RNA Editing Enzymes Modulate the Expression of Hepatic CYP2B6, CYP2C8, and Other Cytochrome P450 Isoforms

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ABSTRACT

A-to-I RNA editing, the most frequent type of RNA editing in mammals, is catalyzed by adenosine deaminase acting on RNA (ADAR) enzymes. Recently, we found that there is a large interindividual variation in the expression of ADAR1 protein in the human livers. In this study, we investigated the possibility that A-to-I RNA editing may modulate the expression of cytochrome P450 (P450), causing interindividual variations in drug metabolism potencies. We found that knockdown of ADAR1 or ADAR2 in HepaRG cells resulted in the decreased expression of CYP2B6 and CYP2C8 mRNA and protein. Knockdown of ADARs significantly decreased the stability of CYP2B6 mRNA but not CYP2C8 mRNA. Luciferase assays revealed that the 3'-untranslated region of CYP2B6 and the promoter region of CYP2C8 would be involved in the decrease in their

expression by the knockdown of ADARs. We found that the decreased expression of the hepatocyte nuclear factor 4 α (HNF4 α) protein by the knockdown of ADARs was one of the reasons for the decreased transactivity of CYP2C8. The mRNA levels of other P450 isoforms, such as CYP2A6, 2C9, 2C19, 2D6, and 2E1, which are known to be regulated by HNF4 α , were also decreased by ADAR1 or ADAR2 knockdown. Exceptionally, the CYP3A4 mRNA level was significantly increased by ADAR1 knockdown, suggesting the possibility that the change could be due to the change in the expression or function of other regulatory factors. In conclusion, this study revealed that the RNA editing enzymes ADAR1 and ADAR2 are novel regulatory factors of P450-mediated drug metabolism in the human liver.

Introduction

RNA editing is a post-transcriptional process that changes nucleic acid sequences on RNA. The most frequent type in mammals is A-to-I RNA editing, catalyzed by the adenosine deaminase acting on RNA (ADAR) enzyme (Kim et al., 1994), which converts adenosine in double-stranded RNA to inosine by hydrolytic deamination (Wagner et al., 1989; Nishikura, 2010). Cellular machineries recognize inosine as a guanosine; thus, the conversion possibly changes the resulting amino acid sequence, splicing, microRNA (miRNA) targeting, and maturation (Farajollahi and Maas, 2010). In mammals, there are three members of ADAR: ADAR1, ADAR2, and ADAR3 (Bass et al., 1997). ADAR1 has two isoforms, ADAR1 p150 (a 150 kDa protein) and ADAR1 p110 (a 110 kDa protein). The former is interferon inducible and present in the nucleus and cytoplasm, whereas ADAR1 p110 (110 kDa protein) is constitutively and ubiquitously expressed and present in the nucleus (Patterson and Samuel, 1995; Desterro et al., 2003). ADAR2 is also ubiquitously expressed and localized in the nucleus, whereas the expression of ADAR3 is limited to the brain (Melcher et al., 1996). Although ADAR1 and ADAR2 have A-to-I RNA editing activity,

ADAR3 does not have editing activity (Kim et al., 1994; Chen et al., 2000).

It has been reported that *ADAR1* knockout mice are embryonic lethal, caused by severe liver damage (Wang et al., 2000), and *ADAR2* knockout mice die within 20 days after birth due to status epilepticus (Higuchi et al., 2000). Thus, RNA editing by ADARs is an essential event for viability. Additionally, the disruption of RNA editing by mutations in the *ADAR* gene is associated with some diseases (Slotkin and Nishikura, 2013). For example, the abnormal editing of GluR2, an ionotropic glutamate receptor, by the decreased expression or activity of ADAR2 causes amyotrophic lateral sclerosis (Feldmeyer et al., 1999). Mutations in the *ADAR1* gene cause dyschromatosis symmetrica hereditaria, a rare genetic pigmentation disorder (Miyamura et al., 2003). These studies highlight the importance of ADARs in biologic phenomena and homeostasis.

Cytochrome P450s (P450s) are the major drug-metabolizing enzymes, accounting for the biotransformation of 70% of all drugs in clinical use (Guengerich, 2008). P450s comprise a superfamily, and the CYP1, 2, and 3 families are responsible for drug metabolism in the liver. There are significant interindividual differences in the expression and activity of hepatic P450s, causing variable therapeutic efficacy or adverse events of drugs. Understanding the factors causing interindividual and intraindividual differences in drug metabolism potencies is required for the practice of personalized or precision medicine and the promotion of efficient drug development. The interindividual differences of P450s result from intrinsic factors, such as genetic polymorphisms, disease states, sex, and age, as well as extrinsic factors, such as

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ABBREVIATIONS: ADAR, adenosine deaminase acting on RNA; AhR, aryl hydrocarbon receptor; HNF4 α , hepatocyte nuclear factor 4 α ; LC, liquid chromatography; miRNA, microRNA; P450, cytochrome P450; PCR, polymerase chain reaction; siADAR, small interfering RNA for human adenosine deaminase acting on RNA; siControl, small interfering control; 3'-UTR, 3'-untranslated region.

exposure to drugs and environmental chemicals, alcohol, smoking, and diet (Zanger and Schwab, 2013). As one of the major mechanisms of the variability in the expression, the transcriptional regulation of P450s by transcription factors, including hepatocyte nuclear factors 4 α (HNF4 α), pregnane X receptor, constitutive androstane receptor, and aryl hydrocarbon receptor (AhR), has greatly been appreciated.

Recently, our research group revealed that there is a large interindividual variation (220-fold) in ADAR1 protein expression in human liver samples (Nakano et al., 2016). This finding prompted us to examine whether the interindividual differences of ADARs may contribute to the hepatic drug metabolism potencies. Recently, we found that ADAR1 negatively regulates AhR expression by creating a binding sequence of miRNA in the 3'-untranslated region (3'-UTR). The downregulation of AhR affected CYP1A1, a downstream gene of AhR (Nakano et al., 2016). This was the first study to reveal that A-to-I RNA editing modulates the xenobiotic metabolism. Thereafter, when we searched a database, REDiportal (<http://srv00.recas.ba.infn.it/atlas/>), we noticed that some P450 genes and transcriptional factors regulating P450s are registered to be subjected to RNA editing (Nakano and Nakajima, 2018). We expanded our study to investigate whether ADARs regulate the expression and activity of P450 isoforms in the CYP1, 2, and 3 families. For this purpose, differentiated HepaRG cells were used because they retain P450 expression and activity (Anthérieu et al., 2010) comparable to that in primary human hepatocytes.

Materials and Methods

Chemicals and Reagents. Phenacetin, acetaminophen, and α -amanitin were obtained from Wako Pure Chemicals (Osaka, Japan). Amodiaquine and diclofenac were obtained from Sigma-Aldrich (St. Louis, MO). Hydroxybupropion, bufuralol, and 1'-hydroxybufuralol were obtained from Corning (Corning, NY). The pGL3-basic vector, pNL1.1 vector, and Nano-Glo Dual-Luciferase Reporter Assay System were obtained from Promega (Madison, WI). Lipofectamine RNAiMAX, Silencer Select small interfering RNA for human ADAR [(siADAR), siADAR1 (s1007) and siADAR2 (s1010)], negative control #1, and Advanced Dulbecco's modified Eagle's medium were obtained from Life Technologies (Carlsbad, CA). Viofectin was purchased from Viogene (Taipei, Taiwan). RNAiso and random hexamers were obtained from Takara (Shiga, Japan). ReverTra Ace was obtained from Toyobo (Osaka, Japan). Primers were commercially synthesized at Integrated DNA Technologies (Coralville, IA). Luna Universal qPCR Master Mix was obtained from New England Biolabs (Ipswich, MA). The mouse anti-human ADAR1, mouse anti-human ADAR2, and mouse anti-human β -actin monoclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit anti-human CYP2B6 antibody and rabbit anti-human CYP2C8 polyclonal antibody were obtained from Corning. The IRDye 680 goat anti-mouse IgG, goat anti-rabbit IgG, and donkey anti-goat IgG antibodies were obtained from LI-COR Biosciences (Lincoln, NE). The restriction enzymes were obtained from Nippon Gene (Tokyo, Japan) and New England Biolabs. All other chemicals and solvents were of the highest grade commercially available.

Cell Cultures. Human hepatocellular carcinoma-derived HepaRG cells, purchased from KAC (Kyoto, Japan), were cultured in William's E medium supplemented with 10% FBS (Invitrogen), 100 U/ml penicillin, 100 μ g/ml streptomycin, 5 μ g/ml insulin, 2 mM glutamine, and 50 μ M hydrocortisone hemisuccinate. After 2 weeks, the medium was replaced by the same medium supplemented with 2% DMSO, and the cell culture was maintained for 2 weeks to differentiate the cells with the maximal functional activities. The medium was exchanged every 2 or 3 days. Human hepatocellular carcinoma-derived Huh-7 cells, obtained from Riken Gene Bank (Tsukuba, Japan), were cultured in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical, Tokyo, Japan) containing 10% FBS. These cells were cultured at 37°C under the atmospheric conditions of 5% CO₂ and 95% air.

Transfection of Small Interfering RNA for ADAR1 or ADAR2 into HepaRG of Huh-7 Cells and the Preparation of Cell Homogenate, Total RNA, and Nuclear Extracts. The differentiated HepaRG cells were seeded into

six-well plates and transfected with 50 nM siADAR1 or siADAR2 using Lipofectamine RNAiMAX. After incubation for 72 hours in DMSO-free medium, the cells were harvested. One-half of the cells were suspended in Tris-Glycine-EDTA buffer [10 mM Tris-HCl, 20% glycerol, and 1 mM EDTA (pH 7.4)], disrupted by freeze/thawing three times, and homogenized. The other half of the cells was used to prepare total RNA using RNAiso. Huh-7 cells were seeded into six-well plates and transfected with 5 nM siADAR1 or siADAR2 using Lipofectamine RNAiMAX. After incubation for 72 hours, the cells were harvested and used to prepare nuclear extracts following the research by Dignam et al. (1983) with slight modification. Briefly, Huh-7 cells were suspended with five volumes of buffer A (10 mM HEPES, pH 7.6, 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, and 0.05% NP40), and centrifuged at 3000 rpm at 4°C for 5 minutes. The supernatant was removed and two volumes of buffer A were added to the pellet. The suspension was centrifuged at 3000 rpm at 4°C for 5 minutes. The supernatant was removed and two volumes of buffer C (20 mM HEPES, pH 7.8, 420 mM KCl, 0.2 mM EDTA, 1.5 mM MgCl₂, 1 mM dithiothreitol, and 10% glycerol) were added to the pellet. After incubation for 30 minutes at 4°C, the suspension was centrifuged at 15,000 rpm at 4°C for 30 minutes and the supernatant was recovered as nuclear extracts.

SDS-PAGE and Western Blot Analysis. The cell homogenates (20 μ g for ADAR1, CYP2B6, 10 μ g for β -actin, and 30 μ g for CYP2C8) and nuclear extracts (20 μ g for HNF4 α) were separated by 7.5% SDS-PAGE and transferred to an Immobilon-P transfer membrane (Millipore, Billerica, MA). For ADAR2 analysis, 50 μ g of the cell homogenate was separated by 7.5% SDS-PAGE and transferred to a Protran nitrocellulose membrane (Whatman GmbH, Dassel, Germany). The membranes were probed with the primary antibody and then with the fluorescent, dye-conjugated secondary antibodies. The bands were detected using the Odyssey Infrared Imaging system (LI-COR Biosciences). The ADAR1, ADAR2, CYP2B6, and CYP2C8 protein levels were normalized to the β -actin protein levels.

Real-Time Reverse Transcription Polymerase Chain Reaction. The cDNA was synthesized from total RNA using ReverTra Ace. A 1 μ l portion of the reverse-transcribed mixture was added to the polymerase chain reaction (PCR) mixture containing 5 pmol of each primer and 10 μ l of the Luna Universal qPCR Mix in a final volume of 20 μ l. The sequences of the primers and PCR conditions are shown in Table 1. Real-time reverse transcription PCR was performed using Mx3000P (Stratagene). Each P450 mRNA level was normalized to the β -actin mRNA level.

Measurement of P450 Activity. The differentiated HepaRG cells were transfected with siADARs as described previously. After 72 hours, the medium was replaced with FBS-free Advanced Dulbecco's modified Eagle's medium containing 100 μ M phenacetin (CYP1A2), 2 μ M amodiaquine (CYP2C8), 50 μ M diclofenac (CYP2C9), and 20 μ M bufuralol (CYP2D6). After incubations at 37°C for 4 hours (CYP1A2), 30 minutes (CYP2C8), 3 hours (CYP2C9), and 1 hour (CYP2D6), the medium was collected and an equal volume of acetonitrile was added to the medium. After removing the protein by centrifugation at 15,000 rpm for 5 minutes, a portion of supernatant was subjected to liquid chromatography (LC)-tandem mass spectrometry. The LC equipment (Shimadzu, Kyoto, Japan) contained a CBM-20A controller, LC-20AD pumps, a SIL-20AC HT autosampler, a CTO-20AC column oven, and an SPD-20A UV detector equipped with a Develosil ODS-UG-3 column (3 μ m, 4.6 $\times$ 150 mm; Nomura Chemical, Seto, Japan). The column temperature was set at 40°C, and the flow rate was 0.2 ml/min. The mobile phase was water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). The conditions for elution were as follows: 20%–80% B (0–2 minutes), 80% B (2–5 minutes), and 20% B (5–7 minutes). The LC was connected to an LC-MS 8040 (Shimadzu). The metabolites were monitored in the multiple reactions monitoring mode. The mass spectrometry conditions are shown in Table 2. The analytical data were processed using LabSolutions (version 5.82 SP1; Shimadzu).

Evaluation of the Stability of P450 mRNA. HepaRG cells were transfected with siADARs as described previously. After 24 hours, the cells were treated with 10 μ g/ml of α -amanitin, an inhibitor of RNA polymerase II. The total RNA was prepared 0, 1, 2, 4, and 6 hours later. The CYP2B6 and CYP2C8 mRNA levels were determined by real-time reverse transcription PCR, as described previously.

Reporter Plasmid Construction. The 3'-UTR of CYP2B6, from +1461 to 3052, was amplified by PCR using genomic DNA as a template. The nucleotide numbers refer to the transcription start site as +1. The following primer set was used: 5'-CGT GGC CCC AGA AGA CAT CGA TC-3' and 5'-GGG AGG TCA

TABLE 1
Sequence of primers used for reverse transcription PCR

Target	Sequence	Annealing Temperature °C	Extension Time s	Step
CYP1A2 ^a	S 5'- CCA ACG TCA TTG GTG CCA TG -3' AS 5'- GTG ATG TCC CGG ACA CTG TTC -3'	57	20	3
CYP2A6 ^b	S 5'- AAA GAG TTC CTG TCA CTG TTG C -3' AS 5'- GCT GAC GGT CTC GGT GCC CC -3'	64	20	2
CYP2B6	S 5'- CTT GCG GGG ATA TGG TGT GA -3' AS 5'- TCA AAC AGC TGG CCG AAT AC -3'	57	20	3
CYP2C8 ^c	S 5'- AGA TCA GAA TTT TCT CAC CC -3' AS 5'- AAC TTC GTG TAA GAG CAA CA -3'	60	20	2
CYP2C9 ^c	S 5'- CAG ATC TGC AAT AAT TTT TCT C -3' AS 5'- CTT TCA ATA GTA AAT TCA GAT G -3'	50	15	3
CYP2C19 ^c	S 5'- ATT GAA TGA AAA CAT CAG GAT TG -3' AS 5'- GAG GGT TGT TGA TGT CCA TC -3'	64	20	2
CYP2D6 ^c	S 5'- GGT GTG ACC CAT ATG ACA TC -3' AS 5'- CTC CCC GAG GCA TGC ACG -3'	60	15	3
CYP2E1 ^d	S 5'- ACG GTA TCA CCG TGA CTG TGG -3' AS 5'- GCA TCT CTT GCC TAT CCT TGA -3'	58	20	2
CYP3A4	S 5'- GTA TGG AAA AGT GTG GGG CT -3' AS 5'- GAC CAT CTC CTT GAG TTT TCC A -3'	60	20	2
CYP3A5 ^e	S 5'- AGT GTT CTT TCC TTC ACT TTA -3' AS 5'- TTC AAC ATC TTT CTT GCA AGT -3'	60	20	2
β -Actin ^e	S 5'- TCA CCC TGA AGT ACC CCA TC -3' AS 5'- GAT AGC ACA GCC TGG ATA GC -3'	64	30	2

S, Sense primer; AS, Antisense primer.

^aIwanari et al. (2002).

^bNakajima et al. (2006).

^cKatoh et al. (2004).

^dMohri et al. (2010).

^eOda et al. (2012).

GGC TTT AGA G-3'. The fragment was inserted into the pGL3p vector at the XbaI site downstream from the luciferase gene. The constructed luciferase reporter plasmid was termed pGL3p/2B6 3'-UTR.

The 5'-flanking region of the *CYP2C8* gene, from -2491 to -1, was amplified by PCR using genomic DNA as a template. The following primer set was used: 5'-CGC GGT ACC TCA CAA CCC TAT CCA AGC TG- 3' and 5'-TTG AAG CTT AAA GTA AAC AAT CAC CTA GAT CCA AT- 3'. The fragment was inserted into the pGL3b vector via the KpnI and *Hind* III restriction sites to construct the pGL3b/2C8-2491 plasmid. This plasmid was digested by SphI and *Hind* III to obtain a fragment containing the sequence from -514 to -1, and the fragment was cloned into the pGL3b vector to construct the pGL3b/2C8-514 plasmid. In the HNF4 α binding site from -57 to -45 base pairs, the mutations were introduced from -53 to -48 base pairs by inverse PCR, followed by an In-Fusion reaction using the In-Fusion HD Enzyme Premix (Takara). For the inverse PCR, the following PCR primer set was used: 5'-TGG GCG TTT CAC TCA GAA AAA AAG TAT AAA -3' and 5'-GTG GTG AAA CGC CCA TGG ATA GAA ATA AAA TGT -3'. The underlined nucleotides represent the mutated sequence. The obtained plasmid was termed the pGL3b/2C8 MT plasmid.

Luciferase Assay. The various pGL3 luciferase reporter plasmids were transfected with the pNL 1.1 plasmid into Huh-7 cells. Briefly, the cells were seeded into 96-well plates. After 24 hours, 5 nM siADAR1 or siADAR2 was transfected using Lipofectamine RNAiMAX. After 24 hours, 100 ng of the pGL3b plasmid and 100 pg of the pNL 1.1 plasmid were transfected using Viofectin. After 48 hours, the luciferase activity was measured with a luminometer (Wallac, Turku, Finland) using the Nano-Glo Dual-Luciferase Reporter Assay System.

Assessment of RNA Editing Level in the *CYP2B6* 3'-UTR. To analyze RNA editing in the 3'-UTR of *CYP2B6*, direct sequence analysis of the PCR product was performed following our previous study (Nakano et al., 2016). The 3'-UTR of *CYP2B6* was amplified by PCR using cDNA derived from HepaRG cells as a template. The following primer sets were used to amplify the 3'-UTR of *CYP2B6*: the forward primer 5'-CGT GGC CCC AGA AGA CAT CGA TC-3' and reverse primer 5'-TGA CAT CAG GAG TTC AAG ACC-3', or the forward primer 5'-GGT GAT TCT CCT AGC TCC AA-3' and reverse primer 5'-CAT GAA AGC AAC CAG ACA CAA-3'. Specific products were purified and subjected to direct sequencing.

Statistical Analyses. Statistical significance was determined by ANOVA followed by Tukey's method. A value of $P < 0.05$ was considered statistically significant.

Results

CYP2B6 Expression Level Was Decreased by the Knockdown of ADARs. To examine the possibility that hepatic P450s are regulated by ADARs, either siADAR1 or siADAR2 was transfected into HepaRG cells. The siADAR1 construct used in this study can target both ADAR1 p110 and ADAR1 p150. As shown in Fig. 1A, ADAR1 p110 was significantly decreased [91% of small interfering control (siControl)] by the transfection of siADAR1 but not siADAR2. The ADAR1 p150 protein was not detected. The ADAR2 protein was significantly decreased (77% and 56% of siControl, respectively) by the transfection of siADAR2 and siADAR1 (Fig. 1B). Thus, we confirmed that either the ADAR1 or ADAR2 protein was successfully knocked down.

Next, we evaluated the expression levels of the P450 isoforms in the ADAR knockdown cells. We found that the *CYP2B6* mRNA (73% and 68% of siControl, respectively) and protein (65% and 53% of siControl, respectively) (Fig. 2) levels were significantly decreased by

TABLE 2
Conditions of liquid chromatography–tandem mass spectrometry

Isoform	Metabolite	<i>m/z</i>		Collision Energy eV
		Precursor Ion	Production	
CYP1A2	Acetaminophen	152.05	110.00	–16
CYP2C8	<i>N</i> -Desethylamodiaquine	328.10	283.05	–19
CYP2C9	4'-Hydroxydiclofenac	311.15	267.10	10
CYP2D6	1'-Hydroxybufuralol	278.35	186.05	–18

m/z, mass-to-charge ratio.

Fig. 1. Knockdown of ADAR1 or ADAR2 in differentiated HepaRG cells. ADAR1 (A) and ADAR2 (B) protein levels in siADAR1- or siADAR2-transfected HepaRG cells were determined by western blotting and normalized to the β -actin levels. The values represent the levels relative to the siControl. Each column represents the mean $\pm$ S.D. of three independent experiments. ** P < 0.01; *** P < 0.001, compared with siControl.

the knockdown of ADAR1 or ADAR2. These results suggest that ADARs positively regulate the expression level of CYP2B6.

ADARs Stabilize CYP2B6 mRNA by Targeting the 3'-UTR. We investigated whether the decrease in CYP2B6 expression by ADAR knockdown was due to enhanced mRNA degradation. In the presence of α -amanitin, a transcriptional inhibitor, the CYP2B6 mRNA level remained unchanged until after 6 hours in the siControl-transfected cells, indicating that the CYP2B6 mRNA is stable. By the knockdown of ADAR1 or ADAR2, the CYP2B6 mRNA levels were significantly decreased (30% and 40% of siControl, respectively) 6 hours after treatment with α -amanitin (Fig. 3A), indicating that ADARs positively regulate the CYP2B6 mRNA expression by stabilizing the mRNA. Next, to examine the involvement of the 3'-UTR in the decrease in CYP2B6 expression by the knockdown of ADARs, a luciferase assay using a reporter plasmid containing the 3'-UTR was performed (Fig. 3B). Because the reporter plasmid could not efficiently be transfected into the differentiated HepaRG cells, we used Huh-7 cells instead of the HepaRG cells. The luciferase activities of pGL3p/2B6 3'-UTR were lower than those of pGL3p/empty. It is common that the luciferase activity of the reporter plasmid containing the 3'-UTR is lower than that of the empty plasmid because 3'-UTR includes miRNA binding sites. Actually, it has been reported that miR-25-3p suppresses the expression

of CYP2B6 in HepaRG cells via binding to the 3'-UTR (Jin et al., 2016). When the reporter plasmid was transfected with small interfering RNA into Huh-7 cells, the luciferase activity of pGL3p/2B6 3'-UTR was decreased (30% of siControl) by the knockdown of ADAR2 but not of ADAR1, suggesting that ADAR2 apparently regulates the CYP2B6 expression by targeting the 3'-UTR.

No A-to-I RNA Edited Sites Were Found in the 3'-UTR of CYP2B6. In REDportal, RNA editing sites are registered within the two Alu elements in the 3'-UTR of CYP2B6. To examine whether the 3'-UTR of CYP2B6 is subjected to RNA editing, we performed direct sequencing. In the results, no edited sites were detected in the two Alu elements located in the 3'-UTR of CYP2B6 (data not shown). Therefore, the change in the CYP2B6 expression by the knockdown of ADAR1 or ADAR2 appears to be not relevant with the RNA editing of CYP2B6 by itself.

CYP2C8 Expression Level and Activity Were Decreased by the Knockdown of ADARs. We investigated whether the knockdown of ADARs affects other P450 expression levels, focusing on CYP2C8 as one example. As shown in Fig. 4, A and B, the CYP2C8 mRNA (66% and 54% of siControl, respectively) and protein (77% and 64% of siControl, respectively) levels were significantly decreased by the knockdown of ADAR1 or ADAR2. To examine if the CYP2C8 activity

Fig. 2. Effects of ADAR1 or ADAR2 knockdown on the expression of CYP2B6 mRNA and protein in differentiated HepaRG cells. CYP2B6 mRNA (A) and protein (B) levels in siADAR1- or siADAR2-transfected HepaRG cells were determined by real-time reverse transcription PCR and western blot analysis, respectively, and normalized to the β -actin levels. It was confirmed that the lower band represents CYP2B6 by comparison with the band observed with a commercially available recombinant CYP2B6 (data not shown). The values represent the levels relative to the siControl. Each column represents the mean $\pm$ S.D. of three independent experiments. ** P < 0.01; *** P < 0.001, compared with siControl.

Fig. 3. Effects of ADAR1 or ADAR2 knock-down on the stability of CYP2B6 mRNA and the use of luciferase assays to examine the significance of the 3'-UTR. (A) siADAR1- or siADAR2-transfected HepaRG cells were treated with 10 μ g/ml of α -amanitin and the CYP2B6 mRNA level was determined by real-time reverse transcription PCR. The values are expressed as relative to the values of the CYP2B6 mRNA level at 0 hours. Each point represents the mean $\pm$ S.D. of three independent experiments. * P < 0.05; ** P < 0.01. (B) Huh-7 cells were transfected with 5 nM siADAR1 or siADAR2. After 24 hours, the reporter plasmids were transfected. The values are expressed as the activity relative to that of the pGL3p plasmid/siControl. Each column represents the mean $\pm$ S.D. of three independent experiments. * P < 0.05, compared with siControl.

is also decreased, the amodiaquine *N*-desethylase activity was evaluated by LC-tandem mass spectrometry. The activity (64% and 40% of siControl, respectively) was significantly decreased by the knockdown of ADAR1 and ADAR2, although the latter case did not reach a statistically significant difference (Fig. 4C). These results suggest that ADARs positively regulate the CYP2C8 expression and activity.

ADARs Positively Regulate the Transactivity of CYP2C8 via HNF4 α . In the same way as CYP2B6, we investigated whether the knockdown of ADARs results in a decrease in the CYP2C8 mRNA stability. Unlike the stability of CYP2B6, the stability of CYP2C8 mRNA was not changed by ADAR1 or ADAR2 knockdown (Fig. 5A). Then, to investigate whether the knockdown of ADARs affects the transactivity of CYP2C8, a luciferase assay using reporter plasmids containing the promoter region of CYP2C8 was performed. When the pGL3b/2C8-2491 plasmid was used, the luciferase activity was decreased (32% of siControl) by ADAR2 knockdown but not by ADAR1 knockdown. In contrast, the luciferase activity of the pGL3b/2C8-514 plasmid (30% and 49% of siControl, respectively) was decreased by ADAR1 or ADAR2 knockdown (Fig. 5B). We noticed that there is a binding site of HNF4 α in the upstream region of -57 to -45 (Ferguson et al., 2005) in the CYP2C8 gene. To examine whether the disruption of

HNF4 α binding may counteract the siADAR-dependent decrease in the transactivity, a luciferase assay using the plasmid containing a mutation in the response element of HNF4 α was performed. When pGL3b/2C8-514MT was transfected, the luciferase activity was not changed by the knockdown of ADAR1 or ADAR2 (Fig. 5B).

Next, we examined whether the decrease in CYP2C8 transactivity was due to the decreased expression of HNF4 α . As shown in Fig. 5, C and D, we found that the HNF4 α protein (63% and 79% of siControl, respectively) level was significantly decreased by ADAR1 or ADAR2 knockdown, although the HNF4 α mRNA level was not decreased. These results suggest that the decrease in CYP2C8 expression by the knockdown of ADAR1 or ADAR2 could be attributed to the repression of HNF4 α expression.

Effects of ADAR Knockdown on the Other P450 mRNA Expression Levels and Activities in Differentiated HepaRG Cells. It is known that HNF4 α transactivates not only CYP2B6 and 2C8 but also CYP2A6, 2C9, 2C19, 2D6, 2E1, and 3A4 (Kamiyama et al., 2007; Chen et al., 2018). Given that the HNF4 α protein expression is regulated by ADARs, we investigated whether the expression of these P450 mRNAs is also modulated in siADAR1- or siADAR2-transfected HepaRG cells. As shown in Fig. 6, A-F and H, the CYP1A2 (20%

Fig. 4. Effects of ADAR1 or ADAR2 knock-down on the CYP2C8 expression and activity in differentiated HepaRG cells. CYP2C8 mRNA (A) and protein (B) levels in siADAR1- or siADAR2-transfected HepaRG cells were determined by real-time reverse transcription PCR and western blotting, respectively, and normalized to the β -actin levels. The values represent the levels relative to the siControl. (C) CYP2C8 activity in siADAR1- or siADAR2-transfected HepaRG cells was measured by LC-tandem mass spectrometry. Each column represents the mean $\pm$ S.D. of three independent experiments. * P < 0.05; ** P < 0.01; *** P < 0.001, compared with siControl.

Fig. 5. Effects of ADAR1 or ADAR2 knockdown on the transactivity of CYP2C8. ADARs positively regulate the transactivity of CYP2C8 via HNF4α. (A) siADAR1- or siADAR2-transfected HepaRG cells were treated with 10 μg/ml of α-amanitin and CYP2C8 mRNA was determined by real-time reverse transcription PCR (RT-PCR). The values are expressed as relative to the values of the CYP2C8 mRNA levels at 0 hours. Each point represents the mean ± S.D. of three independent experiments. (B) Huh-7 cells were transfected with 5 nM siADAR1 or siADAR2. After 24 hours, the reporter plasmids were transfected. The values are expressed as the activity relative to that of the pGL3p plasmid/siControl. (C and D) HNF4α mRNA and protein levels (using nuclear extracts) in siADAR1- or siADAR2-transfected Huh-7 cells were determined by real-time RT-PCR and western blotting, respectively. The HNF4α protein level was evaluated as the sum of the densities of two bands. The values represent the levels relative to the siControl. Each column represents the mean ± S.D. of three independent experiments. **P* < 0.05; ***P* < 0.01; ****P* < 0.001, compared with siControl.

and 78% of siControl, respectively), CYP2A6 (71% and 72% of siControl, respectively), CYP2C9 (60% of siControl), CYP2C19 (76% and 47% of siControl, respectively), CYP2D6 (26% and 40% of siControl, respectively), CYP2E1 (78% and 81% of siControl, respectively), and CYP3A5 (72% and 40% of siControl, respectively) mRNA levels were decreased by ADAR1 or ADAR2 knockdown, although the changes in CYP1A2 and CYP2D6 by ADAR1 knockdown and the changes in CYP3A5 by ADAR knockdown did not show significant differences. The CYP3A4 mRNA was decreased (76% of siControl) by the knockdown of ADAR2, but unexpectedly, it was increased 3-fold by the knockdown of ADAR1 (Fig. 6G). Next, the effects of ADAR knockdown on the P450 activities were evaluated by using LC–tandem mass spectrometry. We measured phenacetin *O*-deethylation, diclofenac 4'-hydroxylation, and bufuralol 1'-hydroxylation as the typical activities of CYP1A2, CYP2C9, and CYP2D6, respectively. The CYP1A2 activity (24% of siControl) was not changed by ADAR knockdown (Fig. 6I), whereas the CYP2C9 (44% siControl)

and CYP2D6 (59% of siControl) activities were decreased by ADAR1 knockdown (Fig. 6, J and K). This phenomenon was consistent with the results of mRNA alteration. Taken together, the results show that the alteration of HNF4α expression affects multiple hepatic P450 mRNA levels. In the case of CYP3A4, there is a possibility that some factors other than HNF4α may also contribute to the change in the expression of ADARs.

Discussion

A-to-I RNA editing has the potential to change gene expression and function. The effects of RNA editing on the pharmacokinetic-related genes have not yet been clarified. In this study, we investigated the possibility that ADARs regulate human P450 expression and activity in the liver.

We found that ADAR knockdown resulted in a decrease in CYP2B6 mRNA via enhancing mRNA stability (Fig. 3A). In REDportal, RNA

Fig. 6. Effects of ADAR1 or ADAR2 knockdown on the other P450 mRNA levels and activities in differentiated HepaRG cells. CYP1A2 (A), CYP2A6 (B), CYP2C9 (C), CYP2C19 (D), CYP2D6 (E), CYP2E1 (F), CYP3A4 (G), and CYP3A5 (H) mRNA levels in siADAR1- or siADAR2-transfected HepaRG cells were determined by real-time reverse transcription PCR and normalized to the β -actin levels. The values represent the levels relative to the siControl. Phenacetin *O*-deethylation (I), diclofenac 4'-hydroxylation (J), and bufuralol 1'-hydroxylation (K) in siADAR1- or siADAR2-transfected HepaRG cells were measured as the CYP1A2, CYP2C9, and CYP2D6 marker activities, respectively. Each column represents the mean $\pm$ S.D. of three independent experiments. * P < 0.05; ** P < 0.01; *** P < 0.001, compared with siControl.

editing sites are registered within the two Alu elements in the 3'-UTR of CYP2B6. It has been reported that ~90% of RNA editing sites are located in Alu elements in the human transcriptome (Picardi et al., 2017).

Because RNA editing in the 3'-UTR may be relevant to the changed stability of mRNA, we examined whether the 3'-UTR of CYP2B6 in HepaRG cells is actually subjected to RNA editing by direct sequencing.

However, no editing sites were detected in this region (data not shown). Recently, it has been reported that ADARs regulate gene expression in an editing-independent manner. For example, ADARs repressed the expression of methyltransferase such as 7A (METTL7A), a tumor suppressor gene, by promoting the processing of miR-27a that targets the 3'-UTR of METTL7A. This phenomenon was also observed in an ADAR mutant lacking enzymatic activity, indicating that ADARs regulate METTL7A in an editing-independent manner (Qi et al., 2017). In another paper, it was reported that ADAR2 enhances the stability of nuclear-retained, Cat2-transcribed nuclear RNA by limiting the access of the RNA binding protein HuR and poly(A) specific ribonuclease deadenylase (Anantharaman et al., 2017). It is worth investigating the possibility that CYP2B6 is regulated in an editing-independent manner. In addition, there is a possibility that ADARs regulate CYP2B6 at the transcriptional level as well as the post-transcriptional level because the alteration of CYP2B6 mRNA stability by knockdown of ADARs was somewhat small (Fig. 3A). It has been reported that CYP2B6 is transactivated by HNF4 α (Kamiyama et al., 2007), whose expression was decreased by knockdown of ADARs (Fig. 5D). Therefore, the regulation of HNF4 α by ADAR1 possibly contributes CYP2B6 expression.

We found that CYP2C8 expression and activity were decreased by ADAR knockdown (Fig. 4), and this alteration occurred in a transcriptional manner (Fig. 5A). It has been reported that CYP2C8 upstream has the response elements of peroxisome proliferator-activated receptor α , glucocorticoid receptor, and HNF4 α (Ferguson et al., 2005; Makia and Goldstein, 2016). The luciferase activity of the pGL3b/2C8-514 plasmid was decreased by ADAR1 or ADAR2 knockdown (Fig. 5B). When mutations were introduced in the response element of HNF4 α , the luciferase activity was not changed by the knockdown of ADAR1 or ADAR2 (Fig. 5B). Additionally, the HNF4 α protein level was also decreased by ADAR1 or ADAR2 knockdown (Fig. 5D). Thus, it was suggested that ADAR1 or ADAR2 positively regulates the expression of CYP2C8 through the upregulation of HNF4 α . Since the HNF4 α mRNA level was not changed by ADAR1 or ADAR2 knockdown (Fig. 5C), it was believed that ADARs may positively regulate HNF4 α expression in a post-transcriptional manner. According to REDIportal, HNF4 α mRNA does not have any RNA editing sites, indicating that ADARs indirectly regulate HNF4 α expression. Some miRNAs have been reported to regulate HNF4 α expression (Takagi et al., 2010; Ramamoorthy et al., 2012; Wang and Burke et al., 2013), and A-to-I changes in an miRNA transcript can alter its processing to cause changes in mature miRNA expression (Ekdahl et al., 2012; Vesely et al., 2012). Therefore, it is possible that ADARs regulate HNF4 α by changing the miRNA processing.

CYP2C8 plays a role in the metabolism of clinically used drugs, such as paclitaxel (Rahman et al., 1994), amodiaquine (Walsky and Obach, 2004), and amiodarone (Ohyama et al., 2000). Since the CYP2C8 activity was decreased by ADAR1 knockdown, the alteration of ADAR1 expression may influence the metabolism of these drugs; this results in alterations to the drug efficacy or causes adverse effects. There are large interindividual differences in the CYP2C8 protein level and activity (Naraharisetti et al., 2010), and the variability is partially attributed to the genetic polymorphism of CYP2C8. Among the 14 CYP2C8 alleles, CYP2C8*2, CYP2C8*3, and CYP2C8*4 are common alleles with amino acid changes. These alleles have been reported to contribute to alterations in CYP2C8 activity. For example, liver samples from heterozygotes of CYP2C8*3 showed significantly lower paclitaxel 6 α -hydroxylase activity compared with wild-type samples (Bahadur et al., 2002). In contrast, another research group reported no difference in the activities between two groups (Taniguchi et al., 2005). There is a large interindividual difference in the CYP2C8 metabolic activities among the

same genotype; therefore, it is possible that the interindividual variation could be due to factors other than genetic factors. The present study revealed that CYP2C8 expression was modulated by ADARs (Fig. 4). The ADAR-dependent regulation of CYP2C8 also contributes to the interindividual differences in CYP2C8.

Since the CYP2A6, 2B6, 2C9, 2C19, 2D6, 2E1, and 3A4 expression levels are also regulated by HNF4 α (Kamiyama et al., 2007; Chen et al., 2018), these P450 mRNA levels were evaluated. We found that the CYP2A6, 2C9, 2C19, 2D6, and 3A4 mRNA levels were decreased by ADAR1 or ADAR2 knockdown (Fig. 6, B–F), and these alterations were consistent with HNF4 α expression (Fig. 5E). Thus, HNF4 α could be involved in the decreases in these P450 mRNA levels by ADAR1 or ADAR2 knockdown. Unlike other P450 isoforms, the CYP3A4 mRNA levels were significantly increased by the knockdown of ADAR1 (Fig. 6G). The change was not consistent with that of HNF4 α expression. In a recent paper, it was shown that the contribution of HNF4 α in the regulation of CYP3A4 is smaller than that of the other P450 isoforms (Chen et al., 2018).

In conclusion, the present study has demonstrated that the RNA editing enzymes, ADARs, are novel factors regulating P450. Our previous study revealed a large interindividual variation (220-fold) in ADAR1 protein expression in human liver samples (Nakano et al., 2016). Therefore, the variation in ADAR1 expression could be one of the factors causing the interindividual variation in P450 expression.

Authorship Contributions

Participated in research design: Nozaki, Nakano, Fukami, Nakajima.

Conducted experiments: Nozaki.

Contributed new reagents or analytic tools: Nozaki, Iwakami.

Performed data analysis: Nozaki, Nakano, Nakajima.

Wrote or contributed to the writing of the manuscript: Nozaki, Nakano, Nakajima.

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