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MicroRNA hsa-miR-29a-3p modulates CYP2C19 in human liver cells

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ABSTRACT

Cytochrome P450 2C19 (CYP2C19) is involved in the metabolism of many drugs. Extensive studies have demonstrated that genetic variants and endogenous and environmental factors play important roles in the expression of *CYP2C19*. However, the role of microRNAs (miRNAs) in controlling *CYP2C19* expression has not been investigated completely. In the present study, we performed *in silico* analysis to rank putative miRNA/*CYP2C19* hybrids with regards to the predicted stabilities of their duplexes and then we applied a series of biochemical and molecular assays to elucidate the underlying functional mechanisms for the regulation of CYP2C19 by miRNAs. *In silico* analysis indicated that hsa-miR-23a-3p and hsa-miR-29a-3p target the coding region of *CYP2C19* with hybrid stabilities of –27.5 kcal/mol and –23.3 kcal/mol, respectively. RNA electrophoresis mobility shift assays showed that both hsa-miR-23a-3p and hsa-miR-29a-3p miRNAs were able to bind directly to their cognate targets in the *CYP2C19* transcript. Further, a significant inverse correlation was found between chemically-induced up-regulation of hsa-miR-29a-3p and CYP2C19 expression in HepaRG cells. In addition, inverse correlations were also observed in human liver tissue samples between the level of *CYP2C19* mRNA expression and both hsa-miR-23a-3p and hsa-miR-29a-3p levels. All these results demonstrated the suppressing role of hsa-miR-29a-3p on *CYP2C19* expression.

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1. Introduction

Human cytochrome P450 2C19 (CYP2C19), an important drug metabolizing enzyme mainly expressed in the liver, plays pivotal roles in the activation or elimination of many therapeutic drugs, endogenous biomolecules, and environmental toxicants, including proton pump inhibitors (*e.g.*, omeprazole, lansoprazole and pantoprazole), antiplatelet drugs (*e.g.*, clopidogrel), anticonvulsants (*e.g.*, phenytoin, methylphenytoin, diazepam, and phenobarbital), antidepressants (*e.g.*, sertraline, citalopram, fluoxetine, and venlafaxine), anti-infective agents (*e.g.*, proguanil, chlorproguanil, and nelfinavir), hormones (*e.g.*, progesterone and testosterone), and pesticides (*e.g.*, chlorpyifos and diazinon) [1–3]. Next to CYP3A4/5 that participates in metabolizing 30% of drugs, CYP2C19 is one of the most important hepatic drug metabolizing enzymes (DMEs), participating in the metabolism of 6–10% of clinically prescribed drugs [4].

Several different factors affect the expression of *CYP2C19* and its functional activity in humans. For instance, it has been reported that genetic variations affect CYP2C19 expression and enzyme activity among humans, with more than an 800-fold difference in expression found in a cohort of 427 human liver samples [1,4,5]. Variation in the ability to catalyze 4′-hydroxylation of the CYP2C19 substrate mephenytoin allows individuals in most ethnicities to be categorized as poor metabolizers (PMs) or extensive metabolizers (EMs) and *CYP2C19* genetic variants have been associated with PM and EM phenotypes [1]. For example, *CYP2C19*1* is an allele found in the majority of people having normal CYP2C19 activity, *i.e.* the principal EM phenotype. *CYP2C19*2* is an allele found in 2–5% Caucasians and 18–23% of Japanese [6] that creates an RNA splicing defect, effectively a null allele, resulting in a PM phenotype. *CYP2C19*3* is also a null allele

Abbreviations: CYP, cytochrome P450; miRNA, microRNA; DMETs, drug metabolizing enzymes and transporters; PM, poor metabolizer; EM, extensive metabolizer; ERα, estrogen receptor α; CAR, constitutive androstane receptor; PXR, pregnane X receptor; RE, response element; 3′-UTR, 3′-untranslated region; SULT, sulfotransferase; Ago, argonaute RISC catalytic component; RISC, RNA-induced silence complex; FSB, fetal bovine serum; EMSA, electrophoretic mobility shift assay.

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that introduces a premature stop codon in exon 4 [7], accounting for another type of PM. In contrast, *CYP2C19*17*, a variant involving the 5′-flanking region that provides for enhanced binding to GATA-binding (GATA) proteins [8], results in an elevated expression of *CYP2C19* among variant carriers, accounting for ultra-rapid metabolizers. To date, more than 34 individual *CYP2C19* variants are listed in its nomenclature system, encompassing a variety of genetic polymorphisms (http://www.cypalleles.ki.se/cyp2c19.htm).

In addition to genetic polymorphisms, endogenous and exogenous stimuli may also affect *CYP2C19* expression dramatically through the mediation of transcription factors, such as the estrogen receptor α (ERα) [9], the constitutive androstane receptor (CAR) and the pregnane X receptor (PXR) which utilize the same DNA sequence binding specificity [10], the glucocorticoid receptor (GR) [10], and the GATA-4 transcription factor [11], by binding their response elements located at the *CYP2C19* promoter. Drug–drug interactions affecting the normal nuclear receptor-mediated regulation of CYP2C19 have a significant impact on clinical pharmacology. For example, the administration of 17β-estradiol or 17α-ethinylestradiol can down-regulate the expression of CYP2C19 through the interaction of ligand-bound ERα with its cognate estrogen response element (ERE) at position −151/−147 in the *CYP2C19* promoter [9], providing a mechanism for impaired CYP2C19 expression associated with the use of oral contraceptives by women. On the other hand, the induction of CYP2C19 by rifampicin or other xenobiotics is due to ligand-dependent activation of transcription through the interaction of PXR/CAR with the CAR response element (CAR-RE) located within the proximal promoter of *CYP2C19* [12], providing a mechanistic explanation for increased clearance of some CYP2C19-metabolized drugs, including warfarin [13], mephenytoin [14], and hexobarbital [15], in humans exposed to PXR/CAR agonists.

Epigenetic modifications that influence the expression of various drug metabolizing enzymes provide another mechanism contributing to inter-individual variability in drug metabolism and efficacy. Thus, the epigenetic regulation of DME gene expression has a tremendous impact on the optimization of drug therapy. Although a few CpG islands were detected in the CYP2C19 gene using *in silico* analysis with the potential to influence gene expression via epigenetic DNA methylation [8], little is known about the actual impact of these sites on CYP2C19 expression [16]. MicroRNAs (miRNAs) provide another epigenetic mechanism for regulating DME gene expression. The miRNAs are ~22 nucleotide small RNA molecules which usually suppress gene expression by targeting partially complementary sequences located in the 3′-untranslated regions (3′-UTR) of mRNA transcripts, resulting in the enhanced degradation of targeted mRNA transcripts or the repression of mRNA translational efficiency [17]. Considerable efforts have been made recently to elucidate the roles of specific miRNA species in regulating DME expression. MiRNAs have been shown to affect the expression of many DMEs, including *CYP1B1* [18],*CYP2E1* [19], *CYP3A4* [20] and *SULT1A1* [21]. In the case of *CYP2C19*, two miRNA binding sites were identified within its 3′-UTR that interacted with miR-103 or miR-107 [22]. Transfection of ectopic miR-103 and miR-107 into human hepatocytes resulted in decreased CYP2C19 expression, suggesting that altered miRNA levels among humans could contribute to the inter-individual variability of CYP2C19 expression.

In the current study, we carried out integrative analyses using *in silico*, *in vivo,* and *in vitro* approaches to investigate the potential interaction and the mechanisms by which miRNAs target *CYP2C19*. First, we identified a miRNA targeting site in the coding region of CYP2C19 using *in silico* methods and then we employed a series of biochemical assays to elucidate the interaction between hsa-miR-29a-3p and *CYP2C19* mRNA transcripts. Our results demonstrate that hsa-miR-29a-3p can suppress CYP2C19 expression in an Ago1-dependent manner in human liver cells.

2. Materials and methods

2.1. Cell lines and materials

293T human embryonic kidney cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). HepaRG cells, terminally differentiated hepatic cells retaining many characteristics of primary human hepatocytes, were obtained from Life Technologies (Carlsbad, CA). These cell lines were maintained according to ATCC and Life Technologies recommendations, respectively.

2.2. In silico *analyses*

Three public databases, microRNA.org (http://www.microrna.org/), PITA (http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html) and TargetScan (Release 6.2, http://www.targetscan.org), were screened to identify potential miRNA response elements resident within the *CYP2C19* 3′-UTR. A fourth database, miRTar.human (http://mirtar.mbc.nctu.edu.tw/human/), was also screened to predict miRNAs that could target sites within the full *CYP2C19* transcript, including the 5′-UTR, the protein coding region, and the 3′-UTR. The CellMiner™ database (version 1.5, http://discover.nci.nih.gov/cellminer), which integrates a variety of data for the NCI-60 cell lines including miRNAs levels, gene transcripts levels, and pharmacological data sets, was used to select candidate miRNAs negatively correlated with CYP2C19 expression. This database also was screened to identify chemical compounds that are positively or negatively correlated with hsa-miR-23a-3p or hsa-miR-29a-3p expression. The RNAhybrid program (http://bibiserv2.cebitec.uni-bielefeld.de/rnahybrid) was used to calculate the minimum free energy of hybridization for candidate miRNAs with putative binding sites detected within the *CYP2C19* mRNA sequence (NM_000769.1).

2.3. Exogenous CYP2C19 expression assays

The pCMV6-CYP2C19 vector, which expresses the full length cDNA for *CYP2C19* (Refseq NM_000769) conjugated with a C-terminal Myc-DDK tag, was purchased from OriGene Technologies (Rockville, MD) and used in the exogenous *CYP2C19* expression assays in 293T cells. The CYP2C19-M1 or CYP2C19-M2 constructs, which include single nucleotide synonymous mutations in the putative hsa-miR-29a-3p target sequence in *CYP2C19* mRNA, were prepared by site-directed mutagenesis of the pCMV6-CYP2C19 plasmid insert. Briefly, CYP2C19-MUT1-F and CYP2C19-MUT1-R primers, or CYP2C19-MUT2-F and CYP2C19-MUT2-R primers, together with the corresponding CYP2C19-F or CYP2C19-R primer, respectively, were used to amplify mutant DNA fragments (detailed primer sequence information is available pre request). The PCR products were then double-digested with *Kpn* I and *Not* I (New England Biolabs, Beverly, MA), and subcloned into *Kpn* I and *Not* I-linearized pCMV6-CYP2C19 vectors. The resultant constructs were sequenced to confirm authenticity.

293T Human embryonic kidney cells, cultured in Dulbecco's Modified Eagle medium with 10% fetal bovine serum (FBS), were seeded at 2×10^5 cells per well in 24-multiwell plates and allowed to grow to 70–80% confluence. Using Lipofectamine 2000 reagent (Life Technologies), 293T cells were transfected with pCMV6-CYP2C19, CYP2C19-Mut1, or CYP2C19-Mut2 plasmids (300 ng) together with 25 nmol/L hsa-miR-29a-3p mimic, hsa-miR-23a-3p mimic, miRNA negative control (all from Thermo Scientific, Tewksbury, MA) or predesigned siRNA oligonucleotide specific