RNA Editing Modulates Human Hepatic Aryl Hydrocarbon Receptor Expression by Creating MicroRNA Recognition Sequence*

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Adenosine to inosine (A-to-I) RNA editing is the most frequent type of post-transcriptional nucleotide conversion in humans, and it is catalyzed by adenosine deaminase acting on RNA (ADAR) enzymes. In this study we investigated the effect of RNA editing on human aryl hydrocarbon receptor (AhR) expression because the AhR transcript potentially forms double-stranded structures, which are targets of ADAR enzymes. In human hepatocellular carcinoma-derived Huh-7 cells, the ADAR1 knockdown reduced the RNA editing levels in the 3′-untranslated region (3′-UTR) of the AhR transcript and increased the AhR protein levels. The ADAR1 knockdown enhanced the ligand-mediated induction of CYP1A1, a gene downstream of AhR. We investigated the possibility that A-to-I RNA editing creates miRNA targeting sites in the AhR mRNA and found that the miR-378-dependent down-regulation of AhR was abolished by ADAR1 knockdown. These results indicated that the ADAR1-mediated down-regulation of AhR could be attributed to the creation of a miR-378 recognition site in the AhR 3′-UTR. The interindividual differences in the RNA editing levels within the AhR 3′-UTR in a panel of 32 human liver samples were relatively small, whereas the differences in ADAR1 expression were large (220-fold). In the human liver samples a significant inverse association was observed between the miR-378 and AhR protein levels, suggesting that the RNA-editing-dependent down-regulation of AhR by miR-378 contributes to the variability in the constitutive hepatic expression of AhR. In conclusion, this study uncovered for the first time that A-to-I RNA editing modulates the potency of xenobiotic metabolism in the human liver.

RNA editing is a post-transcriptional process that alters the nucleotide sequence of RNA transcripts. Among the various types of RNA editing, adenosine-to-inosine (A-to-I) RNA editing is the most frequent type of RNA editing in mammals (1, 2).

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3 The abbreviations used are: A-to-I, adenosine to inosine, ADAR, adenosine deaminase acting on RNA; AhR, aryl hydrocarbon receptor; CYP, cytochrome P450; dsRNA, double-stranded RNA; miRNA, microRNA; MRE, microRNA recognition element; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

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To construct the ADAR1 p150 expression plasmid, the cDNA from exon 1 to exon 3 of human ADAR1 p150 was amplified by PCR using a reverse-transcribed product from human liver RNA, the forward primer 5'-ATG AAT CCG CAG CAG GGG TAT CTC GTT AG-3', and the reverse primer 5'-CAT CCT GCT TGG CCA CTG TCT GTC TCC CA-3'. This cDNA fragment was cloned into the pTARGET/ADAR1 p110 vector, resulting in pTARGET/ADAR1 p150.

The cDNA from exon 3 to exon 5 of human ADAR2 was amplified by PCR using a reverse-transcribed product from human liver RNA, the forward primer 5'-GTC AAG AAA CCC TCA AAA GT-3', and the reverse primer 5'-GAG AAG TTT TCG GTC AGG CTG-3'. This cDNA fragment was cloned into the pTARGET vector, resulting in the pTARGET/ADAR2(ex3-ex5) plasmid. The cDNA from exon 4 to exon 12 of human ADAR2 was amplified by PCR using a reverse-transcribed product from human liver RNA, the forward primer 5'-CTG TCT TAC CAC CAT TCC CA-3', and the reverse primer 5'-TCC CTC CCC AAG GTA TGC AC-3'. This cDNA fragment was cloned into the pTARGET vector, resulting in the pTARGET/ADAR2(ex4-ex12) plasmid. The ADAR2 cDNA from exon 4 to 12 was digested from pTARGET/ADAR2(ex4-ex12) and was cloned into pTARGET/ADAR2(ex3-ex5), resulting in pTARGET/ADAR2.

Transfection of Huh-7 Cells with siRNA, Expression Plasmid, and miRNA Mimics and Preparation of Cell Homogenates and Total RNA—Huh-7 cells were transfected with siRNA and miRNA mimics as follows; the day before transfection, Huh-7 cells were seeded into 6-well plates. After 24 h, the Huh-7 cells were transfected with 5 nm siRNA and/or miRNA mimics using Lipofectamine RNAiMAX. When the transfections were carried out with 1000 ng of the pTARGET vector, Lipofectamine 2000 was used instead of Lipofectamine RNAiMAX. After incubation for 48 h, the cells were harvested and resuspended in a small amount of TGE buffer (10 mM Tris-HCl, 20% glycerol, and 1 mM EDTA (pH 7.4)) and disrupted by freeze-thawing three times. Total RNA was prepared using RNAiso.

Human Liver and Preparation of Homogenates and Total RNA—Human liver samples from 19 donors were obtained from the Human and Animal Bridging (HAB) Research Organization (Chiba, Japan), which is in partnership with the National Disease Research Interchange (NDRI, Philadelphia, PA). Samples from 13 additional donors were obtained from autopsy materials that were discarded after pathological investigation. The use of the human livers was approved by the Ethics Committees of Kanazawa University (Kanazawa, Japan) and Iwate Medical University (Morioka, Japan). Homogenates were prepared from the human liver samples by homogenization with lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, and 1% Nonidet P-40) containing protease inhibitors (0.5 mM; p-aminophenyl)methanesulfonyl fluoride, 2 μg/ml aprotinin, and 2 μg/ml leupeptin). The protein concentration was determined using the Bradford protein assay reagent (Bio-Rad) with γ-globulin as the standard. Total RNA was prepared using RNAiso, and the integrity was assessed by estimating the ratio of the band densities of the 28S and 18S rRNA.

SDS-PAGE and Western Blot Analysis—For the analysis of the AhR, ADAR1, and GAPDH protein levels, cell homogenates

enzymes, including cytochrome P450 (CYP) 1A1, 1A2, and 1B1 (13). In the present study we investigated whether ADARs in the human liver can modulate AhR expression and subsequently affect the potency of xenobiotic metabolism by altering miRNA recognition.

**Experimental Procedures**

*Chemicals and Reagents—*2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) was obtained from Cambridge Isotope Laboratories (Cambridge, MA). The plg3L-promoter vector, pHL-R-TK vector, pTARGET vector, and Dual-Luciferase Reporter Assay System were purchased from Promega (Madison, WI). Lipofectamine 2000, Lipofectamine RNAiMAX, Silencer Select siRNA for human ADAR1 (s1007) (siADAR1), human ADAR2 (s1010) (siADAR2), and negative control #1 and miRNA mimics for miR-29a, miR-140, miR-378, and negative control #1 were purchased from Life Technologies. RNAliso, random hexamer, and SYBR Premix Ex Taq were from Takara (Shiga, Japan). ROX was purchased from Stratagene (La Jolla, CA). ReverTra Ace was purchased from Toyobo (Osaka, Japan). All of the primers were commercially synthesized at RIKAKEN (Nagoya, Japan). The rabbit anti-human AhR polyclonal antibody (sc-5579), mouse anti-human ADAR1 monoclonal antibody (sc-73408), and mouse anti-human ADAR2 monoclonal antibody (sc-73409) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit anti-human GAPDH polyclonal antibody (IMG-5143A) was purchased from IMGENIX (San Diego, CA). IRDye 680 goat anti-rabbit IgG and goat anti-mouse IgG were from LI-COR Biosciences (Lincoln, NE). Restriction enzymes were from New England BioLabs (Ipswich, MA). All other chemicals and solvents were of the highest grade commercially available.

*Cell Cultures—*The human hepatocellular carcinoma-derived cell line Huh-7 was obtained from Riken Gene Bank (Tsukuba, Japan). The HeLa human cervical carcinoma-derived cell line was obtained from American Type Culture Collection (Manassas, VA). Huh-7 and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. All of the cells were maintained at 37 °C under an atmosphere of 5% CO₂ and 95% air.

*Expression Plasmid Construction—*The cDNA from exon 2 to exon 11 of human ADAR1 p110 was amplified by PCR using a reverse-transcribed product from human liver RNA, the forward primer 5’-ATG GCC GAG ATC AAG GAG AA-3’, and the reverse primer 5’-CTG TAG AGA AAC CTG ATG AAG CC-3’. This cDNA fragment was cloned into the pTARGET vector, resulting in the pTARGET/ADAR1 p110(ex2-ex11) plasmid. The cDNA from exon 7 to exon 14 of human ADAR1 p110 was amplified by PCR using a reverse-transcribed product from human liver RNA, the forward primer 5’-GCT TGG GAA CAG GGA ATC G-3’, and the reverse primer 5’-CTA TAC TGG GCA G AAG ATA AA-3’. This cDNA fragment was cloned into the pTARGET vector, resulting in the pTARGET/ADAR1 p110(ex7-ex14) plasmid. The ADAR1 p110 cDNA from exon 7 to 14 was digested from pTARGET/ADAR1 p110(ex7-ex14) and was cloned into pTARGET/ADAR1 p110(ex2-ex11), resulting in pTARGET/ADAR1 p110.
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from Huh-7 cells (20 μg) were separated by 7.5% SDS-PAGE and transferred to an Immobilon-P transfer membrane (Millipore, Billerica, MA). For the analysis of the ADAR2 protein levels, cell homogenates from Huh-7 cells (50 μg) were separated by 7.5% SDS-PAGE and transferred to a Protran nitrocellulose membrane (Whatman GmbH, Dassel, Germany). The membranes were probed with rabbit anti-human AhR polyclonal, mouse anti-human ADAR1 monoclonal, mouse anti-human ADAR2 monoclonal, or rabbit anti-human GAPDH polyclonal antibodies and the corresponding fluorescent dye-conjugated secondary antibodies. The band densities were quantified with an Odyssey Infrared Imaging system (LI-COR Biosciences). The AhR, ADAR1, and ADAR2 protein levels were normalized to GAPDH.

Real-time RT-PCR for AhR—cDNA was synthesized from total RNA using ReverTra Ace. The primers for human AhR and CYP1A1 were described previously (14). A 1-μl portion of the reverse transcription mixture was added to a PCR mixture containing 10 pmol of each primer, 12.5 μl of the SYBR Premix Ex Taq solution, and 75 nM ROX in a final volume of 25 μl. The PCR conditions were as follows; after an initial denaturation at 95 °C for 30 s, amplification was performed by denaturation at 94 °C for 4 s, annealing, and extension at 62 °C for 20 s for 40 cycles. Real-time PCR was performed using the Mx3000P (Stratagene) with the MxPro QPCR software. The AhR and CYP1A1 mRNA levels were normalized to those of GAPDH as described previously (15).

Assessment of RNA Editing Levels in the AhR 3′-UTR—For the RNA editing analysis, direct sequencing was performed on PCR products. The AhR 3′-UTR has an antisense-oriented AluSx and a sense-oriented AluSc. The two Alu elements were individually amplified by PCR using cDNA or genomic DNA as a template. The following primer sets were used to amplify the AluSx or AluSc: AluSx sense (5′/H11032-GAG AGC AAG GTT TGG TGC-3′) and AluSx antisense (5′/H11032-GCT CTT CAG CTC TCA TAT CT-3′) or AluSc sense (5′/H11032-CTG AAG TGC TTA GAC ACA TT-3′) and AluSc antisense (5′/H11032-CCT CAT GCT GGA AAC AAA TT-3′). The PCR mixture contained the cDNA (or genomic DNA), 1 × PCR buffer (67 mM Tris-HCl, pH 8.8, 16 mM (NH₄)₂SO₄, 0.45% Triton X-100, and 0.02% gelatin), 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.4 μM concentrations of each primer, and 0.5 units of Taq polymerase (Greiner, Tokyo, Japan) in a final volume of 25 μl. After an initial denaturation at 94 °C for 3 min, amplification was performed by denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 15 s for 35 cycles followed by a final extension at 72 °C for 5 min. The PCR product was subjected to electrophoresis using 2% agarose gel. Control experiments without the reverse transcriptase were conducted to verify that the amplified products were from the reverse-transcribed cDNA rather than from contaminating genomic DNA. Specific products were gel-purified and subjected to direct sequencing. The extent of editing is represented as a percentage calculated from the ratio of the peak height of G over the sum of the peak heights of G and A in the sequencing electropherograms.

Reporter Plasmid Construction—To construct the luciferase reporter plasmids, a fragment from +5168 to +5226 of the AhR 3′-UTR containing the microRNA recognition element (MRE) for miR-378 was amplified by PCR using Huh-7 cDNA and was inserted into the pGL3p vector at the Xbal site downstream of the luciferase gene. DNA sequencing analyses were performed to determine whether the plasmids carried unedited (adenosine) or edited (guanosine) nucleosides at the editing sites. Such plasmids were called pGL3p/MRE(A) and pGL3p/MRE(G), respectively. The pGL3p/c-miR-378 vector, which has a sequence that perfectly matches that of the mature miR-378, was constructed previously (16).

Luciferase Assay—HeLa cells were transiently transfected with various pGL3 luciferase reporter plasmids and the phRL-TK plasmid. Briefly, the day before the transfection the cells were seeded into 24-well plates. After 24 h, the cells were transfected with 190 ng of the pGL3 plasmid, 10 ng of the phRL-TK plasmid, and 5 nM concentrations of the miR-378 mimic or control using Lipofectamine 2000. After incubation for 48 h, the cells were resuspended in passive lysis buffer, and luciferase activity was measured on a luminometer using the Dual-Luciferase Reporter Assay System.

Determination of Mature miR-378 Levels in Human Livers—The expression levels of mature miR-378 in a panel of 32 human livers were determined using the TaqMan microRNA assay (Applied Biosystems, Foster City, CA) as reported previously (17). The expression levels of miR-378 were normalized to U6 small nuclear RNA (U6 snRNA) levels.

Statistical Analyses—Statistical significance was determined by analysis of variance followed by Dunnett’s multiple comparisons test or Tukey’s method test. The comparison of two groups was made with an unpaired, two-tailed Student’s t test. Correlation analyses were performed by Spearman’s rank method. A value of p < 0.05 was considered statistically significant.

Results

Effect of ADAR1 or ADAR2 Knockdown on AhR Expression—To investigate whether ADAR1 or ADAR2 regulates human AhR expression, ADAR1 or ADAR2 was knocked down in Huh-7 cells by RNA interference. The siADAR1 used in this study targets both ADAR1 p110 and ADAR1L p150. After transfection with siADAR1 and siADAR2, the ADAR1 p110 and ADAR2 protein levels were significantly (p < 0.001) decreased (20 and 23% of the siControl, respectively) (Fig. 1, A and B). The ADAR1 p150 protein was not detected by Western blot analysis. The knockdown of ADAR1 resulted in a significant (p < 0.05) increase in AhR protein levels (2.1-fold over siControl), but not mRNA levels, whereas the silencing of ADAR2 did not affect expression at any level (Fig. 1, C and D). These results suggest that ADAR1 negatively regulates AhR expression in a post-transcriptional manner.

Effect of ADAR1 or ADAR2 Knockdown on RNA Editing Levels in the AhR 3′-UTR—The RADAR database of RNA editing sites indicates the potential for RNA editing in the AhR 3′-UTR. To investigate whether AhR is actually subjected to RNA editing, we performed sequence analyses of AhR genomic DNA and cDNA from Huh-7 cells. A-to-I RNA-edited sites were detected as A-to-G changes. We found 38 edited sites within the Alu elements in the AhR 3′-UTR (Fig. 2). To investigate whether the ADAR1 or the ADAR2 enzyme is responsible for the RNA
editing events, we analyzed the sequence of the AhR cDNA from Huh-7 cells transfected with siADAR1 or siADAR2. The RNA editing level of almost all of the editing sites was reduced by ADAR1 knockdown but not by ADAR2 knockdown (Fig. 1E). Typical sequencing electropherograms of these RNA-edited sites are shown in Fig. 1F. These results indicated that ADAR1 plays a critical role in editing AhR mRNA.

Effect of ADAR1 or ADAR2 Overexpression on AhR Protein and RNA Editing Levels—To further investigate the role of RNA editing in AhR expression, ADAR1 p110, ADAR1 p150, and...
ADAR2 were overexpressed in Huh-7 cells by transfection with expression plasmid. ADAR1 p110, ADAR1 p150, and ADAR2 were successfully overexpressed (Fig. 3, A and B), but the AhR mRNA and protein levels were not changed (Fig. 3, C and D). Through sequencing analysis it was demonstrated that the RNA editing levels were mildly increased by the overexpression of the ADAR isoforms (Fig. 3, E and F). These results suggest that the endogenous levels of ADAR1 are sufficient for the editing of the AhR transcript.

**Effect of ADAR1 Silencing on the Induction of Genes Downstream of AhR**—We investigated whether the ADAR1-mediated down-regulation of AhR might affect the induction of genes downstream of AhR. Treatment of Huh-7 cells with 10 nM TCDD, a ligand of AhR, resulted in a significant ($p < 0.01$) increase in CYP1A1 mRNA levels (3.6-fold). When ADAR1 was knocked down, an enhanced induction of CYP1A1 mRNA (6.0-fold) was observed. It was confirmed that the AhR protein levels were increased by the silencing of ADAR1 in the presence of DMSO (2.0-fold over siControl), whereas the AhR protein levels in the TCDD-treated cells were lower than in control cells (Fig. 4). The latter phenomenon could be explained by the fact that the degradation of the AhR protein is accelerated by ligand binding through the ubiquitin-proteasome pathway (18, 19). Collectively, these data demonstrated that ADAR1 affects the induction of genes downstream of AhR.

**RNA Editing of the AhR Transcript Creates miRNA Target Sites**—We surmised that ADAR1 negatively regulates AhR expression by creating miRNA target sites in the 3′-UTR of AhR mRNA. *In silico* analysis using miRediTar predicted miR-29a, miR-140, and miR-378 as candidate miRNAs whose binding affinity to the edited sequence in the AhR 3′-UTR was predicted to be higher than their affinity to the unedited sequence (Fig. 5A). Transfection of Huh-7 cells with miR-29a mimics significantly ($p < 0.01$) decreased the AhR mRNA levels (40% of miControl), but transfection with miR-140 or miR-378 mimics had no effect (Fig. 5B). The AhR protein levels were significantly ($p < 0.05$) decreased by the overexpression of miR-29a and miR-378 (45 and 53% of miControl, respectively) but were not changed by miR-140 (Fig. 5C). To investigate whether the miR-29a- or miR-378-dependent down-regulation of AhR

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**FIGURE 3. Effect of the overexpression of ADAR1 p110, ADAR1 p150, or ADAR2 on AhR expression.** ADAR1 p110 and ADAR1 p150 protein (A), ADAR2 protein (B), AhR mRNA (C), and AhR protein (D) levels in Huh-7 cells 48 h after transfection with 1000 ng of pTARGET/Empty, pTARGET/ADAR1 p110, pTARGET/ADAR1 p150, or pTARGET/ADAR2. Protein and mRNA levels were determined by Western blot or real-time RT-PCR, respectively, and were normalized to GAPDH. The values represent the levels relative to pTARGET/Empty or pTARGET/ADAR1 p150. ND, not detectable. E, RNA editing levels in the AhR 3′-UTR in Huh-7 cells after transfection with 1000 ng of pTARGET/Empty, pTARGET/ADAR1 p110, pTARGET/ADAR1 p150, or pTARGET/ADAR2. The editing level is represented as a percentage, which was calculated by dividing the peak height of G by the sum of peak heights of G and A in the electropherograms resulting from the direct sequencing. A RNA editing site is shown by numbered arrows. The numbers represent the RNA editing sites in the AhR 3′-UTR indicated in Fig. 2. Each column represents the mean ± S.D. of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. **
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AhR expression in Huh-7 cells was knocked down with siADAR1 and either miR-29a or miR-378 mimics. The AhR mRNA (Fig. 5D) and protein (Fig. 5E) levels were significantly (p < 0.05) reduced (27–51% of miControl) by transfection with miR-29a mimic regardless of whether ADAR1 was knocked down. Interestingly, the overexpression of miR-378 significantly (p < 0.05) decreased (50% of miControl) the AhR protein levels, and the miR-378-dependent down-regulation of AhR was abolished by the knockdown of ADAR1 (Fig. 5E). These results indicated that miR-378 and miR-29a regulate AhR expression in a RNA editing-dependent and -independent manner, respectively. To further examine whether RNA editing affects the binding of miR-378 to the AhR 3′-UTR, we performed luciferase assays using reporter plasmids carrying the edited sequence (pGL3p/MRE(G)). The luciferase activity of the pGL3p/MRE(G) was slightly (78% of control) but significantly (p < 0.05) reduced by the overexpression of miR-378, whereas the luciferase activity of the pGL3p/MRE(A) plasmid was not affected (Fig. 5F), indicating that miR-378 recognizes the edited sequence within the AhR 3′-UTR.

ADAR Protein Levels, AhR RNA Editing and Protein Levels, and miR-378 Levels in Human Livers—We sought to examine the variability in the ADAR protein levels in human liver samples. ADAR1, but not ADAR1p150 or ADAR2, was detected by Western blot analysis (Fig. 5A). A large interindvidual difference (220-fold) in the ADAR1 p110 protein levels was observed in the panel of 32 human liver samples. To investigate whether the large interindividual difference affects the RNA editing levels in the AhR 3′-UTR, we performed direct sequencing using cDNA samples from the human liver samples numbered 1 (which had the lowest ADAR1 p110 protein levels), 6, 16, and 21 (which had the highest ADAR1 p110 protein levels). The RNA editing levels tended to be correlated with ADAR1 p110 expression levels in some RNA editing sites. However the interindvidual difference in the RNA editing levels was relatively small compared with that in ADAR1 expression levels (Fig. 6B). To investigate whether the small interindividual variability in RNA editing levels in the human livers was specific for AhR, we examined the RNA editing levels in filamin B, β, which has been reported to be edited by ADAR1 (11). The RNA editing levels were correlated with ADAR1 p110 expression, and the interindividual variability was relatively large compared with that of AhR (Fig. 6, C and D). These results suggest that low ADAR1 expression levels in the livers would be sufficient to edit the AhR 3′-UTR.

Next we examined the relationship between the expression levels of AhR mRNA, protein, and miR-378 and in 32 individual human liver samples. The AhR protein levels were not positively correlated with the AhR mRNA levels (Rs = 0.14) (Fig. 6E), indicating the involvement of post-transcriptional regulation. Interestingly, as shown in Fig. 6F, the miR-378 levels (46-fold) were inversely correlated with the AhR protein levels (Rs = 0.57, p < 0.001). Collectively, these results suggest that interindividual differences in the RNA editing levels in the AhR transcript are small but that miR-378 has a significant impact on AhR down-regulation and is thus one of the causal factors of the interindividual variability in AhR expression in human livers.

Discussion

A-to-I RNA editing is a post-transcriptional modification, which by causing a discrepancy between genomic DNA and its transcript, contributes to the diversity of the transcriptome. A-to-I modification in the coding regions of miRNAs can lead to functional alterations of the encoded protein (20, 21). However, most A-to-I RNA editing occurs in non-coding regions, such as the UTR and miRNA transcripts (22–24). It is becoming clear that RNA editing can play an important role in the regulation of the RNA interference machinery. miRNAs are transcribed in the nucleus by RNA polymerase II as long primary transcripts (pri-miRNAs) containing a stem-loop structure. The pri-miRNAs are subsequently cleaved into 70–100-nucleotide precursors (pre-miRNAs). After they are exported into the cytoplasm, pre-miRNAs undergo secondary cleavage, leading to mature miRNA duplexes. The stem-loop structures of the pri-miRNAs make them favorable targets for ADARs. A-to-I editing events can alter the processing of miRNA, thereby affecting miRNA expression (25, 26). In other cases, A-to-I editing of the miRNA seed sequence could change its target selection or binding efficiency (27). In this study we sought to examine whether the editing of target mRNA, rather than miRNA, might affect mRNA:miRNA binding by altering seed matches. The possibility that miRNA target sites can be created or deleted by RNA editing has been proposed (23, 28), but studies supporting this hypothesis are very limited (29, 30).

We found that the AhR 3′-UTR in human liver and human hepatocarcinoma-derived cells is predominantly subjected to
RNA editing by ADAR1, rather than ADAR2 (Figs. 1E, 2, and 6B). Because the AhR 3′-UTR has inverted Alu repeats that appear to form matched dsRNA structures, our finding is consistent with a previous report that ADAR2 prefers selective sites in dsRNA containing mismatches, bulges, and internal loops, whereas ADAR1 promiscuously targets adenosines in perfectly base-paired dsRNA (31). Recently, it was revealed that the RNA editing levels within reversely oriented Alu repeats are negatively correlated with the distance between two Alu elements (32). The distance between the two Alu elements in the AhR 3′-UTR is relatively short (107 bp). Considering that together with the fact that the two Alu elements can form highly matched dsRNA structures, AhR mRNA could be a good substrate of the ADAR enzyme. On the other hand, the RNA editing site in filamin B, β is not located in Alu element. In addition, in silico analysis reveals that the RNA sequence of filamin B, β forms a poorly matched and shorter double strand structure than that of AhR, implying that filamin B, β forms mRNA would be a less favorable substrate of the ADAR enzymes. These RNA structural features would explain why the AhR transcript was highly edited, even in the liver samples with very low ADAR1 level (Fig. 6, A and B).

The overexpression of ADAR2 significantly increased the RNA editing levels in 15 of 38 editing sites (no. 4, 9, 10, 11, 14, 19, 20, 21, 23, 30, 31, 32, 33, 34, and 38), although the editing levels in most of the sites were reduced by ADAR1 knockdown (Figs. 1E and 3E). ADAR1 and ADAR2 have distinct but overlapping target specificities (6, 33). In addition to the RNA structural features, the overexpression of ADAR2 significantly increased the RNA editing levels in 15 of 38 editing sites.
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The surrounding nucleotides have some influence on recognition by ADARs. ADAR1 has a 5’ neighbor preference (A = U > C > G) but no apparent 3’ neighbor preference (34). The 5’ neighbor preference of ADAR2 (A = U > C = G) is similar to that of ADAR1; however, ADAR2 has a 3’ neighbor preference (U = G > C = A) (35). 10 of the above 15 RNA editing sites in the AhR 3′-UTR have U or G as the 3′ neighbor (Fig. 2). Therefore, ADAR1 and ADAR2, both, would edit the AhR 3′-UTR. Although a previous study (11) reported that ADAR2 is functionally expressed in normal human livers, hepatic ADAR2 protein was not detected in this study even when an antibody from another vendor was used (data not shown). Because of the lower expression level of ADAR2 compared with ADAR1, the role of ADAR2 in RNA editing in human livers may be only minor.

By computer analysis, miR-29a, miR-140, and miR-378 were predicted to bind to the edited sequences in the AhR 3′-UTR (Fig. 5A). Through co-transfection of Huh-7 cells with miRNA mimics and siADAR1, it was revealed that miR-29a and miR-378 regulate AhR expression in a RNA editing-independent and dependent manner, respectively (Fig. 5, D and E). Using a luciferase assay, it was demonstrated that the A-to-I (artificially G) conversion at editing site 22 was functional and created the binding site for miR-378 (Fig. 5F). No other miR-378 MRE was predicted in the unedited AhR mRNA. These results suggest that the miR-378-dependent down-regulation of AhR requires RNA editing. The AhR 3′-UTR is highly edited not only in Huh-7 cells but also in normal human livers (Fig. 6B), implying that hepatic AhR expression can be regulated by miR-378. This
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**Human AhR mRNA**

![Diagram of human AhR mRNA](image)

**AhR mRNA**

| +1 | +614 | +3160 | +4718 | +5023 | +5131 | +5422 | +6247 |
|----|------|-------|-------|-------|-------|-------|-------|

**MRE29a**

- +3631
- 3’-UUUAAAAAUAAGGGUUGGGCCU-G-3’

**miR-29a**

- 3’-AUUGGCUAAGGGCUACCAGAU-5’

**FIGURE 7. Schematic representation of the human AhR mRNA and a predicted MRE of miR-29a in unedited AhR mRNA.** The numbering denotes the 5’ end of the mRNA as 1. Open arrows indicate Alu elements and directions. The MREs of miR-29a is located in the 3’-UTR of the human AhR mRNA (from +3631 to +3653). Bold letters represent seed sequences.

is supported by the negative correlation between miR-378 and AhR expression in the panel of 32 human livers (Fig. 6F). miR-378 has been shown to function as an oncogene in liver cancer (36). In addition, miR-378 is functionally expressed in normal liver, where it is reported to regulate lipid metabolism (37) and CYP2E1-mediated xenobiotic metabolism (16, 17). The present study adds new insight into the role of miR-378 in the human liver. As for miR-29a, although we did not identify a functional MRE, *in silico* analysis predicted a potential MRE lacking a RNA editing site upstream of the Alu elements (Fig. 7). During the preparation of this manuscript, an independent study reported that the human AhR 3’-UTR was directly recognized by miR-29a, although a functional MRE was not identified (38). miR-29 family members, including miR-29a, are known to be down-regulated in human fibrotic livers (39). Recently, it was reported that activation of AhR induces hepatic fibrosis by directly regulating pro-fibrotic pathways (40). It would be of interest to examine the role of miR-29a-mediated AhR regulation in the development of hepatic fibrosis.

Finally, we sought to clarify the significance of the RNA editing-mediated regulation of AhR expression. AhR is responsible for the transcriptional regulation of xenobiotic-metabolizing enzymes, such as the CYP1 isoforms, UDP-glucuronosyltransferases, and glutathione S-transferases. Our study demonstrated that the regulation of AhR expression by RNA editing affects the induction of its downstream target, CYP1A1 (Fig. 4). It is known that CYP1A1 levels are associated with the levels of AhR and its heterodimeric partner, the AhR nuclear translocator (41). Therefore, RNA editing is one of the key factors regulating the expression of xenobiotic-metabolizing enzymes. AhR plays important roles not only in regulation of xenobiotic-metabolizing enzymes but also in tumor initiation, promotion, and progression (42). Accumulating evidence suggests that disrupted RNA editing or abnormal ADAR expression is associated with cancer (10). It is possible that the altered ADAR expression in cancer affects AhR expression by changing the levels of AhR mRNA editing.

In summary, we found that A-to-I RNA editing regulates the expression of AhR by creating miR-378 binding sites. This mechanism contributes to the interindividual variability in AhR expression in human livers. This study is the first to prove that RNA editing modulates the potency of xenobiotic metabolism in the human liver.

**Author Contributions**—Ma, N., T. F., S. G., and Mi, N. designed the research. Ma, N. conducted most of the experiments and analyzed the results. M. T. and Y. A. provided human liver samples. Ma, N. and Mi, N. wrote the paper.

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