Special Section on Epigenetic Regulation of Drug Metabolizing Enzymes and Transporters

Small Nucleolar RNA-Derived MicroRNA hsa-miR-1291 Modulates Cellular Drug Disposition through Direct Targeting of ABC Transporter ABCC1

Yu-Zhuo Pan, Amy Zhou, Zihua Hu, and Ai-Ming Yu

Department of Pharmaceutical Sciences (Y.-Z.P., A.Z., A.-M.Y.), Center for Computational Research (Z.H.), University at Buffalo, The State University of New York, Buffalo, New York; and Department of Biochemistry & Molecular Medicine, University of California Davis School of Medicine, Sacramento, California (A.-M.Y.)

Received March 20, 2013; accepted May 16, 2013

ABSTRACT

Multidrug resistance-associated protein 1 (MRP1/ABCC1) is an important membrane transporter that contributes to cellular disposition of many endobiotic and xenobiotic agents, and it can also confer multidrug resistance. This study aimed to investigate the role of human noncoding microRNA-1291 (hsa-miR-1291) in regulation of ABCC1 and drug disposition. Bioinformatics analyses indicated that hsa-miR-1291, localized within the small nucleolar RNA H/ACA box 34 (SNORA34), might target ABCC1 3'-untranslated region (3'UTR). Using splinted ligation small RNA detection method, we found that SNORA34 was processed into hsa-miR-1291 in human pancreatic carcinoma PANC-1 cells. Luciferase reporter assays showed that ABCC1 3'-UTR-luciferase activity was decreased by 20% in cells transfected with hsa-miR-1291 expression plasmid, and increased by 40%

in cells transfected with hsa-miR-1291 antagomir. Furthermore, immunoblot study revealed that ABCC1 protein expression was sharply reduced in hsa-miR-1291-stably transfected PANC-1 cells, which was attenuated by hsa-miR-1291 antagomir. The change of ABCC1 protein expression was associated with an alternation in mRNA expression. In addition, hsa-miR-1291-directed downregulation of ABCC1 led to a greater intracellular drug accumulation and sensitized the cells to doxorubicin. Together, our results indicate that hsa-miR-1291 is derived from SNORA34 and modulates cellular drug disposition and chemosensitivity through regulation of ABCC1 expression. These findings shall improve the understanding of microRNA-controlled epigenetic regulatory mechanisms underlying multidrug resistance and interindividual variability in pharmacokinetics.

Introduction

MicroRNAs (miRNAs or miRs) are a large family of short (~22 nt), single-strand, noncoding RNAs transcribed from genome. Usually, miRNAs suppress target gene expression through the inhibition of translation or acceleration of mRNA degradation after imperfectly complementary Watson-Crick base pairings with miRNA response element (MRE) within the 3'-untranslated regions (3'UTRs) of mRNA targets. Over 2000 miRNAs have been identified in humans, and they are predicted to govern posttranscriptional regulation of thousands of protein-coding genes in control of essentially all life processes (Kasinski and Slack, 2011). Nevertheless, there are only

a limited number of studies (Tsuchiya et al., 2006; Kovalchuk et al., 2008; To et al., 2008; Pan et al., 2009a,b; Liang et al., 2010; Mohri et al., 2010; Haenisch et al., 2011; Li et al., 2011; Borel et al., 2012) on miRNA-controlled posttranscriptional gene regulation of the xenobiotic-metabolizing enzymes and transporters that underlie drug metabolism and disposition as well as multidrug resistance (MDR).

ATP-binding cassette, sub-family C (CFTR/MRP), member 1 (ABCC1/MRP1) is a membrane transporter that is expressed ubiquitously in human tissues and contributes to cellular disposition of numerous xenobiotics (e.g., doxorubicin, SN-38, and imatinib) and endobiotics (e.g., glutathione and leukotrienes). Inhibition, genetic variations, and altered expression of ABCC1 may lead to variable drug disposition, cytotoxicity, and clinical outcome (Maeno et al., 2009; Cho et al., 2011; Pajic et al., 2011). ABCC1 is also able to confer resistance to many chemotherapeutic agents, such as anthracyclines (e.g., doxorubicin) and the folate antagonist methotrexate. Indeed, clinical

This project was supported in part by the National Institutes of Health National Institute on Drug Abuse [Grant R01-DA021172].

dx.doi.org/10.1124/dmd.113.052092.

ABBREVIATIONS: 3'UTR, 3'-untranslated region; ADME, absorption, distribution, metabolism, and excretion; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; hsa-miR-1291, *Homo sapiens* microRNA-1291; MDR, multidrug resistance; miRNA, microRNA; MRE, microRNA response element; MRP1/ABCC1, multidrug resistance-associated protein 1/ATP-binding cassette, subfamily C, member 1; MTT, methylthiazolylidiphenyl-tetrazolium bromide; PCR, polymerase chain reaction; RT-qPCR, reverse transcriptase quantitative real-time polymerase chain reaction; SNORA34/ACA34, small nucleolar RNA, H/ACA box 34; snoRNA, small nucleolar RNA.

studies have demonstrated that overexpression of ABCC1 in a variety of solid or invasive tumors, such as breast, ovarian, lung, prostate, and neuroblastoma, is implicated as a high risk factor of MDR and is a negative prognostic biomarker (Filipits et al., 2005; Haber et al., 2006; Triller et al., 2006; Faggad et al., 2009). For example, ABCC1 is readily detectable in all primary neuroblastoma samples studied, and a greater degree of ABCC1 expression is highly predictive of both event-free survival and overall patient survival (Haber et al., 2006).

The 3'UTR of ABCC1, which is around 2 kb in length and contains several computationally predicted MREs, awaits experimental investigation. Recently, the action of miR-326 on ABCC1 3'UTR and its impact on ABCC1-mediated MDR has been successfully demonstrated (Liang et al., 2010). Understanding the miRNA-controlled epigenetic regulatory mechanisms shall provide novel insight into interindividual variability in drug absorption, distribution, metabolism, and excretion (ADME); improved understanding of the mechanisms underlying overexpression of ABC efflux transporters in MDR cancer cells will also further the development of rational or new drug therapy (Gomez and Ingelman-Sundberg, 2009; Yu, 2009; Ingelman-Sundberg and Gomez, 2010; Nakajima and Yokoi, 2011; Yu and Pan, 2012; Yokoi and Nakajima, 2013). Therefore, the present study aimed to delineate the action of a relatively newer human miRNA hsa-miR-1291 on the 3'UTR of ABCC1. Interestingly, we find that hsa-miR-1291 sequences reside within the small nucleolar RNA (snoRNA), H/ACA box 34 (SNORA34) (Watkins and Bohnsack, 2012). We show that SNORA34 indeed can be processed to mature hsa-miR-1291 in PANC-1 cells. Furthermore, we present data suggesting that hsa-miR-1291 regulates *ABCC1* gene expression, and the suppression of ABCC1 protein expression by hsa-miR-1291 is translated into a significantly greater level of intracellular drug accumulation and chemosensitivity.

Materials and Methods

Chemicals and Materials. Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, penicillin sodium, and streptomycin sulfate solution were

purchased from Corning Cellgro (Manassas, VA). G418, fetal bovine serum (FBS), Lipofectamine 2000, and Trizol reagent were bought from Life Technologies (Carlsbad, CA). Actinomycin D, doxorubicin, and methylthiazolyldiphenyl-tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO). Primers and digoxin-labeled oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). Restriction enzymes KpnI, XhoI, NotI, psiCHECK-2 vector, and the dual luciferase assay system were purchased from Promega (Madison, WI). T4 DNA ligase was obtained from New England Biolabs (Ipswich, MA). The antibodies against ABCC1/MRP1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were bought from Abcam (Cambridge, MA), and Santa Cruz Biotechnologies (Santa Cruz, CA), respectively. Horseradish peroxidase (HRP)-labeled anti-digoxin antibody and anti-mouse secondary antibodies were purchased from Jackson Immuno-Research Laboratories (West Grove, PA). Hybond enhanced chemiluminescence (ECL) membrane and Nylon⁺ membrane were from GE Healthcare (Piscataway, NJ). The hsa-miR-1291 antagomirs (anti-miR-1291) and negative control oligonucleotides, ECL substrate, BCA Protein Assay Kit, and Pierce CL-Xposure film were from Thermo Scientific (Rockford, IL). Radio immunoprecipitation assay buffer was purchased from Rockland Immunochemicals (Gilbertsville, PA). Complete protease inhibitor cocktail was purchased from Roche Diagnostics (Mannheim, Germany).

Cell Culture. The human pancreatic carcinoma PANC-1 cells, small lung cancer H69 cells and the drug-resistant subline H69AR, and embryonic kidney HEK-293 cells were purchased from American Type Culture Collection (Manassas, VA) and grown in DMEM medium containing 10% FBS, 100 U/mL of penicillin sodium, and 100 µg/mL of streptomycin sulfate at 37°C in a humidified atmosphere of 5% carbon dioxide.

Plasmids. The coding region (1033 bp) of SNORA34 containing hsa-miR-1291 was amplified from the human genome DNA via polymerase chain reaction (PCR). The PCR products were digested with MluI and XhoI, and inserted into a small RNA expression vector named pCMV-Globin (a gift from Dr. Tamás Kiss, Université Paul Sabatier, France) (Kiss et al., 2002), resulting in a SNORA34/miR-1291 expression plasmid named pCMV-SNORA34/miR-1291. The pCMV empty vector was used as a control. The whole 3'UTR of ABCC1 (NM_004996.3) consisting of 1822 nt was amplified by PCR and inserted into psiCHECK-2 vector after linearization by XhoI and NotI, leading to a ABCC1 3'UTR-luciferase reporter plasmid named psiCHECK-ABCC1-3'UTR. All PCR primer sequences are provided in Table 1.

TABLE 1

Oligonucleotides used for the construction of plasmids, quantitative polymerase chain reaction, and splinted ligation experiments

Application	Oligonucleotide	Sequence (5'→3')
Cloning		
psiCHECK-ABCC1-3'UTR	Forward	CCGCTCGAGCGGAGGGTGATCGTCTTGGACAAAGGAGAAATC
	Reverse	TTGCGGCCGCAACCCCAAACTTAGTTTATCTAATGTGAAAGAATG
pCMV-SNORA34/miR-1291	Forward	CGACGCGTCGTCAACTAATAGAAGTGGGTTCTGGCTTCCC
	Reverse	CCCTCGAGGGGACTTGGATGTTGTGGGTGATGGTATGCGAG
Stem-loop RT-qPCR		
hsa-miR-1291	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACTGCT
	Forward	GCCCTGGCCCTGACUGAAGACC
U74	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAATTGT
	Forward	CCTGTGGAGTTGATCCTAGTCTGGGTG
Universal reverse primer		GTCGAGGGTCCGAGGT
qPCR		
ABCC1	Forward	AACCTGGACCCATTACGCC
	Reverse	GACTGGATGAGGTCGTCCGT
SNORA34	Forward	CTGACTGAAGACCAGCAGTTGTA
	Reverse	TGAAATAGCCATTCCCTACTGAG
18S	Forward	GTAACCCGTTGAACCCATT
	Reverse	CCATCCAATCGGTAGTAGCG
Splinted Ligation		
Detection Probe		5'-Phosphate-CGCTTATGACATTTC-3'-digoxin
Bridge oligo for miR-1291		GAATGTCATAAGCACTGCTGGTCT
Bridge oligo for SNORA34		GAATGTCATAAGCGGTTGTTTCTTC

3'UTR, 3'-untranslated region; RT, reverse transcriptase; RT-qPCR, reverse transcriptase quantitative real-time polymerase chain reaction; SNORA34/ACA34, small nucleolar RNA, H/ACA box 34.

Fig. 1. hsa-miR-1291 and SNORA34 sequences are colocalized within the intron 9 of an uncharacterized gene *C12orf41* on human chromosome 12q13.11.

Bioinformatics Analysis. The 3'UTR of ABCC1 was retrieved from NCBI ENTREZ (<http://www.ncbi.nlm.nih.gov/ENTREZ>) and searched for hsa-miR-1291 MREs by RNAhybrid (<http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/>), TargetScan (<http://www.targetscan.org/>), miRanda (<http://www.microrna.org/microrna/home.do>), and PITA (<http://genie.weizmann.ac.il/pubs/mir07/>) algorithms.

Luciferase Reporter Assay. The luciferase reporter assay was performed as described previously (Pan et al., 2009a,b). Briefly, HEK-293 cells (1×10^4 cells/well)

were co-transfected with psiCHECK-ABCC1-3'UTR luciferase reporter plasmid (0.1 μ g) and pCMV-miR-1291 or control plasmid (0.4 μ g). Likewise, cells were co-transfected with ABCC1 3'UTR-luciferase reporter (0.1 μ g) and 50 nM of anti-miR-1291 or control oligonucleotides. Luciferase activity was determined using the dual luciferase reporter assay system and the Berthold Centro LB960 Luminometer (Berthold Technologies, Oak Ridge, TN). Relative luciferase activities were calculated as the ratios between *Renilla* and firefly luciferase activities, and further normalized to control treatments.

Establishment of miR-1291 Stably Transfected PANC-1 Cell Line. The PANC1 cells in exponential growth were seeded into 6-well plates at a concentration of 2×10^5 cells/well. After 24 hours, cells were transfected with 2.5 μ g of pCMV-SNORA34/miR-1291 or control plasmids using Lipofectamine 2000. Media were replaced after 24 hours, and the cells were selected stepwise with 500 μ g/mL of G418. After 3 to 5 weeks, G418-resistant clones were selected with a cloning ring for amplification in culture.

Splinted Ligation. The splinted ligation was conducted as described (Maroney et al., 2008) with some modifications. Briefly, total RNA was isolated with Trizol reagent, and quantified using NanoDrop (Thermo Scientific). A mixture of 500 ng total RNA, 100 fmol bridge oligonucleotides, and 2 pmol 3'-digoxin-labeled ligation oligonucleotides (Table 1) was denatured at 95°C for 1 minute and then annealed at 65°C for 5 minutes and at 37°C for 15 minutes. Then 50 U of T4 DNA ligase were added to the reaction and incubated at 37°C for 2 hours. The ligase was heat inactivated at 75°C for 15 minutes. After the addition of 2X loading dye (20 mM EDTA and 0.05% xylene cyanole in 95% formamide), the mixture was denatured at 95°C for 5 minutes and immediately transferred onto ice to prevent annealing, then loaded onto a pre-run denaturing 15% urea-polyacrylamide gel. After separation through electrophoresis, nucleic acids were transferred onto Nylon⁺ membrane, fixed onto the membrane by UV cross-linking in a Stratalinker (Stratagene, Santa Clara, CA) for 3 minutes at the highest power, blocked with 5% nonfat milk, and detected by HRP-labeled anti-digoxin antibody and ECL method. The light emitted from HRP-catalyzed oxidation of luminol was captured on film or by the ChemiDoc XRS+ System (Bio-Rad, Hercules, CA).

Reverse Transcription Quantitative Real-Time PCR (qPCR). Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) quantification of mRNA of interest and stem loop RT-qPCR analyses of mature hsa-miR-1291 were conducted using gene selective primers (Table 1) on a Bio-Rad MyIQ real-time PCR system, as described (Li et al., 2011; Rodrigues et al., 2011). The cycle number (C_T) at which the amplicon concentration crossed a defined threshold was determined for each mRNA/miRNA. The relative level of each analyte over internal standard was calculated using the Eq. $2^{-\Delta C_T}$, where ΔC_T was the difference in C_T values between analyte and internal standard (18S or U74), and then compared between different groups or treatments.

RNA Degradation Analysis. Forty-eight hours after transfection with hsa-miR-1291 and control plasmids, PANC-1 cells were treated with actinomycin D at a final concentration of 5 μ g/mL to block de novo RNA synthesis.

Fig. 2. hsa-miR-1291 is produced from SNORA34 in PANC-1 cells. (A) Expression of SNORA34 and miR-1291 was detected by splinted ligation method in H69AR and PANC-1 cells. Mature miR-1291 was clearly detected as a labeled band of 35 nt. (B) SNORA34 could be processed to hsa-miR-1291 in PANC-1 cells following transient transfection with pCMV-SNORA34/miR-1291 plasmid, which might be inhibited by the anti-hsa-miR-1291 oligonucleotide.

Cells were harvested at 0, 24, and 48 hours. ABCC1 mRNA levels were determined by RT-qPCR and normalized to 18S.

Western Blots. The cells were lysed in radioimmunoprecipitation assay buffer with complete protease inhibitors, and protein concentrations were determined with BCA Protein Assay Kit. Whole-cell proteins (50 μ g/lane) were separated on a 10% SDS-PAGE gel and transferred onto Hybond ECL membrane. Membranes were first incubated with selective antibody against ABCC1 or GAPDH, and subsequently with a peroxidase goat anti-mouse IgG. The membranes were then incubated with ECL substrates, and images were acquired by ChemiDoc XRS+ System (Bio-Rad).

Flow Cytometry Analyses. Intracellular drug accumulation was investigated using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ), as reported (Li et al., 2011). Briefly, cells (5×10^5) were incubated with phenol red-free RPMI 1640 medium containing 10% FBS and 50 μ M of doxorubicin at 37°C for 1 hour. Cells incubated in the absence of drug were used as negative controls. Cells were then washed twice with ice-cold medium, incubated with drug-free medium, harvested by centrifugation, and subjected to flow cytometry analyses in the FL-3 channel (650 ± 30 nm). Drug efflux ability was reflected by the fluorescence of drug accumulated within cells. Flow cytometry data were analyzed by WinMDI Version 2.8 software (Scripps Institute, San Diego, CA). Relative intracellular drug accumulation was calculated by normalized geometric mean of hsa-miR-1291 group to the control group after subtraction of the basal fluorescence (negative control).

Chemosensitivity. Cell sensitivity to doxorubicin was carried out as described (Pan et al., 2009a). Briefly, stably transfected PANC-1 cells were plated at 1000 cells/well in 96-well plates in DMEM medium containing various concentrations of doxorubicin. Cell viability was determined using MTT assay. Inhibition (IC_{50} value) of cell growth by the drug was estimated by fitting the percentage of cell growth (vehicle control plus 0 μ M test drug as 100%) to the Hill equation $Y = 100 / (1 + 10^{[(\log IC_{50} - X) * \text{HillSlope}]})$ (GraphPad Prism 5; GraphPad Software, Inc., San Diego, CA) (Pan et al., 2009a). All experiments were carried out in triplicate and repeated once with separate cultures.

Data Analysis. All values were expressed as mean $\pm$ S.D. Different treatments (qPCR, luciferase activity, and drug accumulation data) were

compared by unpaired Student's *t* test, and multiple variances (chemosensitivity) were analyzed by two-way analysis of variance (GraphPad Prism 5). Difference was considered as significant if the probability was less than 0.05 ($P < 0.05$).

Results

Mature hsa-miR-1291 Is Derived from SNORA34. To improve the understanding of miRNA mechanistic functions in regulation of ADME, we employed multiple bioinformatic algorithms to screen potential miRNA targets for cytochrome P450 enzymes and ABC transporters. To identify more promising miRNA candidates, we conducted analyses to see if particular miRNAs were enriched for a list of ADME 106 genes by comparing the number of miRNA target ADME genes to the number of genes for the same miRNA in the whole human genome (unpublished data). Candidate miRNAs showing significant enrichment (Fisher's exact test) were obtained for further analysis. Among a set of putative miRNAs (unpublished data), hsa-miR-1291 is a newer miRNA that might be generated from SNORA34 (Fig. 1 and 2) and act on the 3'UTR of ABCC1 (Fig. 3), critical for xenobiotic disposition and MDR. Thus, we examined the biogenesis of hsa-miR-1291 and possible role in regulation of ABCC1-mediated drug disposition.

Our bioinformatic analyses also showed that hsa-miR-1291 sequences are located within the intron 9 of an uncharacterized gene *C12orf41* on human chromosome 12q13.11. Given the fact that the precursor hsa-miR-1291 overlaps with SNORA34 (Fig. 1), we reasoned that SNORA34 might be processed to 24-nt mature hsa-miR-1291. To test the hypothesis, we adapted a splinted ligation method based on a direct ligation of miRNA and probe (Maroney et al., 2008) and examined the association of SNORA34 and hsa-miR-1291 expression in PANC-1, H69AR, and H69 cells. For our purposes, we

Fig. 3. hsa-miR-1291 acts on the 3'UTR of ABCC1. (A) Bioinformatic analyses revealed four putative MREs for hsa-miR-1291 within the 3'UTR of ABCC1. The seed sequences were underlined. (B) ABCC1 3'UTR-luciferase activity was significantly altered in HEK-293 cells after the manipulation of hsa-miR-1291 function with hsa-miR-1291 expression plasmid or antagomir (anti-miR-1291). Data are mean $\pm$ S.D. ($N = 3$ in each group). * $P < 0.05$, compared with the control (Student's *t* test).

used a nonradioactive digoxin-labeled probe with relative high sensitivity, among which PANC-1 and drug-resistant H69AR cells exhibit a high level expression of ABCC1. Our data showed that SNORA34 was detectable from the total RNAs isolated from PANC-1, H69AR, and H69 cells (Fig. 2A). Among them, H69 cells exhibited the lowest level of SNORA34, and PANC-1 cells had the highest level. Consistent with this finding, miR-1291 showed a greater expression in PANC-1 cells, whereas it was undetectable in H69 cells (Fig. 2A), suggesting a positive relationship between hsa-miR-1291 and SNORA34 expression.

To investigate the processing of SNORA34, we created a SNORA34/miRNA-1291 expression plasmid. A 1033-bp fragment of SNORA34 was inserted into the intron 2 of globin within the snoRNA expression vector pCMV-globin to maintain the intronic feature of SNORA34. This design also proved to ensure a good expression of snoRNA (Darzacq et al., 2002; Kiss et al., 2002). After a transient transfection with SNORA34 expression plasmids, the expression of SNORA34 was increased in H69AR cells but unchanged in PANC-1 cells (Fig. 2B). However, the hsa-miR-1291 expression level remained undetectable in SNORA34-transfected H69AR cells but seemed to be higher in SNORA34-transfected PANC-1 cells (Fig. 2B), suggesting a fine processing of SNORA34 to hsa-miR1291 in PANC-1 cells. Therefore, the PANC-1 cells were further transfected with an anti-miR-1291 oligonucleotide. As expected, the expression of mature hsa-miR-1291 was markedly reduced by the hsa-miR-1291 antagomir (Fig. 2B). The results suggest that mature hsa-miR-1291 may be generated from SNORA34 in PANC-1 cells.

hsa-miR-1291 Acts on the 3'UTR of ABCC1. The ABCC1 3'UTR consists of four putative hsa-miR-1291 MRE sites (Fig. 3A). The first site (position 32-38) is well conserved, and the others are relatively more selective for human ABCC1. The values of binding energy (ΔG_{duplex}) of hsa-miR-1291 with the target sequences estimated by RNAhybrid were -29.3 , -24.1 , -24.7 , and -27.0 kcal/mol, respectively. To evaluate the potential interaction between hsa-miR-1291 and ABCC1 3'UTR, we conducted a luciferase reporter study in cells with gained and lost hsa-miR-1291 function. Our data showed that ABCC1 3'UTR luciferase reporter activities were reduced by $\sim 20\%$ in cells transfected with miR-1291 expression plasmid, and increased by $\sim 40\%$ in cells transfected with anti-miR-1291 oligonucleotide (Fig. 3B). These data support the actions of hsa-miR-1291 on the 3'UTR of ABCC1.

hsa-miR-1291 Regulates the Expression of ABCC1. To examine the potential role of hsa-miR-1291 in regulation of ABCC1, we first generated a SNORA34/hsa-miR-1291 stably transfected PANC-1 cell line (Fig. 4). Quantitative PCR analyses showed that hsa-miR-1291 expression was approximately 18-fold higher in SNORA34/hsa-miR-1291 stably transfected PANC-1 cells than in the control, whereas SNORA34 expression was unchanged (Fig. 4). Overexpression of hsa-miR-1291 led to a reduced ABCC1 protein expression in hsa-miR-1291 stably transfected cells, as revealed by immunoblot analyses (Fig. 5A). This was associated with approximately 5-fold lower ABCC1 mRNA expression (data not shown). Furthermore, the suppression of ABCC1 protein expression by gain of hsa-miR-1291 function could be rescued; ABCC1 mRNA expression was approximately 11-fold higher in hsa-miR-1291 stably transfected cells (data not shown) after transient transfection with anti-miR-1291 oligonucleotide (Fig. 5B).

In addition, an mRNA degradation study was performed to test if mRNA decay mechanism was involved in the hsa-miR-1291 controlled posttranscriptional regulation of ABCC1. When actinomycin D blocked de novo RNA synthesis, ABCC1 mRNA stability did not differ between cells transfected with hsa-miR-1291 and control

Fig. 4. A hsa-miR-1291 stably transfected PANC-1 cell line (A) was established, which showed 18-fold higher hsa-miR-1291 expression (B) but no change in SNORA34 (C). Data are mean $\pm$ S.D. ($N = 6$ in each group). * $P < 0.05$, compared with the control (Student's t test).

plasmids (Fig. 6). This might exclude the involvement of mRNA degradation mechanism, and suggest the presence of other possible means, such as translation inhibition or targeting of transcription factors.

hsa-miR-1291 Modulates Cellular Drug Disposition. To examine whether the regulation of ABCC1 protein expression by hsa-miR-1291

Fig. 5. hsa-miR-1291 regulates ABCC1 protein expression. Immunoblots showed a lower ABCC1 protein level in hsa-miR-1291 stably transfected PANC-1 cells (A), which was reversed when the cells were transfected with hsa-miR-1291 antagomir (anti-miR-1291). (B) GAPDH was used as a loading control.

Fig. 6. hsa-miR-1291 does not affect ABCC1 mRNA stability. ABCC1 mRNA levels in PANC-1 cells treated with actinomycin D were determined by qPCR analyses and normalized to 18S in corresponding samples. Data are mean $\pm$ S.D. ($N = 3$ in each group).

can be translated into significant difference in xenobiotic disposition, we conducted flow cytometry analyses to determine intracellular doxorubicin accumulation. Our data showed that there was a greater level of doxorubicin fluorescence intensity in hsa-miR-1291 stably transfected PANC-1 cells (Fig. 7A). Overall, doxorubicin accumulation within hsa-miR-1291 stably transfected PANC-1 cells was approximately 2.5-fold higher than the control cells (Fig. 7B), which is likely due to a lower ABCC1 expression (Fig. 5A) that enables the antineoplastic drug doxorubicin to be pumped out of the cells.

Gain of hsa-miR-1291 Function Sensitizes Human Carcinoma Cells to Xenobiotics. To evaluate the impact of hsa-miR-1291-controlled regulation of ABCC1 on chemosensitivity, we employed the MTT assay to define the cytotoxicity of doxorubicin in PANC-1 cells stably transfected with hsa-miR-1291 and control plasmids.

Fig. 7. hsa-miR-1291 modulates cellular drug disposition. Flow cytometry analyses showed that intracellular doxorubicin accumulation was increased significantly in PANC-1 cells stably transfected with hsa-miR-1291. (A) Fluorescence histograms of doxorubicin content. (B) Relative drug accumulation indicated by the geometry mean of doxorubicin fluorescence. Data are mean $\pm$ S.D. ($N = 3$ in each group). * $P < 0.05$, compared with the control (Student's t test).

Fig. 8. hsa-miR-1291 sensitizes cancer cells to the anticancer drug. Cytotoxicity study revealed that hsa-miR-1291 stably transfected PANC-1 cells were more sensitive to doxorubicin, compared with the control cells ($P < 0.05$; two-way analysis of variance). This is also manifested by a lower IC_{50} value ($137 \pm 1 \mu M$) and steeper Hill slope (-1.08 ± 0.13) in hsa-miR-1291-transfected cells than in the control ($373 \pm 2 \mu M$ and -0.50 ± 0.05 , respectively). Data are mean $\pm$ S.D. ($N = 6$ in each group).

Cytotoxicity was measured after 48 hours of treatment with various concentrations of doxorubicin. The data showed that hsa-miR-1291 stably transfected cells were much more sensitive to doxorubicin (Fig. 8). This was also demonstrated by a significantly lower IC_{50} value ($137 \pm 1 \mu M$) and steeper Hill slope (-1.08 ± 0.13) in hsa-miR-1291-transfected cells than in the control cells ($373 \pm 2 \mu M$ and -0.50 ± 0.05 , respectively). The results indicate that gain of hsa-miR-1291 function sensitizes PANC-1 cells to doxorubicin.

Discussion

There is increasing evidence supporting that noncoding miRNAs may contribute to epigenetic regulation of ADME processes through their actions on xenobiotic metabolizing enzymes and transporter and nuclear receptors (Gomez and Ingelman-Sundberg, 2009; Yu, 2009; Ingelman-Sundberg and Gomez, 2010; Nakajima and Yokoi, 2011; Yu and Pan, 2012; Yokoi and Nakajima, 2013). In this study, we demonstrate that snoRNA-derived hsa-miR-1291 targets the 3'UTR of membrane transporter ABCC1 and negatively regulates the expression of ABCC1. Gain of hsa-miR-1291 function can sensitize carcinoma cells to anticancer drugs via increasing ABCC1-mediated intracellular drug accumulation.

The snoRNAs are another class of noncoding regulatory RNAs that are 60–300 nt in length and mainly responsible for the posttranscriptional modification of ribosomal RNAs in cells (Kiss, 2002; Watkins and Bohnsack, 2012). There are two major groups of snoRNAs, box C/D snoRNAs and box H/ACA snoRNAs, which function as guide RNAs for the 2'-O-methylation and pseudouridylation of target RNA sequences, respectively. Most interestingly, evolutionarily conserved box H/ACA snoRNAs share structural similarities with miRNA precursors. Consisting of two hairpin domains, H/ACA snoRNAs can be processed by the RNase III-type endoribonuclease Dicer to produce mature miRNAs (Scott et al., 2009) or miRNA-like small RNAs (Ender et al., 2008; Saraiya and Wang, 2008). Recent studies have also demonstrated the derivation of small RNAs from box C/D snoRNAs, which exhibit miRNA-like functions (Brameier et al., 2011). The hsa-miR-1291 precursor (Fig. 1) has been shown to structurally resemble an H/ACA snoRNA, SNORA34 (Scott et al., 2009). In the present study, we show the detection of mature hsa-miR-1291 using a splinted ligation method. Mature hsa-miR-1291 of 24 nt in length is readily processed from the SNORA34/miR-1291 construct in PANC-1 cells, which may be reduced by the anti-SNORA/miR-1291 oligonucleotide. In addition,

the detection of other RNAs longer than hsa-miR-1291/SNORA34 in H69AR and PANC-1 cells (Fig. 2) might indicate the presence of long noncoding RNAs (lncRNAs) containing miR-1291/SNORA34 sequences. Actually, the abundance of lncRNAs in mammalian cells has been demonstrated by various techniques, including unbiased deep sequencing, and there is an increasing interest in understanding the functions and clinical importance of lncRNAs in gene regulation (Wang and Chang, 2011; Kung et al., 2013).

The processing of SNORA34 to hsa-miR-1291 might be tissue- or cell type-dependent. The hsa-miR-1291 was originally discovered via massively parallel sequencing of small RNA libraries generated from stem cells (Morin et al., 2008), and SNORA34 was cloned from HeLa cells (Kiss et al., 2004). We first identified and cloned hsa-miR-1291 sequences from LS-180 cells (unpublished data) when studying miRNA-controlled regulation of CYP3A4 (Pan et al., 2009a). Our recent report showed a lower expression of hsa-miR-1291 than some other ADME regulatory miRNAs in MCF-7 and Caco-2 cells, which might be altered by xenobiotics (Rodrigues et al., 2011). The current study revealed that hsa-miR-1291 was undetectable in H69 cells, which was associated with a lower level of SNORA34. In contrast, both SNORA34 and hsa-miR-1291 were found to be present at a higher level in PANC-1 cells (Fig. 2). These observations suggest a cell specific biogenesis of hsa-miR-1291, and the underlying machinery awaits further exploration.

Computational identification of putative MRE sites for hsa-miR-1291 within ABCC1 3'UTR (Fig. 3) led to the findings on hsa-miR-1291-controlled suppression of ABCC1 mRNA and protein expression (Fig. 5). Although the mRNA degradation study suggests that hsa-miR-1291 does not change ABCC1 mRNA stability, the experiment might be confounded by the fact that actinomycin D is also a substrate of ABCC1 (Hill et al., 2013). Additional investigations are needed to determine whether hsa-miR-1291-mediated suppression of ABCC1 indeed involves translation inhibition. In addition, because there is a lack of hsa-miR-1291 MRE within ABCC1 5'UTR, hsa-miR-1291 might affect ABCC1 expression through the targeting of its transcription factor. Nevertheless, the importance of miRNA regulatory mechanisms is nicely exemplified by its significant impact on ABCC1-mediated cellular drug disposition and chemosensitivity (Figs. 7 and 8). Recent studies have also revealed a critical role for miR-134 and miR-326 in regulation of ABCC1 and in association with MDR (Guo et al., 2010; Liang et al., 2010). Other examples of miRNA functions in regulation of ABC transporters include the control of ABCB1 expression by miR-451 and miR-27 (Kovalchuk et al., 2008; Zhu et al., 2008); ABCG2 expression by miR-328, miR-519c, and miR-520h (To et al., 2008; Pan et al., 2009b; Wang et al., 2010; Li et al., 2011); and ABCC2 expression by miR-379 (Haenisch et al., 2011). These findings support the importance of regulatory miRNAs in modulation of cellular ADME processes and their potential influence on pharmacokinetics and pharmacodynamics (Gomez and Ingelman-Sundberg, 2009; Yu, 2009; Ingelman-Sundberg and Gomez, 2010; Nakajima and Yokoi, 2011; Yu and Pan, 2012; Yokoi and Nakajima, 2013).

Overexpression of ABCC1 is frequently observed in MDR cancers and closely related to the outcome of chemotherapy (Filipits et al., 2005; Haber et al., 2006; Faggad et al., 2009), which is often associated with abnormal expression or miRNAs. Indeed, we have found that hsa-miR-1291 is significantly downregulated in human pancreatic ductal adenocarcinoma, compared with normal pancreas (unpublished data). Very recent studies also show a lower expression of hsa-miR-1291 in acute myocardial infarction (Meder et al., 2011) and renal cell carcinoma (Hidaka et al., 2012), suggesting a common downregulation of hsa-miR-1291 in cancerous tissues. Given the finding on hsa-miR-1291-mediated suppression of ABCC1, novel

approaches might be developed to intervention of hsa-miR-1291 pathways toward combating MDR. Nevertheless, the clinical significance of hsa-miR-1291 warrants additional critical studies.

In summary, our results indicate that SNORA34 may serve as a hsa-miR-1291 precursor. Our data also show that hsa-miR-1291 targets the 3'UTR of ABCC1 and consequently regulates the expression of ABCC1. The impact of hsa-miR-1291 on ABCC1-mediated drug disposition and chemosensitivity support our hypothesis that interference of miRNA regulatory pathway can sensitize human carcinoma cells to anticancer drugs. An improved understanding of miRNA biogenesis and functions in control of ABC transporters will not only provide new insights into variable drug disposition but also offer novel clue to develop rational drug therapy and combat MDR.

Authorship Contributions

Participated in research design: Yu, Pan, Hu.

Conducted experiments: Pan, Zhou.

Contributed to new reagents or analytic tools: Pan.

Performed data analysis: Pan, Yu, Hu, Zhou.

Wrote or contributed to the writing of the manuscript: Yu, Pan, Hu.

References

- Borel F, Han R, Visser A, Petry H, van Deventer SJ, Jansen PL, and Konstantinova P; Réseau Centre de Ressources Biologiques Foie (French Liver Biobanks Network), France (2012) Adenosine triphosphate-binding cassette transporter genes up-regulation in untreated hepatocellular carcinoma is mediated by cellular microRNAs. *Hepatology* **55**:821–832.
- Brameier M, Herwig A, Reinhardt R, Walter L, and Gruber J (2011) Human box C/D snoRNAs with miRNA like functions: expanding the range of regulatory RNAs. *Nucleic Acids Res* **39**: 675–686.
- Cho S, Lu M, He X, Ee PL, Bhat U, Schneider E, Miele L, and Beck WT (2011) Notch1 regulates the expression of the multidrug resistance gene ABCC1/MRP1 in cultured cancer cells. *Proc Natl Acad Sci USA* **108**:20778–20783.
- Darzacq X, Jádý BE, Verheggen C, Kiss AM, Bertrand E, and Kiss T (2002) Cajal body-specific small nuclear RNAs: a novel class of 2'-O-methylation and pseudouridylation guide RNAs. *EMBO J* **21**:2746–2756.
- Ender C, Krek A, Friedländer MR, Beitzinger M, Weinmann L, Chen W, Pfeffer S, Rajewsky N, and Meister G (2008) A human snoRNA with microRNA-like functions. *Mol Cell* **32**: 519–528.
- Faggad A, Darb-Esfahani S, Wirtz R, Sinn B, Schouli J, Könsgen D, Lage H, Noske A, Weichert W, and Buckendahl AC, et al. (2009) Expression of multidrug resistance-associated protein 1 in invasive ovarian carcinoma: implication for prognosis. *Histopathology* **54**:657–666.
- Filipits M, Pohl G, Rudas M, Dietze O, Lax S, Grill R, Pirker R, Zielinski CC, Hausmaninger H, and Kubista E, et al. (2005) Clinical role of multidrug resistance protein 1 expression in chemotherapy resistance in early-stage breast cancer: the Austrian Breast and Colorectal Cancer Study Group. *J Clin Oncol* **23**:1161–1168.
- Gomez A and Ingelman-Sundberg M (2009) Pharmacogenetics: its role in interindividual differences in drug response. *Clin Pharmacol Ther* **85**:426–430.
- Guo L, Liu Y, Bai Y, Sun Y, Xiao F, and Guo Y (2010) Gene expression profiling of drug-resistant small cell lung cancer cells by combining microRNA and cDNA expression analysis. *Eur J Cancer* **46**:1692–1702.
- Haber M, Smith J, Bordow SB, Flemming C, Cohn SL, London WB, Marshall GM, and Norris MD (2006) Association of high-level MRP1 expression with poor clinical outcome in a large prospective study of primary neuroblastoma. *J Clin Oncol* **24**:1546–1553.
- Haenisch S, Laechelt S, Bruckmueller H, Werk A, Noack A, Bruhn O, Remmler C, and Cascorbi I (2011) Down-regulation of ATP-binding cassette C2 protein expression in HepG2 cells after rifampicin treatment is mediated by microRNA-379. *Mol Pharmacol* **80**:314–320.
- Hidaka H, Seki N, Yoshino H, Yamasaki T, Yamada Y, Nohata N, Fuse M, Nakagawa M, and Enokida H (2012) Tumor suppressive microRNA-1285 regulates novel molecular targets: aberrant expression and functional significance in renal cell carcinoma. *Oncotarget* **3**: 44–57.
- Hill CR, Jamieson D, Thomas HD, Brown CD, Boddy AV, and Veal GJ (2013) Characterisation of the roles of ABCB1, ABCC1, ABCC2 and ABCG2 in the transport and pharmacokinetics of actinomycin D in vitro and in vivo. *Biochem Pharmacol* **85**:29–37.
- Ingelman-Sundberg M and Gomez A (2010) The past, present and future of pharmacogenomics. *Pharmacogenomics* **11**:625–627.
- Kasinski AL and Slack FJ (2011) Epigenetics and genetics. MicroRNAs en route to the clinic: progress in validating and targeting microRNAs for cancer therapy. *Nat Rev Cancer* **11**: 849–864.
- Kiss AM, Jádý BE, Bertrand E, and Kiss T (2004) Human box H/ACA pseudouridylation guide RNA machinery. *Mol Cell Biol* **24**:5797–5807.
- Kiss AM, Jádý BE, Darzacq X, Verheggen C, Bertrand E, and Kiss T (2002) A Cajal body-specific pseudouridylation guide RNA is composed of two box H/ACA snoRNA-like domains. *Nucleic Acids Res* **30**:4643–4649.
- Kiss T (2002) Small nucleolar RNAs: an abundant group of noncoding RNAs with diverse cellular functions. *Cell* **109**:145–148.
- Kovalchuk O, Filkowski J, Meservy J, Ilnytsky Y, Tryndyak VP, Chekhun VF, and Pogribny IP (2008) Involvement of microRNA-451 in resistance of the MCF-7 breast cancer cells to chemotherapeutic drug doxorubicin. *Mol Cancer Ther* **7**:2152–2159.

- Kung JT, Colognori D, and Lee JT (2013) Long noncoding RNAs: past, present, and future. *Genetics* **193**:651–669.
- Li X, Pan YZ, Seigel GM, Hu ZH, Huang M, and Yu AM (2011) Breast cancer resistance protein BCRP/ABCG2 regulatory microRNAs (hsa-miR-328, -519c and -520h) and their differential expression in stem-like ABCG2+ cancer cells. *Biochem Pharmacol* **81**:783–792.
- Liang Z, Wu H, Xia J, Li Y, Zhang Y, Huang K, Wagar N, Yoon Y, Cho HT, and Scala S, et al. (2010) Involvement of miR-326 in chemotherapy resistance of breast cancer through modulating expression of multidrug resistance-associated protein 1. *Biochem Pharmacol* **79**: 817–824.
- Maeno K, Nakajima A, Conseil G, Rothnie A, Deeley RG, and Cole SP (2009) Molecular basis for reduced estrone sulfate transport and altered modulator sensitivity of transmembrane helix (TM) 6 and TM17 mutants of multidrug resistance protein 1 (ABCC1). *Drug Metab Dispos* **37**: 1411–1420.
- Maroney PA, Chamnongpol S, Souret F, and Nilsen TW (2008) Direct detection of small RNAs using splinted ligation. *Nat Protoc* **3**:279–287.
- Meder B, Keller A, Vogel B, Haas J, Sedaghat-Hamedani F, Kayvanpour E, Just S, Borries A, Rudloff J, and Leidinger P, et al. (2011) MicroRNA signatures in total peripheral blood as novel biomarkers for acute myocardial infarction. *Basic Res Cardiol* **106**:13–23.
- Mohri T, Nakajima M, Fukami T, Takamiya M, Aoki Y, and Yokoi T (2010) Human CYP2E1 is regulated by miR-378. *Biochem Pharmacol* **79**:1045–1052.
- Morin RD, O'Connor MD, Griffith M, Kuchenbauer F, Delaney A, Prabhu AL, Zhao Y, McDonald H, Zeng T, and Hirst M, et al. (2008) Application of massively parallel sequencing to microRNA profiling and discovery in human embryonic stem cells. *Genome Res* **18**: 610–621.
- Nakajima M and Yokoi T (2011) MicroRNAs from biology to future pharmacotherapy: regulation of cytochrome P450s and nuclear receptors. *Pharmacol Ther* **131**:330–337.
- Pajic M, Murray J, Marshall GM, Cole SP, Norris MD, and Haber M (2011) ABCC1 G2012T single nucleotide polymorphism is associated with patient outcome in primary neuroblastoma and altered stability of the ABCC1 gene transcript. *Pharmacogenet Genomics* **21**:270–279.
- Pan YZ, Gao W, and Yu AM (2009a) MicroRNAs regulate CYP3A4 expression via direct and indirect targeting. *Drug Metab Dispos* **37**:2112–2117.
- Pan YZ, Morris ME, and Yu AM (2009b) MicroRNA-328 negatively regulates the expression of breast cancer resistance protein (BCRP/ABCG2) in human cancer cells. *Mol Pharmacol* **75**: 1374–1379.
- Rodrigues AC, Li X, Radecki L, Pan YZ, Winter JC, Huang M, and Yu AM (2011) MicroRNA expression is differentially altered by xenobiotic drugs in different human cell lines. *Biopharm Drug Dispos* **32**:355–367.
- Saraiya AA and Wang CC (2008) snoRNA, a novel precursor of microRNA in *Giardia lamblia*. *PLoS Pathog* **4**:e1000224.
- Scott MS, Avolio F, Ono M, Lamond AI, and Barton GJ (2009) Human miRNA precursors with box H/ACA snoRNA features. *PLOS Comput Biol* **5**:e1000507.
- To KK, Zhan Z, Litman T, and Bates SE (2008) Regulation of ABCG2 expression at the 3' untranslated region of its mRNA through modulation of transcript stability and protein translation by a putative microRNA in the S1 colon cancer cell line. *Mol Cell Biol* **28**: 5147–5161.
- Triller N, Korosec P, Kern I, Kosnik M, and Debeljak A (2006) Multidrug resistance in small cell lung cancer: expression of P-glycoprotein, multidrug resistance protein 1 and lung resistance protein in chemo-naïve patients and in relapsed disease. *Lung Cancer* **54**:235–240.
- Tsuchiya Y, Nakajima M, Takagi S, Taniya T, and Yokoi T (2006) MicroRNA regulates the expression of human cytochrome P450 1B1. *Cancer Res* **66**:9090–9098.
- Wang F, Xue X, Wei J, An Y, Yao J, Cai H, Wu J, Dai C, Qian Z, and Xu Z, et al. (2010) hsa-miR-520h downregulates ABCG2 in pancreatic cancer cells to inhibit migration, invasion, and side populations. *Br J Cancer* **103**:567–574.
- Wang KC and Chang HY (2011) Molecular mechanisms of long noncoding RNAs. *Mol Cell* **43**: 904–914.
- Watkins NJ and Bohnsack MT (2012) The box C/D and H/ACA snoRNPs: key players in the modification, processing and the dynamic folding of ribosomal RNA. *Wiley Interdiscip Rev RNA* **3**:397–414.
- Yokoi T and Nakajima M (2013) microRNAs as mediators of drug toxicity. *Annu Rev Pharmacol Toxicol* **53**:377–400.
- Yu AM (2009) Role of microRNAs in the regulation of drug metabolism and disposition. *Expert Opin Drug Metab Toxicol* **5**:1513–1528.
- Yu AM and Pan YZ (2012) Noncoding microRNAs: Small RNAs play a big role in regulation of ADME? *Acta Pharmaceutica Sinica B* **2**:93–101.
- Zhu H, Wu H, Liu X, Evans BR, Medina DJ, Liu CG, and Yang JM (2008) Role of MicroRNA miR-27a and miR-451 in the regulation of MDR1/P-glycoprotein expression in human cancer cells. *Biochem Pharmacol* **76**:582–588.

Address correspondence to: Dr. Ai-Ming Yu, Department of Biochemistry & Molecular Medicine, UC Davis School of Medicine, 2700 Stockton Boulevard, Sacramento, CA 95817. E-mail: aimyu@ucdavis.edu