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Modulation of ALDH5A1 and SLC22A7 by microRNA hsa-miR-29a-3p in human liver cells

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ABSTRACT

Observed variations in drug responses among patients may result from differences in heritable genetic traits or from alterations in the epigenetic regulation of drug metabolizing enzymes and transporters (DMETs). MicroRNAs (miRNAs), a group of small non-coding RNAs, provide an epigenetic mechanism for fine-tuning the expression of targeted DMET genes by regulating the efficiency of protein translation and by decreasing mRNA stability *via* enhanced degradation. In the current study we systematically screened 374 important genes encoding DMETs for potential response elements to hsa-miR-29a-3p, a highly abundant miRNA in human liver. RNA electrophoresis mobility shift assays displayed direct interactions between hsa-miR-29a-3p and its cognate targets within the mRNA transcripts for the *ABCC6*, *SLC22A7* and *ALDH5A1* genes. The expression of luciferase reporter genes containing the 3′-UTRs of *SLC22A7* or *ALDH5A1* and the expression of endogenous *SLC22A7* and *ALDH5A1* were each suppressed by transfection with hsa-miR-29a-3p mimics. Importantly, chemically-induced up-regulation of hsa-miR-29a-3p correlated inversely with the expression of *SLC22A7* and *ALDH5A1*. However, our studies failed to detect suppressive effects of hsa-miR-29a-3p on *ABCC6* expression, which might be explained by the notion that the interaction of hsa-miR-29a-3p and *ABCC6* mRNA was unable to recruit ribonucleoproteins to form a RNA-induced silencing complex.

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1. Introduction

Individual differences in drug efficacy and safety are a serious concern for public health. In the United States, over 770,000 serious adverse drug reactions (ADRs), including more than 111,000 deaths, were reported during 2013 in the FDA Adverse Events Reporting System (http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Surveillance/AdverseDrugEffects/ucm070461.htm). Results from numerous studies published during last half century show that variable drug responses in the human population are associated with functional genetic variants (*i.e.*, single nucleotide polymorphisms, SNPs) that affect the expression of drug metabolizing enzymes and transporters (DMETs), key proteins for the uptake, metabolism, and efflux of drugs [1,2]. In addition, individual variations in epigenetic regulatory machinery also contribute to variable DMET expression *via* altered DNA methylation, histone modifications, and other post-transcriptional effects [3,4]. Furthermore, endogenous factors [5,6], environmental factors, and drug-drug interactions are also involved in ADRs [7,8].

It is well known that inter-individual variability in DMET expression has a major impact on drug efficacy and side effects [1,9–11]; and the functional genomics of DMETs has been applied in rational therapeutics [12]. However, the prediction of drug response based on pharmacogenomics is not yet available or is far from reliable for most clinical drugs, indicating the complexity of individual differences in drug efficacy and safety.

Epigenetic regulation of DMET gene expression provides an additional genotype-independent molecular mechanism that may contribute to individual differences in drug response [13]. MicroRNAs (miRNAs), a group of small non-coding RNAs with a

Abbreviations: miRNA, microRNA; DMETs, drug metabolizing enzymes and transporters; 3′-UTR, 3′-untranslated region; miRNP, microRNA ribonucleoprotein; GABA, neurotransmitter 4-aminobutyric acid; GHB, gamma-hydroxybutyric acid; Ago, argonaute RISC catalytic component; RISC, RNA-induced silence complex; FBS, fetal bovine serum; EMSA, electrophoretic mobility shift assay.

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length of approximately 22 nucleotides, are a newly recognized type of epigenetic post-transcriptional regulator. Typically, miRNAs suppress the expression of targeted genes either by inhibiting protein translation or by stimulating mRNA degradation *via* sequence–specific interactions with response elements present in the 3′-UTRs or coding regions of the targeted mRNA transcripts [4,14]. Mechanistic studies have demonstrated the involvement of miRNAs in regulating the expression of a number of DMET genes, such as *CYP1B1* [15], *CYP2E1* [16], *CYP2C9* [17], *CYP3A4* [18], and *SULT1A1* [19]. However, additional studies are needed to elucidate specific mechanisms underlying the regulation of DMET gene expression by miRNAs to provide a more complete pharmacogenomic perspective on individual drug responses.

The miR-29 family, containing miR-29a, miR-29b and miR-29c members, shares a common seed region sequence that is predicted to target a large number of genes associated with diverse biological functions, including the regulation of cell proliferation, secretion and maintenance of extracellular matrix, cell differentiation and apoptosis [20], adaptive immune system functions [21], and bone remodeling [22]. miR-29 has also been reported to play critical roles in the pathogenesis of a variety of human diseases, including cancer [23], liver fibrosis [24], heart injury [20], and pulmonary fibrosis [25]. Liver fibrosis, a common feature of liver dysfunction caused by liver diseases (*e.g.*, hepatitis C viral infection) or drug-induced liver injury (DILI), is usually associated with down-regulated DMET gene expression, resulting in decreased hepatic capacity for drug metabolism and disposition [26]. Therefore, miR-29 could serve as a therapeutic target for these diseases [27]. For example, recent evidence proved that the miR-29 family is activated by celecoxib [28] and by other chemotherapeutics that are involved in the suppression of metastasis in gastric cancer [29].

We recently showed that hsa-miR-29a-3p suppresses *CYP2C19* expression in liver cells by targeting its coding region [30], suggesting that the miR-29 family might also modulate the expression of additional DMET genes. In this study, putative hsa-miR-29a-3p response elements in 374 commonly expressed DMET genes [31], were screened systematically using *in silico* analyses; and then a series of biochemical assays were applied to characterize the interactions between hsa-miR-29a-3p and the cognate sequences detected within DMET genes.

2. Materials and methods

2.1. Culture of cell lines

HepaRG cells, which express many key DMETs highly at levels similar to those found in primary hepatocytes, were selected to detect the regulatory roles of hsa-miR-29a-3p on endogenous DMETs production, while HepG2 and 293T cells were used to test the roles of hsa-miR-29a-3p on exogenous reporter genes expression. All media and supplements for cell culture were obtained from Life Technologies (Carlsbad, CA).

HepaRG cells (terminally differentiated hepatic cells) were obtained from Life Technologies. Terminally differentiated HepaRG cells were cultured in Williams' E medium supplemented with thaw, plate, & general purpose medium supplement for one day, and then maintained in Williams' E medium supplemented with the maintenance/metabolism medium supplement for additional seven days to prepare them for further experiments.

HepG2 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA), and grown in Rosewell Park Memorial Institute 1640 (RPMI 1640) medium supplemented with 10% fetal bovine serum (FBS). The 293T cells were obtained from Biosettia (San Diego, CA) and maintained in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% FBS, 1 mM sodium pyruvate, and non-essential amino acids.

2.2. In silico *analyses*

A total of 374 DMET genes [31] were screened using the CellMiner™ database (version 1.5.1, http://discover.nci.nih.gov/cellminer) to select candidate genes whose expression levels correlated negatively ($r < -0.250$) with hsa-miR-29a-3p levels among the panel of NCI-60 cell lines [32]. Potential hsa-miR-29a-3p response elements were detected in the candidate DMET genes using the databases miRTar.human (http://mirtar.mbc.nctu.edu.tw/human/) and microRNA.org (http://www.microrna.org/). RNAhybrid, a RNA hybridization algorithm (http://bibiserv2.cebitec.uni-bielefeld.de/rnahybrid), was used to predict the free energy of potential miRNA:mRNA duplexes. The GSE22058 public dataset was used to extract the RNA expression data for selected DMET genes and the expression level for hsa-miR-29a-3p in a set of 96 non-tumor liver tissues.

2.3. RNA electrophoretic mobility shift assays

All synthetic oligonucleotides or primers, conventional or covalently modified used in this study were obtained from Integrated DNA Technologies (Coraville, IA). The miRNA oligonucleotide hsa-miR-29a-3p: 5′-UAG CAC CAU CUG AAA UCG GUU A-3′ was labeled with cy5.5™ dye on its 5′ end, while the 2′-*O*-methyl-modified mRNA oligonucleotides, miR-29a-ABCC6-target: 5′-CAA AGC CAA GAU GGU GCU U-3′, miR-29a-ALDH5A1-target: 5′-UUU CUG UCC UGG UGU GGU GCU G-3′, and miR-29a-SLC22A7-target: 5′-GCU UCU UCU AGA GAU GGU GCU A-3′, corresponding to hsa-miR-29a-3p response elements in *ABCC6*, *ALDH5A1*, and *SLC22A7*, were labeled with IRDye®800 dye on their 5′ ends. Unlabeled miRNA negative control Cold-NC: 5′-UCA CAA CCU CCU AGA AAG AGU AGA-3′ or unlabeled hsa-miR-29a-3p oligonucleotides were utilized in the competition assays. Cytoplasmic extracts were prepared from terminally differentiated HepaRG cells using NE-PER Nuclear and Cytoplasmic extraction reagents (Thermo Scientific, Tewksbury, MA), following the manufacturer's instructions.

As described in our previous study [17], RNA electrophoretic mobility shift assays (RNA EMSAs) were carried out according to the manufacturer's protocol for the LightShift Chemiluminescent RNA EMSA Kit (Thermo Scientific). Briefly, the basic reaction mixtures contained 1x REMSA Binding Buffer, 5% glycerol, 200 mM KCl, 100 mM $MgCl_2$, and 2 mM miRNA or/and cognate mRNA oligonucleotides. HepaRG cytoplasmic extracts (2 μg) and non-specific tRNA (1 μg) were added to the 20 μL basic reaction mixtures to form the RNA:protein complexes. Antibodies against Ago1 and Ago2 were obtained from Abcam (Cambridge, MA) and utilized in the supershift assays. Unlabeled oligonucleotides were used at 50-fold molar excesses in competition reactions. The reaction mixtures were incubated at room temperature for 20 min, separated by 12% native polyacrylamide gel electrophoresis (PAGE) at 4 °C, and labeled complexes were detected according to the manufacturer's protocol for the Odyssey CLx Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

2.4. Luciferase reporter gene assays

The pGL3-CU plasmid containing the *firefly* luciferase reporter gene [17] was used to create reporter gene vectors. Briefly, the core 3′-UTRs of *ALDH5A1* or *SLC22A7*, which harbor the response elements for hsa-miR-29a-3p, were created by PCR with the cloning primers ALDH5A1-F: 5′-GGG AAA GU TCC CAT CCC ATG GAT ACA GT-3′ and ALDH5A1-R: 5′-GGA GAC AU AGG TGC TTC GTC TCC ACC TA-3′, or SLC22A7-F: 5′-GGG AAA GU TGA CTT CCC AGA ATG CAG TG -3′ and SLC22A7-R: 5′-GGA GAC AU ATG AGA CCA GTG GGT TGG AG -3′, with extension oligonucleotides 5′-GGA GAC AU-