3, white arrow), indicating the formation of the duplex, while the intensity of each probe alone was decreased

in comparison to that in lane 1 and lane 2. In the presence of cold-miR-486-5p (lane 5), but not cold-NC (lane 4), the duplex formation represented by the yellow band was reduced. The duplex formed by the interaction between cold-hsa-miR-486-5p and “hot” miR-486-MRE caused a shift of the green band that migrated faster than the yellow band (the duplex formed by the interaction between “hot” hsa-miR-486-5p and “hot” miR-486-MRE), which was likely due to the lack of flhot” miR- on the cold-hsa-miR-486-5p probe.

We then performed luciferase gene reporter assays to analyze whether hsa-miR-495-3p or hsa-miR-486-5p targets the SULT2A1 3'-UTR to cause gene silencing. We generated a reporter construct by inserting the SULT2A1 3'-UTR at the 3'-end of the luciferase gene in the pmiRGLO dual-luciferase vector (pmiRGLO-SULT2A1-3'-UTR). HepG2 cells were transfected with pmiRGLO-SULT2A1-3'-UTR or pmiRGLO-EV along with mimics for hsa-miR-495-3p, hsa-miR-486-5p, or mimic-NC. Cells were harvested 48 h after transfection for dual-luciferase assays. Our results showed that the introduction of hsa-miR-495-3p or hsa-miR-486-5p mimics reduced luciferase activity by 39% ($p < 0.05$) and 49% ($p < 0.05$), respectively, in HepG2 cells transfected with pmiRGLO-SULT2A1-3'-UTR, compared to the mimic-NC (Fig. 3C). Transfection of hsa-miR-495-3p or hsa-miR-486-5p mimics had little effect on the luciferase activity in HepG2 cells transfected with pmiRGLO-EV. Our data suggest that hsa-miR-495-3p or hsa-miR-486-5p directly interacts with their cognate targets on the 3'-UTR of SULT2A1 mRNA.

3.3. The overexpression of hsa-miR-495-3p or hsa-miR-486-5p down-regulates SULT2A1 at the mRNA and protein levels

To evaluate whether hsa-miR-495-3p or hsa-miR-486-5p is able to regulate SULT2A1 expression, we performed gain-of-function assays using miRNA mimics. HepG2 cells were transfected with 5 nM and 10 nM of mimics for hsa-miR-495-3p or hsa-miR-486-5p or 10 nM of mimic-NC. The level of hsa-miR-495-3p or hsa-miR-486-5p was significantly increased in a concentration-dependent manner upon introduction of their corresponding exogenous miRNA mimics into HepG2 cells. (Fig. 4A and C). The mRNA levels of SULT2A1 were decreased by 9% and 25% ($p < 0.05$) in cells transfected with 5 nM and 10 nM of the hsa-miR-486-5p mimic compared to control cells, and by 18% ($p < 0.05$) and 23% ($p < 0.05$) in cells with the same concentrations of the hsa-miR-495-3p mimic (Fig. 4B and D). Over-expression of miRNA mimics had no significant effect on the mRNA expression of SULT2B1a or SULT2B1b (Fig. 4E–H), as predicted by miRanda and TargetScan, suggesting that inhibition by hsa-miR-495-3p or hsa-miR-486-5p is specific for SULT2A1. Overexpression of hsa-miR-495-3p or hsa-miR-486-5p also resulted in concentration-dependent decreases of SULT2A1 protein as detected by western blotting (Fig. 4I–L). Our results show that hsa-miR-495-3p or hsa-miR-486-5p suppresses SULT2A1 expression at both the mRNA and protein levels.

3.4. The inhibition of hsa-miR-495-3p or hsa-miR-486-5p upregulates SULT2A1 at the mRNA and protein levels

We conducted a loss-of-function experiment by reducing the levels of endogenous hsa-miR-495-3p or hsa-miR-486-5p in HepG2 cells using the corresponding sequence-specific hairpin inhibitors. HepG2 cells were transfected with 10 nM and 25 nM of inhibitors for

hsa-miR-495-3p, hsa-miR-486-5p, or 25 nM of inhibitor-NC. In response to inhibitor transfection at 10 nM and 25 nM, the levels of hsa-miR-495-3p were decreased by 40% ($p < 0.05$) and 80% ($p < 0.05$), while the levels of hsa-miR-486-5p were reduced by 64% ($p < 0.05$) and 80% ($p < 0.05$) (Fig. 5 A and C). The levels of SULT2A1 mRNA were significantly increased by 1.8-fold ($p < 0.05$) and 1.5-fold ($p < 0.05$) with 25 nM of hsa-miR-495-3p or hsa-miR-486-5p inhibitors, respectively (Fig. 5B and D). The inhibition of either miRNA also significantly upregulated the protein level of SULT2A1 (Fig. 5I–L). The upregulation of SULT2A1 expression at the mRNA and protein levels upon miRNA inhibition was concentration-dependent, which further indicates that hsa-miR-495-3p or hsa-miR-486-5p negatively regulated the expression of SULT2A1. miRNA inhibition had no significant effects on the expression levels of mRNA transcripts for the two SULT2B1 isoforms (Fig. 5E–H), suggesting hsa-miR-495-3p or hsa-miR-486-5p specifically targets SULT2A1.

3.5. hsa-miR-495-3p or hsa-miR-486-5p suppresses SULT2A1 expression through mRNA degradation

To assess whether hsa-miR-495-3p or hsa-miR-486-5p affects the stability of SULT2A1 mRNA, we performed an mRNA stability assay by treating mimics-transfected HepG2 cells with 5 μ g/mL actinomycin D to inhibit gene transcription and measuring the stability of SULT2A1 mRNA. Our data showed that 12 h after starting the actinomycin D treatment, SULT2A1 mRNA was less stable in cells transfected with the hsa-miR-495-3p mimic than in cells transfected with the mimic-NC and that SULT2A1 mRNA was less stable in cells with either hsa-miR-486-5p or hsa-miR-495-3p mimics compared to the mimic-NC 24 h after adding actinomycin D (Fig. 6A). This result suggests that hsa-miR-486-5p or hsa-miR-495-3p suppresses the expression of SULT2A1 by decreasing the stability of SULT2A1 mRNA.

3.6. hsa-miR-495-3p or hsa-miR-486-5p inhibits rifampicin-induced SULT2A1 expression

Although our results showed that hsa-miR-495-3p or hsa-miR-486-5p suppressed the basal expression of endogenous SULT2A1 in HepG2 cells, it was unclear whether either miRNA also inhibits drug-induced expression of SULT2A1. To address this question, we transfected HepG2 cells with 10 nM of mimics for hsa-miR-495-3p, hsa-miR-486-5p, or mimic-NC and then treated the cells with 0.05% DMSO or 50 μ M of rifampicin, a documented inducer of SULT2A1. In the cells treated with DMSO, a condition in which the basal expression of SULT2A1 is present, the level of SULT2A1 mRNA was decreased by the treatment of hsa-miR-486-5p mimics or hsa-miR-495-3p mimics (Fig. 6B), which is consistent with our results in Fig. 4. Rifampicin (dissolved in the vehicle DMSO) treatment induced the expression of SULT2A1 mRNA by 1.7-fold in cells transfected with mimic-NC. However, the inductive effect by rifampicin was significantly reduced by the overexpression of hsa-miR-486-5p or hsa-miR-495-3p, compared to that in rifampicin-treated and mimic-NC transfected cells. These results suggest that hsa-miR-495-3p or hsa-miR-486-5p inhibits not only the basal expression but also rifampicin induction of SULT2A1.

4. Discussion

The role of miRNAs in post-transcriptional regulation of gene expression has been widely explored in many physiological and pathological processes, including drug metabolism. DMEs, for example CYP2B6 [23], CYP 2C19 [24], SULT1A1[18], and UGT2B [16,25,26], have been shown to be targeted by miRNAs for gene silencing [27]. In the current study, we utilized a workflow that integrated various *in silico* and *in vitro* analyses to systematically identify miRNAs that may regulate SULT2A1 expression (Fig. 7). We found that the target-site prediction from two different prediction tools shared 10 miRNA candidates with > 6-mer seed match sequences on the SULT2A1 3'-UTR. hsa-miR-495-3p and hsa-miR-486-5p were selected for further investigation because they showed the highest and most significant negative correlations with SULT2A1 mRNA levels in human liver tissues. Using a series of biochemical, cellular, and molecular assays, we demonstrated that hsa-miR-495-3p and hsa-miR-486-5p suppress SULT2A1 mRNA and protein expression by directly binding to the SULT2A1 3'-UTR for mRNA degradation. We also presented evidence showing that hsa-miR-495-3p and hsa-miR-486-5p inhibit both the basal and rifampicin-induced expression of SULT2A1. In a previous study, we showed that SULT2A1 was indirectly regulated by miR-877-5p via nuclear receptor NR1I2 [19], unlike the mechanism underlying this study by which SULT2A1 is directly suppressed by hsa-miR-495-3p and hsa-miR-486-5p. To rule out the possibility that hsa-miR-495-3p or hsa-miR-486-5p may also indirectly regulate SULT2A1, we assessed the potential binding sites of hsa-miR-495-3p and hsa-miR-486-5p in nuclear receptors, which resulted in zero hits for all nuclear receptors by *in silico* analysis (data not shown).

Numerous miRNA prediction tools are available with different prediction algorithms. We selected miRanda and TargetScan because they are regularly maintained and updated, and they take into consideration the conservation of miRNAs and miRNA targeting sites across species [13]. It is common for a prediction algorithm to generate a large number of false positive candidates. Therefore, comparing the prediction results from two different tools increases confidence in the consensus prediction. Publicly available databases provide an excellent resource for computational analysis of expression profiles to examine the relationship between miRNA candidates and their cognate mRNA targets. We chose the LIHC dataset from the TCGA database, the world's largest and richest collection of genomic data, to investigate a liver-enriched DME, SULT2A1. Due to the heterogeneity of miRNA expression in tumor tissues, we used the non-tumor human liver tissues dataset to perform the correlation analysis between the expression levels of miRNAs and SULT2A1 mRNA.

RNAhybrid has been widely used to calculate the minimum free energy of hybridization between a miRNA and mRNA transcript [28]. In this work, the minimum free energy generated by RNAhybrid was measured based on every base-pair between the miRNA and MRE, regardless of continuous base-pairing at the seed region. Using RNAhybrid, hsa-miR-495-3p showed a higher calculated ΔG with miR-495-MRE-1 than with miR-495-MRE-2, results that conflict with our empirical observations of stable duplex formation using FREMSA. In contrast, IDT OligoAnalyzer analyzes the heterodimer between two sequences based on the longest stretch of continuous base-pairing. Additionally, sequence complementarity within the seed region has been shown to be critical for target recognition

and interaction by miRNAs [29]. This further supports our strategy of using more than one tool for computational analyses. Furthermore, the *in silico* calculations of the minimum free energy of hybridization between a miRNA and an mRNA transcript are primarily based on the base-pairing in a cell-free condition. In reality, stable miRNA/MRE hybridizations are more complicated in the presence of cellular components, such as proteins, that are involved in the formation of RNA-induced silencing complex and that may participate in or mediate miRNA-mRNA interactions [30]. Therefore, the magnitude of the minimum free energy of hybridization between a miRNA and an mRNA transcript is a suggestive indicator predicted by *in silico* calculation, whereas *in vivo* and *in vitro* experimental data provide stronger evidence that validate the interaction between miRNAs and their cognate mRNA targets.

Although miRNAs are only ~ 22 nt long, it has been reported that mature miRNAs display secondary structures [31]. The two bands detected in the FREMSA assay for hsa-miR-495-3p may imply that the hsa-miR-495-3p probe exists in two different conformations under our experimental conditions for RNA binding reactions. IDT OligoAnalyzer analyses also indicated the possibilities of hairpin and self-dimer formation with the hsa-miR-495-3p probe (data not shown). In the presence of miR-495-MRE-1, it is likely that both forms of hsa-miR-495-3p bind to the miR-495-MRE-1 probe and produce a single band. The introduction of 50-fold cold hsa-miR-495-3p probe largely reduced the amount of miR-495-MRE-1 that was available for binding to the “hot” probe, therefore, the “hot” hsa-miR-495-3p probe re-appeared in two forms in the gel. IDT OligoAnalyzer indicated that the ΔG for heterodimer formation between hsa-miR-495-3p and miR-495-MRE-1 was lower than that for homodimer formation of hsa-miR-495-3p probe (data not shown).

Utilizing miRNA mimics and inhibitors, we demonstrated that the concentration-dependent suppressive effects of hsa-miR-495-3p or hsa-miR-486-5p were specific for the expression of SULT2A1, but not its family members SULT2B1a and SULT2B1b. Although hsa-miR-486-5p has a higher expression level than hsa-miR-495-3p in human liver tissues (Fig. 2), we observed a stronger suppression efficiency of hsa-miR-495-3p than that of hsa-miR-486-5p. It is possible that the regulation of SULT2A1 expression by hsa-miR-486-5p is hampered or buffered by other regulators or mechanisms of SULT2A1 expression in the cells. It would be interesting to understand the crosstalk between miRNA regulation, transcriptional regulation by nuclear receptors, DNA methylation, and potentially long non-coding RNA regulation, which have been implicated in modulating DME expression [14]. Further studies to investigate the crosstalk between miRNAs and long non-coding RNAs for modulating SULT2A1 expression are ongoing in our laboratory.

We think that miRNA-modulated suppression of SULT2A1 may affect SULT2A1-dependent chemical sulfation and ultimately sensitivity of the liver to toxicants. The expression of SULT2A1 is closely associated with its sulfation capability and chemical metabolism. For example, both hepatic Sult2A1 expression and the level of dehydroepiandrosterone (DHEA) sulfation by Sult2A1 are reduced in mice treated with lipopolysaccharide during the acute-phase response [32]. Besides DHEA, bile acid is another major substrate of SULT2A1, and sulfation is an important pathway for bile acid elimination [33]. In patients with primary sclerosing cholangitis (PSC), a chronic liver disease associated with bile acid accumulation, SULT2A1 sulfation capacity has been found to be impaired [34]. This finding is in

concordance with the reduction of hepatic SULT2A1 expression in PSC patients in a disease-specific fashion [35]. Additionally, a systematic analysis using kinetic experiments has revealed SULT2A1 as the major human SULT responsible for sulfation of opioid drugs butorphanol and levorphanol. Some opioid drugs can pass through the blood-brain barrier, whereas sulfated metabolites of opioid drugs cannot, which may contribute to pharmacological inactivation of these drugs [36]. Most of the published studies, including the aforementioned, have focused on transcriptional regulation of SULT2A1 expression mediated by nuclear receptors [37–39, 32, 35]. Our findings identified hsa-miR-495-3p and hsa-miR-486-5p as post-transcriptional regulators of SULT2A1 expression; such miRNA-modulated SULT2A1 suppression may affect SULT2A1-dependent chemical detoxification.

It is widely known that exposure of xenobiotics, such as drugs, may lead to altered expression of miRNAs. We showed that the increased levels of hsa-miR-495-3p and hsa-miR-486-5p also inhibited rifampicin-induced expression of SULT2A1. Moreover, the down-regulated or up-regulated expression levels of miR-495-3p and miR-486-5p have been reported in many types of cancer tissue, such as acute myeloid leukemia [40], gastric cancer [41], hepatocellular carcinoma [42], breast cancer [43, 44], lung cancer [45, 46], and pancreatic cancer [47]. It has also been demonstrated that the circulating level of miR-486 (i.e. miR-486-5p) significantly decreases in response to acute and chronic exercise in young men [48]. Therefore, xenobiotic exposures or physical conditions that alter the levels of hsa-miR-495-3p and hsa-miR-486-5p may indirectly modulate the expression of SULT2A1 and influence SULT2A1-dependent metabolism of endogenous compounds and drugs such as acetaminophen. This could potentially affect drug efficacy and drug toxicity, suggesting important pharmacogenomic roles for miRNAs.

A few studies [49, 50] have investigated the functions of miRNAs on drug metabolism at the systemic level *in vivo*. Using a practical single-mouse pharmacokinetics (PK) model, Jilek et al. [49] analyzed miR-34a to understand its impact on the PK of several CYP probe drugs, including midazolam, dextromethorphan, phenacetin, diclofenac, and chlorzoxazone [49]. miR-34a was known to regulate nuclear receptors and several DMEs; however, intravenous administration into mice of human miR-34a as a cancer therapeutic had no or minor effect on the PK of the probe drugs, while *in vitro* experiments indicated that miR-34a had a slight effect on dextromethorphan clearance [49]. The lack of phenotypic effects *in vivo* may be attributed to differences in miRNA target genes due to poor conservation of miRNA target regions across species. Additionally, overexpression of ectopic miRNA mimics in *in vitro* assays may cause an “exaggerated” effect that is not achievable by the endogenous miRNAs. On the other hand, the compartment concentration of a miRNA may influence the overall suppressing effect on target genes, although the compartment concentration is very difficult to measure. Disparity between *in vitro* and *in vivo* studies of miRNAs in drug metabolism should be further investigated.

In summary, we identified hsa-miR-495-3p and hsa-miR-486-5p as two miRNAs that suppress the expression of SULT2A1 through facilitating mRNA degradation by directly binding to SULT2A1 3'-UTR. The combination of *in silico* and *in vitro* analyses and the use of different tools for the same computational analysis enabled a comprehensive evaluation, which could be used as a template for future studies.

Acknowledgements

This study was supported by the NCTR/FDA project E0753201 of the USA.

Abbreviations:

CYP	cytochrome p450
DME	drug metabolizing enzyme
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
FREMSA	fluorescence-based RNA electrophoretic mobility shift assay
miRNA	microRNA
MRE	microRNA recognition element
NC	negative control
PAGE	polyacrylamide gel electrophoresis
PAPS	3'-phosphoadenosine 5'-phosphosulfate
PBS	phosphate-buffered saline
SDS	sodium dodecyl sulfate
SULT	sulfotransferase
RT-qPCR	reverse transcription-quantitative polymerase chain reaction
UGT	UDP-glucuronosyltransferase
UTR	untranslated region

References

- [1]. Sheweita SA, Drug-metabolizing enzymes: mechanisms and functions, *Curr. Drug Metab* 1 (2) (2000) 107–132. [PubMed: 11465078]
- [2]. Coughtrie MWH, Function and organization of the human cytosolic sulfotransferase (SULT) family, *Chem. Biol. Interact* 259 (Pt A) (2016) 2–7. [PubMed: 27174136]
- [3]. Gamage N, Barnett A, Hempel N, Duggleby RG, Windmill KF, Martin JL, McManus ME, Human sulfotransferases and their role in chemical metabolism, *Toxicol. Sci* 90 (1) (2006) 5–22. [PubMed: 16322073]
- [4]. Lindsay J, Wang LL, Li Y, Zhou SF, Structure, function and polymorphism of human cytosolic sulfotransferases, *Curr. Drug Metab* 9 (2) (2008) 99–105. [PubMed: 18288952]
- [5]. Falany CN, He D, Dumas N, Frost AR, Falany JL, Human cytosolic sulfotransferase 2B1: isoform expression, tissue specificity and subcellular localization, *J. Steroid Biochem. Mol. Biol* 102 (1–5) (2006) 214–221. [PubMed: 17055258]
- [6]. Riches Z, Stanley EL, Bloomer JC, Coughtrie MW, Quantitative evaluation of the expression and activity of five major sulfotransferases (SULTs) in human tissues: the SULT “pie”, *Drug Metab. Dispos* 37 (11) (2009) 2255–2261. [PubMed: 19679676]

- [7]. Shibutani S, Shaw PM, Suzuki N, Dasaradhi L, Duffel MW, Terashima I, Sulfation of alpha-hydroxytamoxifen catalyzed by human hydroxysteroid sulfotransferase results in tamoxifen-DNA adducts, *Carcinogenesis* 19 (11) (1998) 2007–2011. [PubMed: 9855017]
- [8]. Meloche CA, Sharma V, Swedmark S, Andersson P, Falany CN, Sulfation of budesonide by human cytosolic sulfotransferase, dehydroepiandrosterone-sulfotransferase (DHEA-ST), *Drug Metab. Dispos* 30 (5) (2002) 582–585. [PubMed: 11950791]
- [9]. Pai TG, Sugahara T, Suiko M, Sakakibara Y, Xu F, Liu MC, Differential xenoestrogen-sulfating activities of the human cytosolic sulfotransferases: molecular cloning, expression, and purification of human SULT2B1a and SULT2B1b sulfotransferases, *BBA* 1573 (2) (2002) 165–170. [PubMed: 12399026]
- [10]. Falany CN, Comer KA, Dooley TP, Glatt H, Human dehydroepiandrosterone sulfotransferase. Purification, molecular cloning, and characterization, *Ann. N. Y. Acad. Sci* 774 (1995) 59–72. [PubMed: 8597484]
- [11]. Ekstrom L, Rane A, Genetic variation, expression and ontogeny of sulfotransferase SULT2A1 in humans, *Pharmacogenomics J* 15 (4) (2015) 293–297. [PubMed: 25802089]
- [12]. Nahar MS, Kim JH, Sartor MA, Dolinoy DC, Bisphenol A-associated alterations in the expression and epigenetic regulation of genes encoding xenobiotic metabolizing enzymes in human fetal liver, *Environ. Mol. Mutagen* 55 (3) (2014) 184–195. [PubMed: 24214726]
- [13]. Li D, Tolleson WH, Yu D, Chen S, Guo L, Xiao W, Tong W, Ning B, MicroRNA-Dependent Gene Regulation of the Human Cytochrome P450, *Pharmacoeugenetics*, Elsevier, 2019, pp. 129–138.
- [14]. Li D, Tolleson WH, Yu D, Chen S, Guo L, Xiao W, Tong W, Ning B, Regulation of cytochrome P450 expression by microRNAs and long non coding RNAs: Epigenetic mechanisms in environmental toxicology and carcinogenesis, *J. Environ. Sci. Health C Environ. Carcinog. Ecotoxicol. Rev* (2019) 1–35.
- [15]. Sutliff AK, Watson CJW, Chen G, Lazarus P, Regulation of UGT2A1 by miR-196a-5p and miR-196b-5p, *J. Pharmacol. Exp. Ther* 369 (2) (2019) 234–243. [PubMed: 30850392]
- [16]. Papageorgiou I, Court MH, Identification and validation of the microRNA response elements in the 3'-untranslated region of the UDP glucuronosyltransferase (UGT) 2B7 and 2B15 genes by a functional genomics approach, *Biochem. Pharmacol* 146 (2017) 199–213. [PubMed: 28962835]
- [17]. Wijayakumara DD, Hu DG, Meech R, McKinnon RA, Mackenzie PI, Regulation of Human UGT2B15 and UGT2B17 by miR-376c in Prostate Cancer Cell Lines, *J. Pharmacol. Exp. Ther* 354 (3) (2015) 417–425. [PubMed: 26163549]
- [18]. Yu X, Dhakal IB, Beggs M, Edavana VK, Williams S, Zhang X, Mercer K, Ning B, Lang NP, Kadlubar FF, Kadlubar S, Functional genetic variants in the 3'-untranslated region of sulfotransferase isoform 1A1 (SULT1A1) and their effect on enzymatic activity, *Toxicol. Sci* 118 (2) (2010) 391–403. [PubMed: 20881232]
- [19]. Yu D, Wu L, Gill P, Tolleson WH, Chen S, Sun J, Knox B, Jin Y, Xiao W, Hong H, Wang Y, Ren Z, Guo L, Mei N, Guo Y, Yang X, Shi L, Chen Y, Zeng L, Dreval K, Tryndyak V, Pogribny I, Fang H, Shi T, McCullough S, Bhattacharyya S, Schnackenberg L, Mattes W, Beger RD, James L, Tong W, Ning B, Multiple microRNAs function as self-protective modules in acetaminophen-induced hepatotoxicity in humans, *Arch. Toxicol* 92 (2) (2018) 845–858. [PubMed: 29067470]
- [20]. Betel D, Wilson M, Gabow A, Marks DS, Sander C, The microRNA.org resource: targets and expression, *Nucleic Acids Res* 36 (Database issue) (2008) D149–53. [PubMed: 18158296]
- [21]. Lewis BP, Burge CB, Bartel DP, Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets, *Cell* 120 (1) (2005) 15–20. [PubMed: 15652477]
- [22]. Zeng L, Chen Y, Wang Y, Yu LR, Knox B, Chen J, Shi T, Chen S, Ren Z, Guo L, Wu Y, Liu D, Huang K, Tong W, Yu D, Ning B, MicroRNA hsa-miR-370-3p suppresses the expression and induction of CYP2D6 by facilitating mRNA degradation, *Biochem. Pharmacol* 140 (2017) 139–149. [PubMed: 28552654]
- [23]. Jin Y, Yu D, Tolleson WH, Knox B, Wang Y, Chen S, Ren Z, Deng H, Guo Y, Ning B, MicroRNA hsa-miR-25-3p suppresses the expression and drug induction of CYP2B6 in human hepatocytes, *Biochem. Pharmacol* 113 (2016) 88–96. [PubMed: 27311985]

- [24]. Yu D, Green B, Tolleson WH, Jin Y, Mei N, Guo Y, Deng H, Pogribny I, Ning B, MicroRNA hsa-miR-29a-3p modulates CYP2C19 in human liver cells, *Biochem. Pharmacol* 98 (1) (2015) 215–223. [PubMed: 26296572]
- [25]. Wijayakumara DD, Mackenzie PI, McKinnon RA, Hu DG, Meech R, Regulation of UDP-glucuronosyltransferase 2B15 by miR-331-5p in prostate cancer cells involves canonical and noncanonical target sites, *J. Pharmacol. Exp. Ther* 365 (1) (2018) 48–59. [PubMed: 29367276]
- [26]. Dluzen DF, Sutliff AK, Chen G, Watson CJ, Ishmael FT, Lazarus P, Regulation of UGT2B expression and activity by miR-216b-5p in liver cancer cell lines, *J. Pharmacol. Exp. Ther* 359 (1) (2016) 182–193. [PubMed: 27474751]
- [27]. Nakano M, Nakajima M, Current knowledge of microRNA-mediated regulation of drug metabolism in humans, *Expert Opin. Drug Metab. Toxicol* (2018).
- [28]. Kruger J, Rehmsmeier M, RNAhybrid: microRNA target prediction easy, fast and flexible, *Nucl. Acids Res* 34 (Web Server issue) (2006) W451–4. [PubMed: 16845047]
- [29]. Bartel DP, MicroRNAs: target recognition and regulatory functions, *Cell* 136 (2) (2009) 215–233. [PubMed: 19167326]
- [30]. Macfarlane LA, Murphy PR, MicroRNA: biogenesis, function and role in cancer, *Curr. Genomics* 11 (7) (2010) 537–561. [PubMed: 21532838]
- [31]. Belter A, Gudanis D, Rolle K, Piwecka M, Gdaniec Z, Naskret-Barciszewska MZ, Barciszewski J, Mature miRNAs form secondary structure, which suggests their function beyond RISC, *PLoS One* 9 (11) (2014) e113848. [PubMed: 25423301]
- [32]. Kim MS, Shigenaga J, Moser A, Grunfeld C, Feingold KR, Suppression of DHEA sulfotransferase (Sult2A1) during the acute-phase response, *Am. J. Physiol. Endocrinol. Metab* 287 (4) (2004) E731–E738. [PubMed: 15198932]
- [33]. Alnouti Y, Bile Acid sulfation: a pathway of bile acid elimination and detoxification, *Toxicol. Sci* 108 (2) (2009) 225–246. [PubMed: 19131563]
- [34]. Trottier J, Bialek A, Caron P, Straka RJ, Heathcote J, Milkiewicz P, Barbier O, Metabolomic profiling of 17 bile acids in serum from patients with primary biliary cirrhosis and primary sclerosing cholangitis: a pilot study, *Dig. Liver Dis* 44 (4) (2012) 303–310. [PubMed: 22169272]
- [35]. Wunsch E, Klak M, Wasik U, Milkiewicz M, Blatkiewicz M, Urasinska E, Barbier O, Bielicki D, Bogdanos DP, Elias E, Milkiewicz P, Liver Expression of sulphotransferase 2A1 enzyme is impaired in patients with primary sclerosing cholangitis: lack of the response to enhanced expression of PXR, *J. Immunol. Res* 2015 (2015) 571353. [PubMed: 26504856]
- [36]. Kurogi K, Chepak A, Hanrahan MT, Liu MY, Sakakibara Y, Suiko M, Liu MC, Sulfation of opioid drugs by human cytosolic sulfotransferases: metabolic labeling study and enzymatic analysis, *Eur. J. Pharm. Sci* 62 (2014) 40–48.
- [37]. Fang HL, Strom SC, Ellis E, Duanmu Z, Fu J, Duniec-Dmuchowski Z, Falany CN, Falany JL, Kocarek TA, Runge-Morris M, Positive and negative regulation of human hepatic hydroxysteroid sulfotransferase (SULT2A1) gene transcription by rifampicin: roles of hepatocyte nuclear factor 4alpha and pregnane X receptor, *J. Pharmacol. Exp. Ther* 323 (2) (2007) 586–598. [PubMed: 17687072]
- [38]. Sonoda J, Xie W, Rosenfeld JM, Barwick JL, Guzelian PS, Evans RM, Regulation of a xenobiotic sulfonation cascade by nuclear pregnane X receptor (PXR), *Proc. Natl. Acad. Sci. U.S.A* 99 (21) (2002) 13801–13806. [PubMed: 12370413]
- [39]. Echchgadda I, Song CS, Roy AK, Chatterjee B, Dehydroepiandrosterone sulfotransferase is a target for transcriptional induction by the vitamin D receptor, *Mol. Pharmacol* 65 (3) (2004) 720–729. [PubMed: 14978251]
- [40]. Jiang X, Huang H, Li Z, He C, Li Y, Chen P, Gurbuxani S, Arnovitz S, Hong GM, Price C, Ren H, Kunjamma RB, Neilly MB, Salat J, Wunderlich M, Slany RK, Zhang Y, Larson RA, Le Beau MM, Mulloy JC, Rowley JD, Chen J, MiR-495 is a tumor-suppressor microRNA down-regulated in MLL-rearranged leukemia, *Proc. Natl. Acad. Sci. U.S.A* 109 (47) (2012) 19397–19402. [PubMed: 23132946]
- [41]. Li Z, Cao Y, Jie Z, Liu Y, Li Y, Li J, Zhu G, Liu Z, Tu Y, Peng G, Lee DW, Park SS, miR-495 and miR-551a inhibit the migration and invasion of human gastric cancer cells by directly interacting with PRL-3, *Cancer Lett* 323 (1) (2012) 41–47. [PubMed: 22469786]

- [42]. Huang XP, Hou J, Shen XY, Huang CY, Zhang XH, Xie YA, Luo XL, MicroRNA-486-5p, which is downregulated in hepatocellular carcinoma, suppresses tumor growth by targeting PIK3R1, FEBS J 282 (3) (2015) 579–594. [PubMed: 25475121]
- [43]. Rask L, Balslev E, Sokilde R, Hogdall E, Flyger H, Eriksen J, Litman T, Differential expression of miR-139, miR-486 and miR-21 in breast cancer patients sub-classified according to lymph node status, Cell Oncol. (Dordr) 37 (3) (2014) 215–227. [PubMed: 25027758]
- [44]. Hwang-Verslues WW, Chang PH, Wei PC, Yang CY, Huang CK, Kuo WH, Shew JY, Chang KJ, Lee EY, Lee WH, miR-495 is upregulated by E12/E47 in breast cancer stem cells, and promotes oncogenesis and hypoxia resistance via downregulation of E-cadherin and REDD1, Oncogene 30 (21) (2011) 2463–2474. [PubMed: 21258409]
- [45]. Chu H, Chen X, Wang H, Du Y, Wang Y, Zang W, Li P, Li J, Chang J, Zhao G, Zhang G, MiR-495 regulates proliferation and migration in NSCLC by targeting MTA3, Tumour Biol 35 (4) (2014) 3487–3494. [PubMed: 24293376]
- [46]. Peng Y, Dai Y, Hitchcock C, Yang X, Kassir ES, Liu L, Luo Z, Sun HL, Cui R, Wei H, Kim T, Lee TJ, Jeon YJ, Nuovo GJ, Volinia S, He Q, Yu J, Nana-Sinkam P, Croce CM, Insulin growth factor signaling is regulated by microRNA-486, an underexpressed microRNA in lung cancer, Proc. Natl. Acad. Sci. U.S.A 110 (37) (2013) 15043–15048. [PubMed: 23980150]
- [47]. Ali S, Saleh H, Sethi S, Sarkar FH, Philip PA, MicroRNA profiling of diagnostic needle aspirates from patients with pancreatic cancer, Br. J. Cancer 107 (8) (2012) 1354–1360. [PubMed: 22929886]
- [48]. Aoi W, Ichikawa H, Mune K, Tanimura Y, Mizushima K, Naito Y, T.J.F.i.p. Yoshikawa, Muscle-enriched microRNA miR-486 decreases in circulation in response to exercise in young men, Front. Physiol 4 (2013) 80. [PubMed: 23596423]
- [49]. Jilek JL, Tian Y, Yu AM, Effects of microRNA-34a on the pharmacokinetics of cytochrome P450 probe drugs in mice, Drug Metab. Dispos 45 (5) (2017) 512–522. [PubMed: 28254952]

Fig. 1.

In silico identification of human miRNA candidates that target the SULT2A1 3'-UTR. A. Schematics of mRNA sequences of SULT2 family members. SULT2A1 mRNA (NM_003167) contains a 988-bp long 3'-UTR, while the transcripts of the *SULT2B1* gene, SULT2B1a (NM_004605) and SULT2B1b (NM_177973), have short 3'-UTRs that are 49 bp and 17 bp long, respectively. Transcript schematics were drawn proportional to lengths of the mRNA sequences. B. Venn diagram comparing the miRanda and TargetScan predictions of human miRNAs that target the SULT2A1 3'-UTR. miRanda identified 12 miRNAs that were conserved with a good miRSVR score (< -0.1), while 309 miRNAs of various conservation levels were predicted by TargetScan. 10 miRNAs were among both prediction results. C. Seed match positions of the 10 miRNAs predicted by both miRanda and TargetScan within the SULT2A1 3'-UTR. miR-383-5p, miR-411-5p, and miR-129-5p were predicted to have two MREs in the SULT2A1 3'-UTR, whereas miR-324-5p, miR-494-3p, miR-196a, miR-196b, miR-495-3p, miR-590-3p, and miR-486-5p were shown to have one MRE in the SULT2A1 3'-UTR. Seed match sequences for all 10 miRNAs but miR-590-3p were in shade; seed match sequence of miR-590-3p was underlined to be distinguished from that of miR-486-5p.

Fig. 2.

Correlation analysis of the expression levels of SULT2A1 mRNA and the 10 miRNA candidates in non-tumor human liver samples. Scatter plots showing the correlation between the level of each of the 10 miRNA candidates and that of SULT2A1 mRNA were shown in an order with the r value from the lowest to the highest, and concomitantly with the p value from the lowest to the highest. All 10 miRNAs have a negative r value in correlation with SULT2A1 mRNA on the expression level; however, only hsa-miR-495-3p ($r = -0.4267$, $p = 0.0022$) and hsa-miR-486-5p ($r = -0.3480$, $p = 0.0143$) were in significantly inverse correlation with SULT2A1 mRNA on the expression level, and they have the highest inverse correlation reflected by the lowest r values.

Fig. 3.

hsa-miR-495-3p and hsa-miR-486-5p directly bind to the SULT2A1 3'-UTR for gene silencing. A. IDT OligoAnalyzer and RNAhybrid calculation of minimum free energy for RNA hybridization. IDT OligoAnalyzer and RNAhybrid showed variation in the minimum free energy for miRNA and MRE binding. Solid lines indicate the base pairs considered for energy calculation, while semi-colons indicate the base-pairing that was excluded for calculation. B. FREMSA. Cy5.5-tagged miRNA probes (red) and IRdye800-tagged SULT2A1 mRNA sequences (green) containing the predicted MREs were incubated alone or together, in the absence or presence of cold miRNA probes or cold-NC. RNA binding reaction mixtures were then subjected to a native PAGE gel. The incubation of hsa-miR-495-3p and miR-495-MRE-1 resulted in a yellow band (white arrow, lane 4), indicating the merge of the red hsa-miR-495-3p and green miR-495-MRE-1 due to the duplex formation. The yellow band was reduced in intensity in the presence of the 50-fold cold hsa-miR-495-3p probe (lane 6) but not cold-NC (lane 5). Hollow arrowheads pointed to the two different conformations of the hsa-miR-495-3p probe (lane 1). No yellow bands were observed when hsa-miR-495-3p was incubated with miR-495-MRE-2 (lanes 7–9), suggesting hsa-miR-495-3p specifically binds to miR-495-MRE-1 on SULT2A1 mRNA. hsa-miR-486-5p directly interacted with its predicted MRE on the SULT2A1 3'-UTR,

which led to a yellow band (lane 3, white arrow) that was reduced in intensity by the addition of cold hsa-miR-486-5p probe (lane 5) instead of the cold-NC (lane 4). C. hsa-miR-495-3p and hsa-miR-486-5p overexpression significantly decreased luciferase activity in HepG2 cells via SULT2A1 3'-UTR. HepG2 cells were transfected with pmiRGLO-SULT2A1-3'-UTR or the empty vector along with mimics for hsa-miR-495-3p, hsa-miR-486-5p, and mimic-NC, respectively. Three independent experiments were conducted, each in triplicate. Data are shown as mean with standard deviation. * $p < 0.05$ compared to mimic-NC. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. hsa-miR-495-3p and hsa-miR-486-5p overexpression down-regulated SULT2A1 mRNA and protein expression in a concentration-dependent manner. HepG2 cells were transfected with mimics for hsa-miR-495-3p, hsa-miR-486-5p, and mimic-NC, respectively. The levels of hsa-miR-486-5p (A), hsa-miR-495-3p (C), SULT2A1 mRNA (B and D), SULT2B1a mRNA (E and G), and SULT2B1b mRNA (F and H) were quantified using RT-qPCR. SULT2A1 protein levels were detected and measured via western blot (I-L). Three independent experiments were conducted. Data are shown as mean with standard deviation. * $p < 0.05$ compared with mimic-NC or 5 nM hsa-miR-495-3p and hsa-miR-486-5p mimic treatment.

Fig. 5.

hsa-miR-495-3p and hsa-miR-486-5p inhibition upregulated SULT2A1 mRNA and protein expression in a concentration-dependent manner. HepG2 cells were transfected with inhibitors for hsa-miR-495-3p, hsa-miR-486-5p, and inhibitor-NC, respectively. The levels of hsa-miR-486-5p (A), hsa-miR-495-3p (C), SULT2A1 mRNA (B and D), SULT2B1a mRNA (E and G), and SULT2B1b mRNA (F and H) were quantified using RT-qPCR.

SULT2A1 protein levels were detected and measured via western blot (I-L). Three independent experiments were conducted. Data are shown as mean with standard deviation.

* $p < 0.05$ compared with NC or 10 nM of inhibitors for hsa-miR-495-3p and hsa-miR-486-5p treatment.

Fig. 6.

hsa-miR-495-3p and hsa-miR-486-5p decreased the stability of SULT2A1 mRNA and inhibited rifampicin-induced SULT2A1 expression. A. The stability of SULT2A1 mRNA was reduced upon overexpression of hsa-miR-495-3p and hsa-miR-486-5p in HepG2 cells. HepG2 cells were transfected with mimics for hsa-miR-495-3p, hsa-miR-486-5p, or mimic-NC, 24 h before the onset of actinomycin D treatment (5 μ g/mL) for 0, 8, 12, and 24 h. SULT2A1 mRNA was less stable in cells transfected with mimics for hsa-miR-486-5p or hsa-miR-495-3p. * $p < 0.05$ compared to NC. B. Overexpression of hsa-miR-495-3p and hsa-miR-486-5p inhibited induction of SULT2A1 by rifampicin. HepG2 cells were transfected with mimics for hsa-miR-495-3p, hsa-miR-486-5p, or mimic-NC, 24 h before the start of rifampicin treatment at 50 μ M. 24 h of rifampicin treatment induced an increase of SULT2A1 mRNA, which could be significantly inhibited by overexpression of hsa-miR-495-3p and hsa-miR-486-5p. Three independent experiments were conducted. Data are shown as mean with standard deviation. * $p < 0.05$ compared to NC treated with DMSO or rifampicin.

Fig. 7.

Schematics of the integrated strategy for miRNA identification. Various tools and resources were used to compare different computational analyses to ensure a high confidence during miRNA prediction. Wet-lab experimental validation was carried out based on the *in silico* analyses results, which showed that hsa-miR-495-3p and hsa-miR-486-5p target SULT2A1 for gene repression via mRNA degradation.

Table 1

Sequences of primers and probes.

Oligonucleotide	Sequence	Application
SULT2A1-3UTR-F	ccgctcgagcggCGTCCAAAACACTCTGGATCT	Reporter construct cloning
SULT2A1-3UTR-R	gctctagagcCTCCATAAACACCGTTTATTGTATATGG	Reporter construct cloning
GAPDH-F	GAAATCCCATCACCATCTTCCAGG	RT-qPCR
GAPDH-R	GAGCCCCAGCCTTCTCCATG	RT-qPCR
SULT2A1-F	TGAGTTCGTGATAAGGGATGAA	RT-qPCR
SULT2A1-R	CAGATGGGCACAGATTGGAT	RT-qPCR
SULT2B1a-RT-F	CACCTCCCTACTCTCCCTCAT	RT-qPCR
SULT2B1a-RT-R	GTCGTGCCTGACTTGGGGTAG	RT-qPCR
SULT2B1b-RT-F	GGCTTGTGGGACACCTATGA	RT-qPCR
SULT2B1b-RT-R	TGTTCTCCGCCAAGCTGATG	RT-qPCR
miR-495-3p	AAACAAACAUGGUGCACUUCUU	EMSA
miR-495-MRE-1	AGGAUGUCUAAAUGUUUGUUA	EMSA
miR-495-MRE-2	CAGGCACGGUGGUUCAUGCCUGUA	EMSA
miR-486-5p	UCCUGUACUGAGCUGCCCCGAG	EMSA
miR-486-MRE	AAUAAGAAAUAAAUUACAGGA	EMSA
Cold-NC	UCACAACCUCCUAGAAAGAGUAGA	EMSA