Dissertation

Regulation of human aryl hydrocarbon receptor and dihydrofolate reductase expression by RNA editing

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ABBREVIATIONS

A	Adenosine
ADAR	Adenosine deaminases acting on RNA
AhR	Aryl hydrocarbon receptor
AsO	Antisense locked nucleic acid/DNA oligonucleotides
A-to-I	Adenosine-to-inosine
C	Cytidine
CCK-8	Cell counting kit-8
cDNA	Complementary deoxyribonucleic acid
CYP	Cytochrome P450
DHFR	Diydrofolate reductase
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded ribonucleic acid
EDTA	Ethylenediamine-*N,N,N',N'*-tetraacetic acid
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G	Guanosine
IgG	Immunogloblin G
I	Inosine
mRNA	Messenger ribonucleic acid
miRNA	MicroRNA
MRE	MicroRNA recognition element
NADPH	Nicotinamide adenine dinucleotide phosphate
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
Pre-miRNA	Precursor microRNA

Pri-miRNA	Primary microRNA
RNA	Ribonucleic acid
RT	Reverse transcription
SD	Standard deviation
SDS	Sodium dodecyl sulfate
siRNA	Small interfering RNA
snRNA	Small nuclear RNA
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
Tris	Tris(hydroxymethyl)aminomethane
WST-8	2-(2-Methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt
U	Uridine
UTR	Untranslated region

CHAPTER 1

General introduction

RNA editing is a post-transcriptional process that alters the nucleotide sequence of RNA transcripts. In animals, the most common type of RNA editing is deamination of adenosine (A) into inosine (I), A-to-I RNA editing (Wagner et al., 1989; Gallo and Galardi, 2008). Since much of the cellular machinery treats 'I' as a guanine (G) nucleotide, the conversion of nucleotides possibly changes amino acid sequence, splicing, microRNA (miRNA) targeting or miRNA maturation (Farajollahi and Maas, 2010). A-to-I RNA editing is catalyzed by adenosine deaminases acting on RNA (ADAR) enzymes (Kim et al., 1994; Gerber et al., 1997). They convert 'A' in double-stranded RNA (dsRNA) structures into 'I' by hydrolytic deamination (Fig. 1). There are three members of the ADAR family in vertebrates: ADAR1, ADAR2, and ADAR3 (also called ADAR, ADARB1, and ADARB2, respectively) (Bass et al., 1997). ADAR1 and ADAR2 are ubiquitously expressed in various tissues and have RNA editing activity. Whereas, there is no evidence to support the enzymatic activity of ADAR3. (Nishikura, 2010). The *ADAR1* gene produces two protein isoforms, ADAR1 p110 (110 kDa protein) and ADAR1 p150 (150 kDa protein) from different transcription initiation sites and start codons. The former is constitutively expressed in the nucleus, whereas the latter is localized in both nuclear and cytoplasmic, and induced by interferon (Patterson and Samuel, 1995; Desterro et al., 2003).

In early studies, it was revealed that RNA editing plays important roles in the central nervous system (Tariq and Jantsch, 2012). For example, glutamate receptor subtype A2 and 5-hydroxytryptamine receptor subtype 2C were known to be subjected to RNA editing (Sommer et al., 1991; Lomeli et al., 1994; Burns et al., 1997), and the disruption of RNA editing in these genes lead to amyotrophic lateral sclerosis and Prader-Willi syndrome, respectively (Kawahara et al., 2004; Morabito et al., 2010). In

these cases, A-to-I editing occurs within exons, changing crucial amino acids for protein function. In recent years, global RNA editing sites were identified by the availability of next generation sequencing-based RNA-Seq in normal and cancer tissues (Liu et al., 2014). The information is compiled in some databases such as RADAR (http://rnaedit.com/). However, the biological significance of RNA editing in humans has not been completely elucidated.

It became apparent that disrupted RNA editing or abnormal ADAR expression are associated with several diseases, including cancer, neurological disorders, metabolic diseases, viral infections, and autoimmune disorders (Slotkin and Nishikura, 2013). However, the biological significance of RNA editing in pharmacokinetics and pharmacodynamics is not fully understood. This study sought to clarify the role of RNA editing in drug response. In **chapter 2**, it was investigated whether RNA editing can affect aryl hydrocarbon receptor (AhR) and its downstream cytochrome P450 (CYP) expression in human liver. In **chapter 3**, the possibility that RNA editing modulates dihydrofolate reductase (DHFR) expression and subsequently affects the cell proliferation and sensitivity to methotrexate of breast cancer cells was examined.

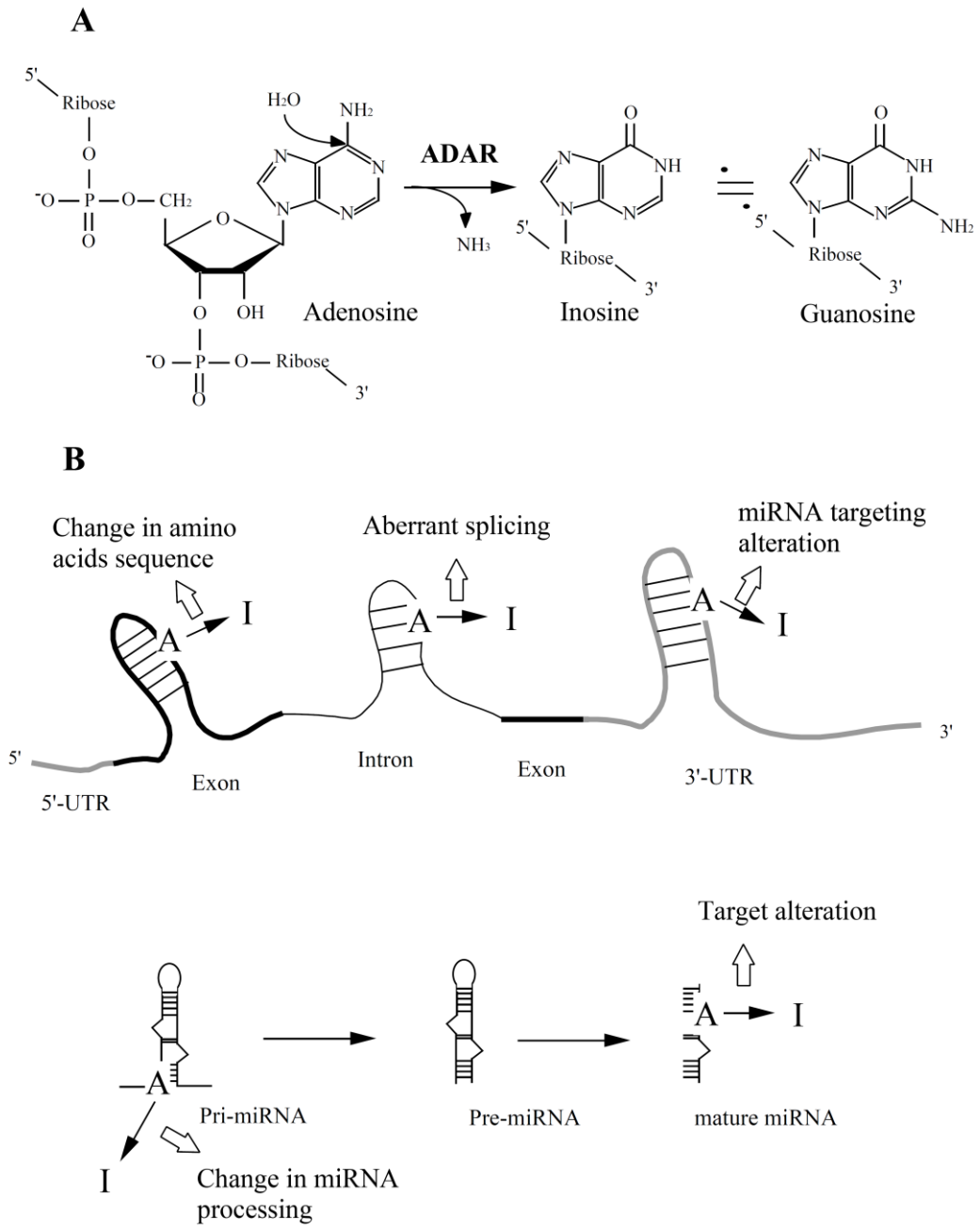

Fig. 1. A-to-I RNA editing. (A) ADAR enzymes convert 'A' to 'I' by hydrolytic deamination. Inosine was recognized by cellular machinery as if it was guanosine. (B) A-to-I editing occurs in dsRNA and possibly changes amino acid sequence, splicing, miRNA targeting or miRNA maturation.

CHAPTER 2

Down-regulation of human aryl hydrocarbon receptor expression by RNA editing in human liver

ABSTRACT

A-to-I RNA editing is the most frequent type of post-transcriptional nucleotide conversion in humans, and it is catalyzed by ADAR enzymes. In this study, the effect of RNA editing on human AhR expression was investigated because the AhR transcript potentially forms double-stranded structures, which are targets of ADAR enzymes. In human hepatocellular carcinoma-derived Huh-7 cells, the knockdown of ADAR1 reduced the RNA editing levels in the 3'-untranslsted region (3'-UTR) of AhR transcript and increased the AhR protein levels. Interestingly, the knockdown of ADAR1 enhanced the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-mediated induction of CYP1A1, a gene downstream of AhR. It was investigated the possibility that A-to-I RNA editing creates miRNA targeting sites in the AhR mRNA, and it was found that the miR-378-dependent down-regulation of AhR was abolished by the knockdown of ADAR1. 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 3'-UTR of AhR. The inter-individual differences in the RNA editing levels within the 3'-UTR of AhR in a panel of 32 human liver samples were relatively small, whereas the differences in ADAR1 expression were large (220-fold). In the panel of human livers, a significant inverse association was observed between the miR-378 and AhR protein levels. These results suggested that the RNA editing-dependent down-regulation of AhR by miR-378 contributes to the variability in the constitutive expression of AhR in the human liver. 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.

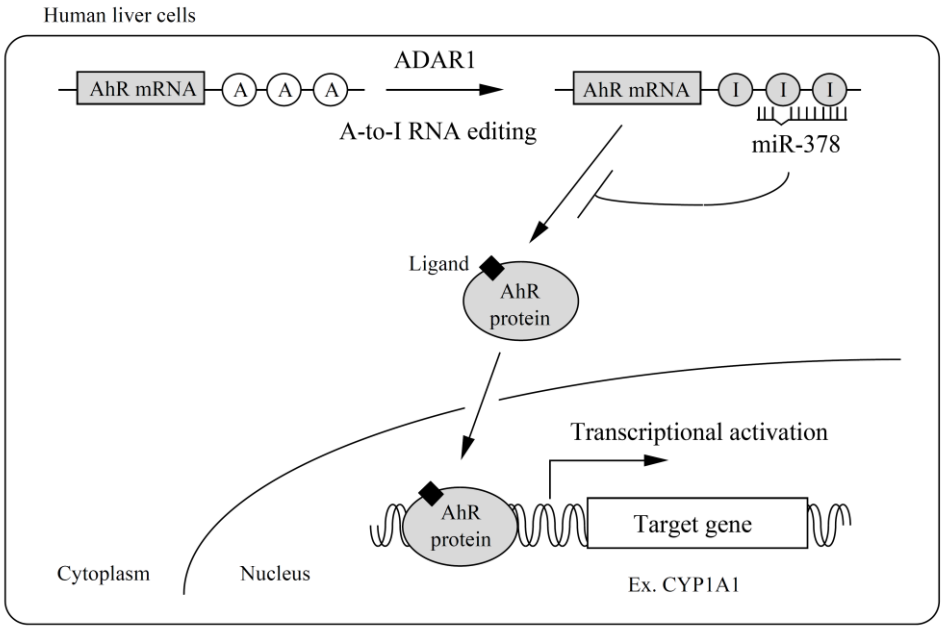

Fig. 2. Graphical abstract of chapter 2. ADAR1 down-regulates AhR expression in human liver cells by creating a miR-378 targeting site through A-to-I RNA editing. The down-regulation of AhR affects ligand-mediated induction of downstream gene of AhR.

INTRODUCTION

As for RNA editing, early studies were mainly for central nervous system (Tariq and Jantsch, 2012). There is little information about the significance of RNA editing in the liver. This study investigated the extent of the inter-individual variability in ADAR expression in normal human livers and its impact on the expression or function of drug metabolizing-related genes, focusing on AhR. The RADAR database identified RNA editing sites within its inverted Alu repeats, which can form dsRNA structures, in the 3'-UTR of human AhR. Because the 3'-UTR generally includes binding sites for miRNA, which silences gene expression via translational repression or mRNA degradation (Bartel, 2004), it is possible that RNA editing creates or destroys miRNA binding site(s), thereby altering expression of target mRNA. AhR is a ligand-activated transcription factor that regulates the expression of xenobiotic-metabolizing enzymes, including CYP1A1, CYP1A2, and CYP1B1 (Ramadoss et al., 2005). In the present study, it was investigated whether ADARs in the human liver can modulate AhR expression and subsequently affect the potency of xenobiotic metabolism by altering miRNA recognition.

MATERIALS AND METHODS

Chemicals and Reagents. TCDD was purchased from Cambridge Isotope Laboratories (Cambridge, MA). The pGL3-promoter vector, phRL-TK vector, pTARGET vector and Dual-Luciferase Reporter Assay System were obtained 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 obtained from Life Technologies (Carlsbad, CA). RNAiso, random hexamer, and SYBR Premix Ex Taq were purchased from Takara (Shiga, Japan). ROX was from Stratagene (La Jolla, CA). ReverTra Ace was obtained from Toyobo (Osaka, Japan). All of the primers were commercially synthesized at RIKAKEN (Nagoya, Japan). Rabbit anti-human AhR polyclonal antibody, mouse anti-human ADAR1 monoclonal antibody, and mouse anti-human ADAR2 monoclonal antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit anti-human GAPDH polyclonal antibody was from IMGENIX (San Diego, CA). IRDye 680 goat anti-rabbit IgG and goat anti-mouse IgG were obtained from LI-COR Biosciences (Lincoln, NE). Restriction enzymes were from New England Biolabs (Ioswich, MA). All other chemicals and solvents were of the highest grade commercially available.

Cell Cultures. Huh-7, the human hepatocellular carcinoma-derived cell line, was obtained from Riken Gene Bank (Tsukuba, Japan). HeLa, the human cervical carcinoma-derived cell line, was obtained from American Type Culture Collection (Rockville, MD). Huh-7 and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). All of the cells were maintained at 37°C under an atmosphere of 5% CO_2 and 95% air.

Expression Plasmid Construction. The cDNA from exon 2 to exon 14 of human

ADAR1 p110 was cloned into the pTARGET vector, resulting in the pTARGET/ADAR1 p110 plasmid. To construct the ADAR1 p150 expression plasmid, the cDNA fragment from exon 1 to exon 3 of human ADAR1 p150 was cloned into the pTARGET/ADAR1 p110 vector, resulting in pTARGET/ADAR1 p150. The cDNA fragment from exon 3 to exon 12 of human ADAR2 was cloned into the pTARGET vector, resulting in pTARGET/ADAR2.

Transfection of siRNA, Expression Plasmid and miRNA Mimics into Huh-7 Cells, and Preparation of Cell Homogenates or Total RNA. Transfection of siRNA and miRNA mimics into Huh-7 cells were carried out as follows: Huh-7 cells were seeded into 6-well plates. After 24 h, 5 nM siRNA and/or miRNA mimics were transfected into Huh-7 cells using Lipofectamine RNAiMAX. When 1000 ng of the pTAEGET vector was transfected, Lipofectamine 2000 was used instead of Lipofectamine RNAiMAX. After incubation for 48 h, the cells were harvested and re-suspended 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 Livers and Preparation of Genomic DNA, Homogenate 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 a partnership with the National Disease Research Interchange (NDRI, Philadelphia, PA). Samples from 13 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*-amidinophenyl)

methanesulfonyl fluoride, 2 μg/ml aprotinin, and 2 μg/ml leupeptin]. The protein concentration was determined by the Bradford protein assay reagent (Bio-Rad, Hercules, CA) 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. The analysis of the AhR, ADAR1 and GAPDH protein levels was performed as follows: cell homogenates from Huh-7 cells (20 μg) were separated by 7.5% SDS-PAGE and transferred to an Immobilon-P transfer membrane (Millipore, Billerica, MA). The analysis of the ADAR2 protein levels was performed as follows: 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 (Iwanari et al., 2002). A 1-μL portion of the reverse-transcription product 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, La Jolla, CA) with the

MxPro QPCR software. The AhR and CYP1A1 mRNA levels were normalized to those of GAPDH as described previously (Tsuchiya et al., 2004).

Assessment of RNA Editing Levels in the 3'-UTR of AhR. To evaluate the extent of RNA editing, direct sequence analysis was performed on PCR products. The 3'-UTR of AhR has everted Alu repeats. 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-S (5'-AGC AAG GTT TGG TGC AAA GT-3') and AluSx-AS (5'-GCT CTT CAG CTC TCA TAT CT-3') or AluSc-S (5'-CTG AAG AGC TTA GAC ACA TT-3') and AluSc-AS (5'-CCT CAT GCT GGA AAC AAA TT-3'). The PCR mixture consists of the cDNA or genomic DNA, 1× PCR buffer [67 mM Tris-HCl, pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100, and 0.02% gelatin], 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM of each primer, and 0.5 U 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 with denaturation at 94°C for 30 s, annealing at 58°C for 40 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 a 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 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. The luciferase reporter plasmids were constructed as follows: a fragment from +5168 to +5226 of the 3'-UTR of AhR 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 *Xba* I site

downstream of the luciferase gene. DNA sequence analyses were performed to determine whether the plasmids carried unedited (A) or edited (G) nucleosides at the editing sites. Such plasmids were named as 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 (Mohri et al., 2010).

Luciferase Assay. Various pGL3 luciferase reporter plasmids were transfected into HeLa cells with the phRL-TK plasmid. Briefly, the cells were seeded into 24-well plates. After 24 h, 190 ng of the pGL3p plasmid, 10 ng of the phRL-TK plasmid and 5 nM of the miR-378 mimic or control were transfected using Lipofectamine 2000. After incubation for 48 h, the cells were re-suspended 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 (Nakano et al., 2015). 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

Down-regulation of AhR Expression by ADAR1. To examine whether ADARs regulate human AhR expression, ADAR1 or ADAR2 in Huh-7 cells was knocked down by using siRNA. Both ADAR1 p110 and ADAR1 p150 is targeted by siADAR1 used in this study. Following transfection with siADAR1 and siADAR2, the knockdown of ADAR1 p110 and ADAR2 protein were confirmed (Fig. 3A and B). The ADAR1 p150 protein was not detected by Western blot analysis. The knockdown of ADAR1 significantly increased AhR protein levels, but not mRNA levels, whereas the silencing of ADAR2 did not affect expression at any level (Fig. 3C and D). These results suggest that ADAR1 negatively regulates AhR expression in a post-transcriptional manner.

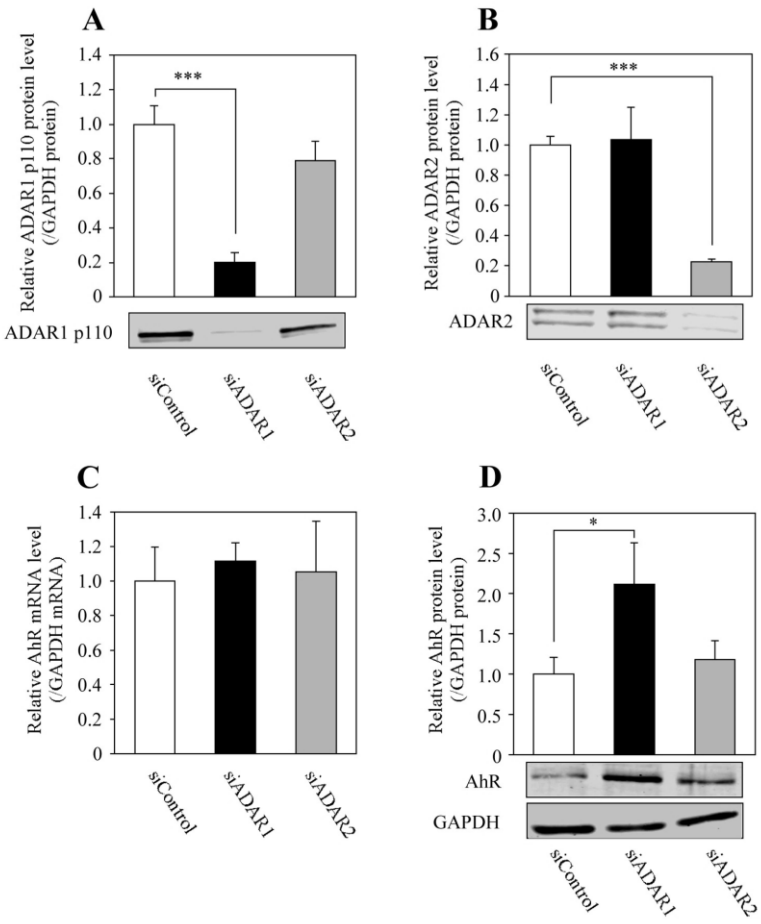

Fig. 3. Effects of ADAR1 or ADAR2 knockdown on AhR expression levels in Huh-7 cells. ADAR1 p110 protein (**A**), ADAR2 protein (**B**), AhR mRNA (**C**), and AhR protein (**D**) levels in Huh-7 cells 48 hr after transfection with 5 nM siADAR1, siADAR2 or siControl were determined by Western blotting or real-time RT-PCR, and were normalized to the GAPDH. The values represent the levels relative to the siControl. Each column represents the mean ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

ADAR1 Edits the 3'-UTR of AhR. According to RADAR, there are potential RNA editing sites in the 3'-UTR of AhR. To examine whether AhR is actually subjected to RNA editing, direct sequencing was performed using AhR genomic DNA and cDNA from Huh-7 cells. Because 'I' is transcribed as 'G' by reverse transcriptase used in RT-PCR, A-to-I RNA edited sites were detected as overlapping peak of 'A' and 'G'. Within the Alu elements in the 3'-UTR of AhR, 38 edited sites were identified (Fig. 4). To investigate whether ADAR1 or ADAR2 is responsible for the RNA editing events, direct sequence analysis of the AhR cDNA from siADAR1- or siADAR2-transfected Huh-7 cells was performed. The RNA editing level of almost all of the editing sites was reduced by ADAR1 knockdown, but not by ADAR2 knockdown (Fig. 5A). Typical sequencing electropherograms of these RNA edited sites are shown in Fig. 5B. These results indicated that ADAR1 plays a critical role in editing AhR mRNA.

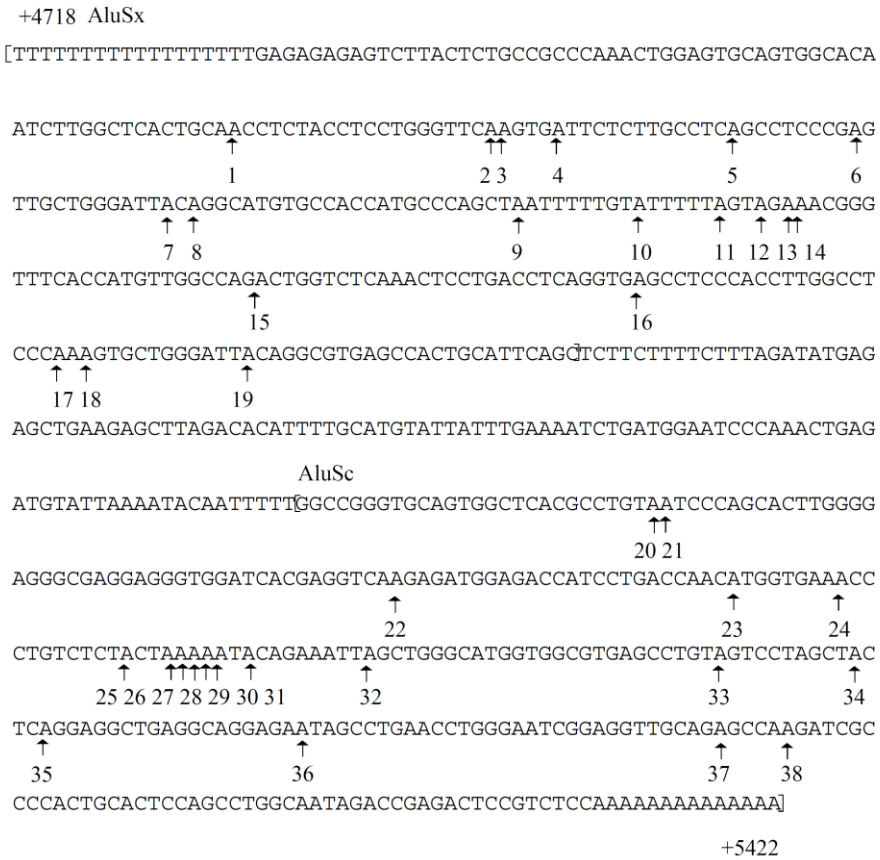

Fig. 4. RNA editing sites in the 3'-UTR of AhR. The nucleotide numbering refers to the 5' end of the mRNA as 1. Square brackets represent Alu elements. Arrows show the RNA editing sites, numbered as 1-38 from 5' to 3'.

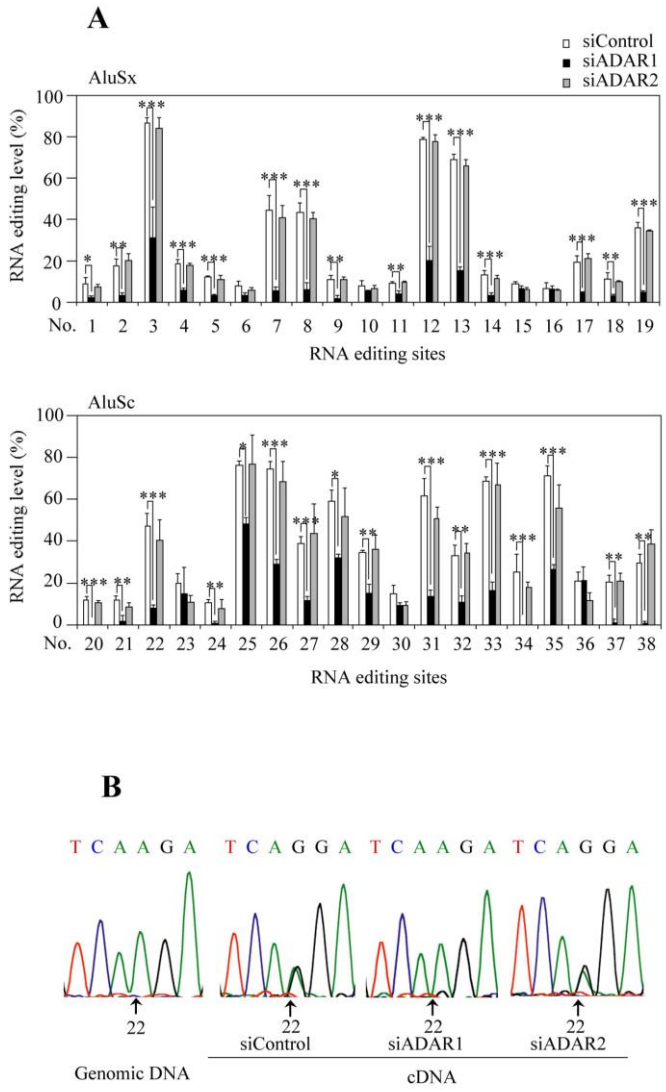

Fig. 5. Effects of ADAR1 or ADAR2 knockdown on RNA editing levels in the 3'-UTR of AhR in Huh-7 cells. (**A**) RNA editing levels in the 3'-UTR of AhR in Huh-7 cells 48 hr after transfection with 5 nM siADAR1, siADAR2 or siControl were calculated as described in Materials and Methods (**B**) Representative electropherograms from the direct sequencing. A RNA editing site is shown by numbered arrows. The numbers represent the RNA editing sites in the 3'-UTR of AhR indicated in Fig. 4. The values represent the levels relative to the siControl. Each column represents the mean ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

AhR Expression Was Not Changed by ADAR1 or ADAR2 Over-expression.

To further investigate the role of RNA editing in AhR expression, ADAR1 p110, ADAR1 p150, and ADAR2 were over-expressed in Huh-7 cells by transfection of expression plasmid. ADAR1 p110, ADAR1 p150, and ADAR2 were successfully over-expressed (Fig. 6A and B), but the AhR mRNA and protein levels were not changed (Fig. 6C and D). Through sequencing analysis, it was demonstrated that the RNA editing levels were mildly increased by the over-expression of the ADAR isoforms (Fig. 7A and B). These results suggest that the endogenous levels of ADAR1 are sufficient for the editing of the AhR transcript.

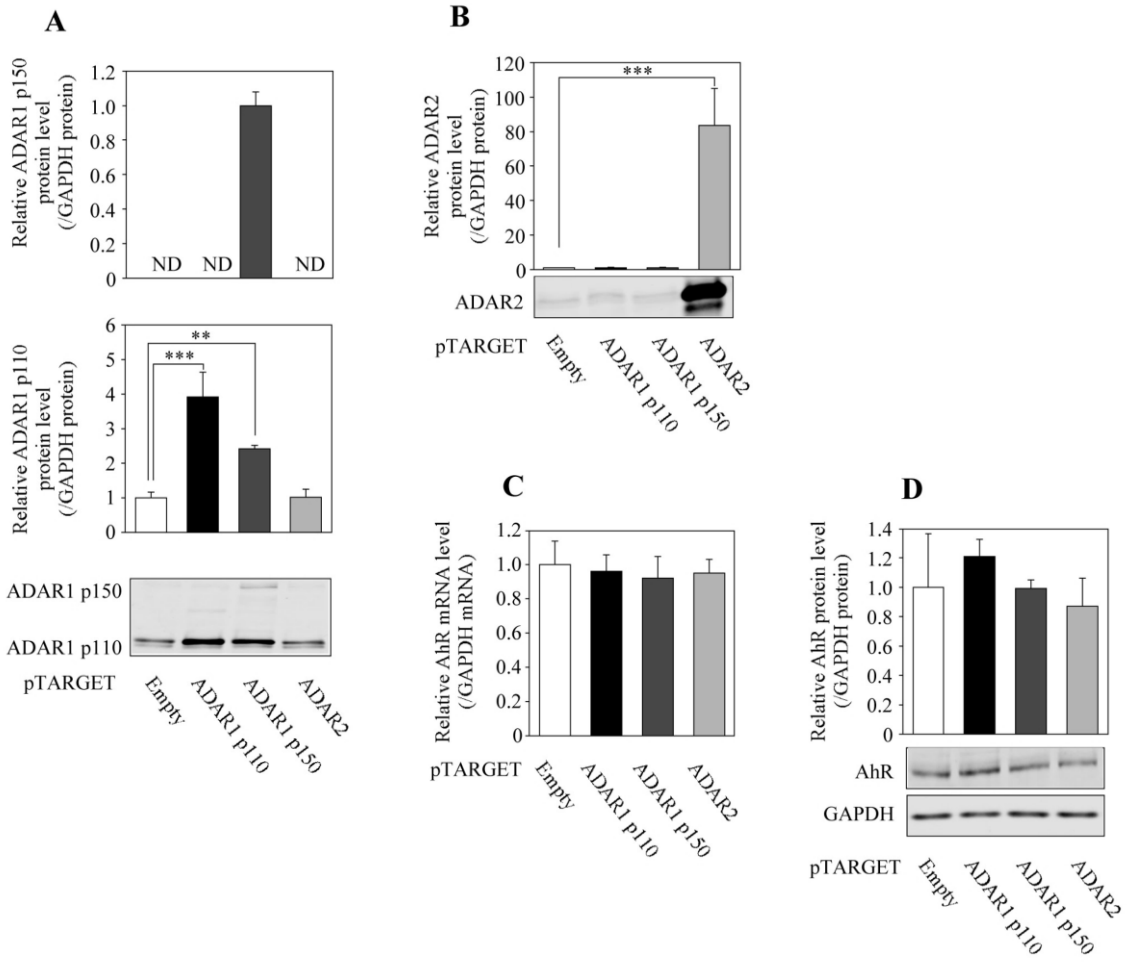

Fig. 6. Effects of the over-expression of ADAR1 p110, ADAR1 p150, or ADAR2 on AhR expression. ADAR1 p110 and ADAR1 p150 protein (**A**), ADAR2 protein (**B**), AhR mRNA (**C**), and AhR protein (**D**) levels in Huh-7 cells 48 hr after transfection with 1000 ng of pTARGET/Empty, pTARGET/ADAR1 p110, pTARGET/ADAR1 p150, or pTARGET/ADAR2 were determined by Western blot or real-time RT-PCR, and were normalized to GAPDH. The values represent the levels relative to pTARGET/Empty or pTARGET/ADAR1 p150. The numbers represent the RNA editing sites in the 3'-UTR of AhR indicated in Fig. 4. Each column represents the mean ± SD of three independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$.

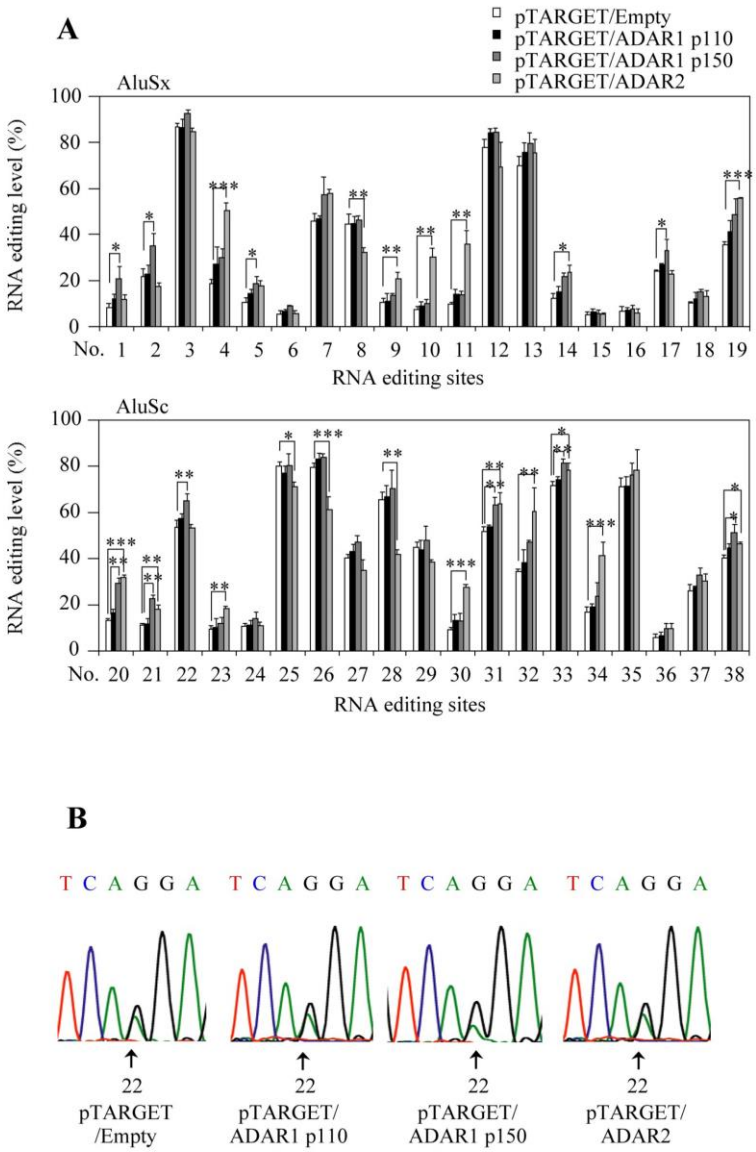

Fig. 7. Effects of over-expression of ADAR1 p110, ADAR1 p150, or ADAR2 on RNA editing levels in the 3'-UTR of AhR in Huh-7 cells. (**A**) RNA editing levels in the 3'-UTR of AhR in Huh-7 cells after transfection with 1000 ng of pTARGET/Empty, pTARGET/ADAR1 p110, pTARGET/ADAR1 p150, or pTARGET/ADAR2. The editing level was calculated as described in Materials and Methods. (**B**) Representative electropherograms resulting from the direct sequencing. A RNA editing site is shown by numbered arrows. The numbers represent the RNA editing sites in the 3'-UTR of AhR indicated in Fig. 4. Each column represents the mean ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

ADAR1 Represses the Induction of Downstream Gene of AhR. It was investigated whether the ADAR1-mediated down-regulation of AhR might affect the induction of genes downstream of AhR. As shown in Fig. 8, treatment of Huh-7 cells with 10 nM TCDD, a ligand of AhR, resulted in a significant increase in CYP1A1 mRNA levels (3.6-fold). When ADAR1 was knocked down, an enhanced induction (6.0-fold) of CYP1A1 mRNA was observed. It was confirmed that the AhR protein levels were increased by the silencing of ADAR1 in the presence of DMSO, whereas the AhR protein levels in the TCDD-treated cells were lower than in control cells. 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 (Davarinos and Pollenz, 1999: Ma and Baldwin, 2000). Collectively, these data demonstrated that ADAR1 affects the induction of genes downstream of AhR.

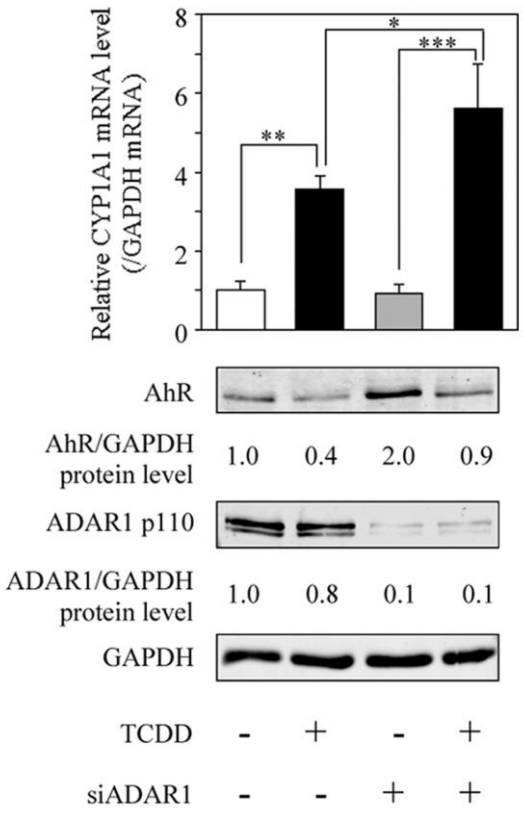

Fig. 8. Effects of ADAR1 knockdown on the induction of CYP1A1 in Huh-7 cells. Twenty-four hours after transfection with 5 nM siADAR1 (+) or negative control #1 (-), Huh-7 cells were exposed to 10 nM TCDD (+) or 0.1% DMSO (-). CYP1A1 mRNA, AhR and ADAR1 protein levels were determined by real-time RT-PCR or Western blot analysis and normalized to the GAPDH levels. The values are expressed relative to the levels observed in the siControl- and DMSO- treated cells. Each column represents the mean ± SD of three independent experiments.*P < 0.05, **P < 0.01 and ***P < 0.001.

RNA Editing of the AhR Transcript Creates miRNA Target Sites. It was surmised that ADAR1 negatively regulates AhR expression by creating miRNA target sites in the 3'-UTR of AhR mRNA. *In silico* analysis using miRediTar (http://microrna.osumc.edu/mireditar) predicted miR-29a, miR-140, and miR-378 as candidate miRNAs whose binding affinity to the edited sequence in the 3'-UTR of AhR was predicted to be higher than their affinity to the unedited sequence (Fig. 9A). Transfection of miR-29a mimics significantly decreased the AhR mRNA levels, but transfection with miR-140 or miR-378 mimics had no effect (Fig. 9B). The AhR protein levels were significantly decreased by the over-expression of miR-29a and miR-378, but were not changed by miR-140 (Fig. 9C). To investigate whether the miR-29a- or miR-378-dependent down-regulation of AhR requires ADAR1-mediated RNA editing, Huh-7 cells were co-transfected with siADAR1 and either miR-29a or miR-378 mimics. The AhR mRNA (Fig. 9D) and protein (Fig. 9E) levels were significantly reduced by transfection of miR-29a mimic regardless of whether ADAR1 was knocked down. Interestingly, the over-expression of miR-378 significantly decreased the AhR protein levels, and the miR-378-dependent down-regulation of AhR was abolished by the knockdown of ADAR1 (Fig. 9E). 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, luciferase assay was performed using reporter plasmids carrying either an unedited sequence (pGL3p/MRE(A)) or an edited sequence (pGL3p/MRE(G)). The luciferase activity of the pGL3p/MRE(G) plasmid was slightly but significantly reduced by the over-expression of miR-378, whereas the luciferase activity of the pGL3p/MRE(A) plasmid was not affected (Fig. 9F), indicating that miR-378 recognizes the edited sequence within the 3'-UTR of AhR.

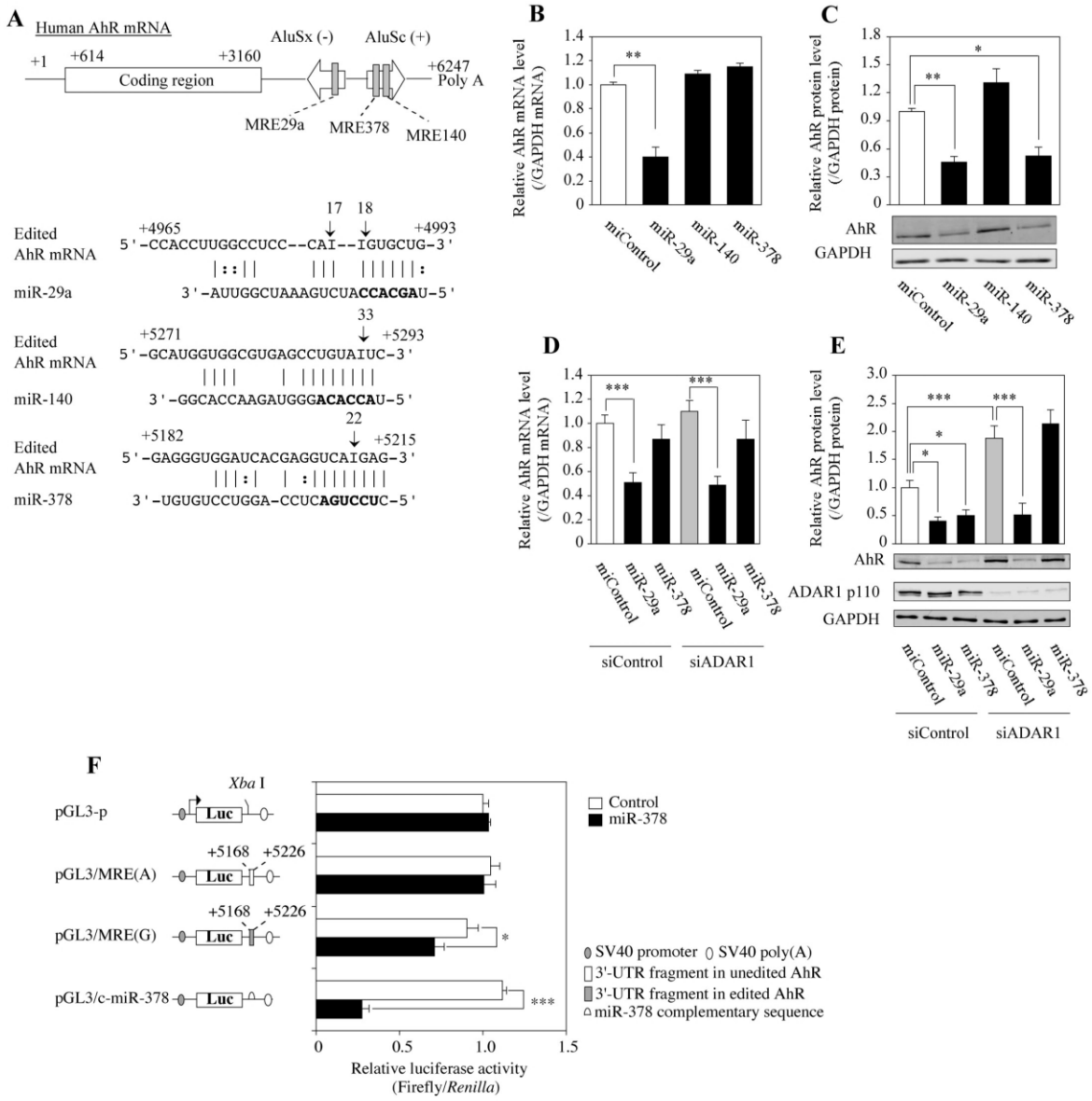

Fig. 9. Effects of RNA editing on the miRNA-dependent down-regulation of AhR in Huh-7 cells. (**A**) Schematic representation of human AhR mRNA, and the predicted MREs of miR-29a, miR-140 and miR-378 in edited AhR mRNA. The numbering denotes the 5' end of the mRNA as 1. Open arrows indicate the direction of the Alu elements. Bold letters represent seed sequences. RNA editing sites are shown by numbered arrows. AhR mRNA (**B** and **D**) and protein (**C** and **E**) levels in Huh-7 cells 48 hr after transfection with 5 nM miR-29a, miR-140, miR-378 mimic or miControl (**B** and **C**) and co-transfection with 5 nM siRNA (siADAR1 or siControl) and 5 nM miRNA mimic (miR-29a, miR-140, miR-378 mimic, or miControl) (**D** and **E**) were determined by real time RT-PCR or Western blot analysis, and were normalized to GAPDH. The values are expressed relative to the levels in the control cells. (**F**) Luciferase assay using plasmids containing a fragment of the 3'-UTR of AhR. HeLa cells were transfected with the reporter plasmids (190 ng) along with the phRL-TK plasmid (10 ng) and the miR-378 mimic or control. Firefly luciferase activity for each construct was normalized to *Renilla* luciferase activity. The values are expressed relative to the pGL3-p plasmid. Each column represents the mean ± SD of three independent experiments. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001.

ADAR Protein Levels, AhR RNA Editing and Protein Levels, and miR-378 Levels in Human Livers. The variability in the ADAR protein levels in the panel of 32 human liver samples was examined. ADAR1 p110, but not ADAR1 p150 or ADAR2, were detected by Western blot analysis (Fig. 10A). A large inter-individual difference (220-fold) in the ADAR1 p110 protein levels was observed in human liver samples. To investigate whether the large inter-individual difference affects the RNA editing levels in the 3'-UTR of AhR, direct sequencing was performed 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 inter-individual difference in the RNA editing levels was relatively small compared to that in ADAR1 expression levels (Fig. 10B). To investigate whether the small inter-individual variability in RNA editing levels in the human livers was specific for AhR, the RNA editing levels in filamin B, β, which has been reported to be edited by ADAR1 (Chan et al., 2014), was examined (Fig. 11A). The RNA editing levels were correlated with ADAR1 p110 expression, and the inter-individual variability was relatively large compared to that of AhR (Fig. 11B). These results suggest that the low ADAR1 expression levels in the livers would be sufficient to edit the 3'-UTR of AhR.

Next, the relationship between the expression levels of AhR mRNA, protein, and miR-378 in 32 individual human liver samples was examined. The AhR protein levels were not positively correlated with the AhR mRNA levels (Rs = -0.14) (Fig. 12A), indicating the involvement of post-transcriptional regulation. Interestingly, as shown in Fig. 12B, the miR-378 levels (46-fold) were inversely correlated with the AhR protein levels (Rs = -0.57). Collectively, these results suggest that inter-individual 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 inter-individual variability in AhR expression in human livers.

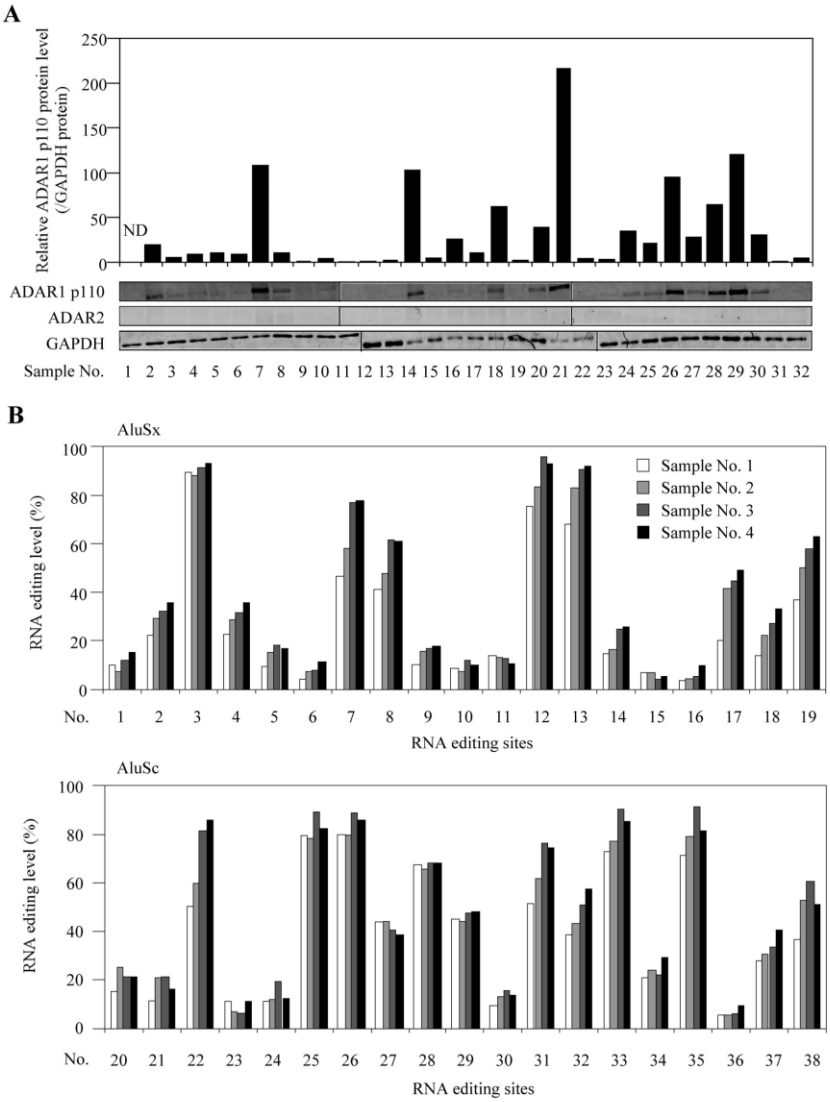

Fig. 10. ADAR protein levels, RNA editing levels in the 3'-UTR of AhR in human livers. (**A**) ADAR protein levels in a panel of 32 human livers were determined by Western blot analysis, and were normalized to GAPDH. ND: not detectable. The values are expressed relative to the sample with the lowest levels. (**B**) RNA editing levels in the 3'-UTR of AhR in 4 human liver samples (No.1, 6, 16, and 21). The editing level was calculated as described in Materials and Methods. Each column represents the mean of two independent experiments.

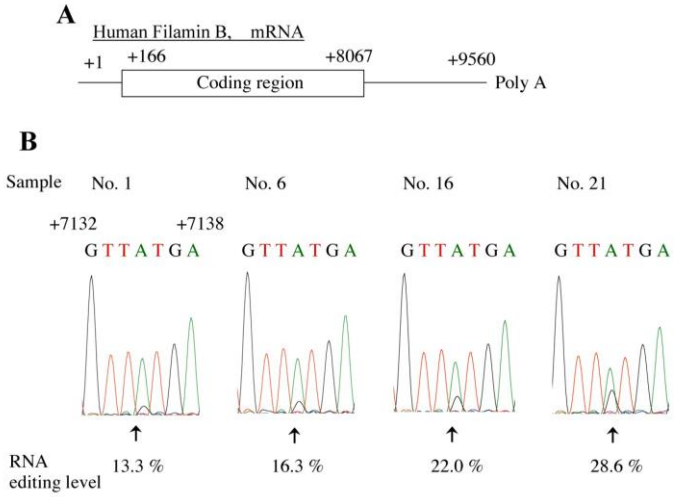

Fig. 11. RNA editing in filamin B, β mRNA in human livers (**A**) Schematic representation of human filamin B, β mRNA. (**B**) RNA editing levels in filamin B, β exon 42 in human liver samples (No. 1, 6, 16 and 21). The editing level was calculated as described in Materials and Methods. The numbering denotes the 5' end of the mRNA as 1. A RNA editing site is shown by arrows.

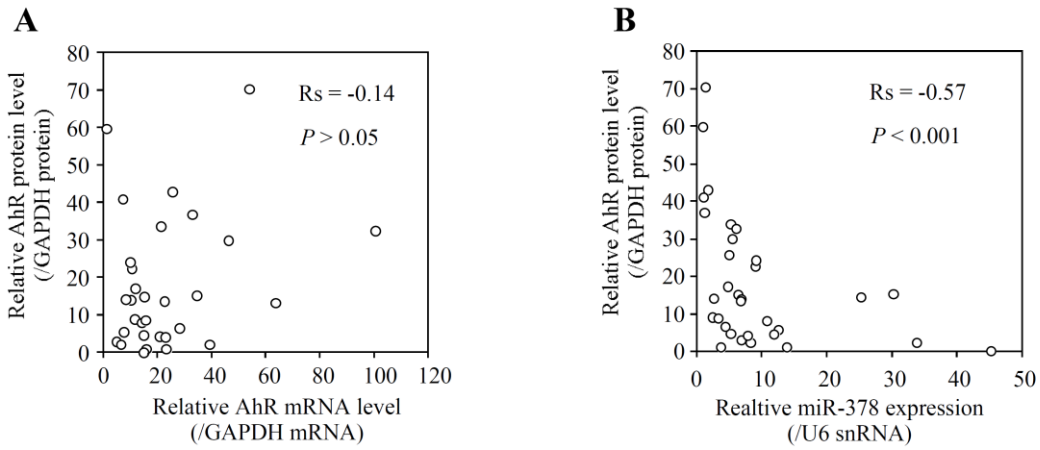

Fig. 12. The relationships between (**A**) AhR protein and AhR mRNA levels; (**B**) AhR protein levels and miR-378 levels in 32 human livers. AhR mRNA or protein levels were determined by real time RT-PCR or Western blot analysis and normalized to GAPDH. The levels of mature miR-378 in the human livers were determined by real time RT-PCR and normalized to U6 snRNA levels. Data represent the mean of two independent experiments. The values are expressed relative to the sample with the lowest levels.

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 mRNAs can lead to functional alterations of the encoded protein (Paschen et al., 1996; Chen et al., 2013). However, most A-to-I RNA editing occurs in non-coding regions, such as the UTR and miRNA transcripts (Levanon et al., 2004; Peng et al., 2012; Wang et al., 2013). 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 nt 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 (Yang et al., 2006; Kawahara et al., 2008). In other cases, A-to-I editing of the miRNA seed sequence could change its target selection or binding efficiency (Kawahara et al., 2007). In this study, it was examined 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 (Peng et al., 2012; Liang and Landweber, 2007), but studies supporting this hypothesis are very limited (Borchert et al., 2009; Wang et al., 2013).

It was found that the 3'-UTR of AhR in human liver and human hepatocarcinoma-derived cells is predominantly subjected to RNA editing by ADAR1, rather than ADAR2 (Fig. 4, Fig. 5A, and Fig. 10B). Because the 3'-UTR of AhR has everted Alu repeats that appear to form matched dsRNA structures, this finding is consistent with a previous report that ADAR1 promiscuously targets 'A' in perfectly base-paired dsRNA, whereas ADAR2 prefers selective sites in dsRNA containing mismatches, bulges, and

internal loops (Källman., 2003). Recently, it was revealed that the RNA editing levels within reversely oriented Alu repeats are negatively correlated with the distance between two Alu elements (Bazak et al., 2014). The distance between the two Alu elements in the the 3'-UTR of AhR 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. In Fig. 11, RNA editing level in Filamin B, β mRNA was determined to investigate whether the small inter-individual variability in RNA editing levels in the human livers was specific for AhR (Fig. 10B). As the result, the RNA editing level was correlated with ADAR1 p110 level, and the inter-individual variability was larger than that of AhR (Fig. 11B). 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 poorly matched and shorter double strand structure than that of AhR, implying that Filamin B, β mRNA would be less favorable substrate of the ADAR enzymes. These RNA structural features would explain the difference of the inter-individual variability of RNA editing levels between AhR and Filamin B, β transcript.

The over-expression of ADAR2 significantly increased the RNA editing levels in 15 out 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 (Fig. 5A and Fig. 7A). ADAR1 and ADAR2 have distinct but overlapping target specificities (Bass et al., 1997; Lehmann and Bass, 2007). In addition to the RNA structure, 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 (Riedmann et al., 2010). 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) (Polson and Bass, 1994). Ten of the above 15 RNA editing sites in the 3'-UTR of AhR have U or G as the 3' neighbor (Fig. 4). Therefore, ADAR1 and ADAR2 both would edit the 3'-UTR of AhR. Although a previous study (Chan et al.,2011)

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 to 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 3'-UTR of AhR (Fig. 9A). 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. 9D and E). Using a luciferase assay, it was demonstrated that the A-to-I (artificially G) conversion at editing site No. 22 was functional and created the binding site for miR-378 (Fig. 9F). 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 3'-UTR of AhR is highly edited not only in Huh-7 cells but also in normal human livers (Fig. 10B), implying that hepatic AhR expression can be regulated by miR-378. This is supported by the negative correlation between miR-378 and AhR expression in the panel of 32 human livers (Fig. 12B). The miR-378 has been shown to function as an oncogene in liver cancer (Ma et al., 2014). In addition, miR-378 is functionally expressed in normal liver, where it is reported to regulate lipid metabolism (Jeon et al., 2013) and CYP2E1-mediated xenobiotic metabolism (Mohri et al., 2010; Nakano et al., 2016). The present study adds new insight into the role of miR-378 in the human liver. As for RNA editing-independent down-regulation by miR-29a, *in silico* analysis predicted a potential MRE for miR-29a lacking a RNA editing site upstream of the Alu elements (Fig. 13). In support of this prediction, a recent study reported that the 3'-UTR of human AhR was directly recognized by miR-29a in Huh-7 cells (Kurtz et al., 2015), although a functional MRE for miR-29a has not been experimentally identified. miR-29 family members, including miR-29a, are known to be down-regulated in human fibrotic livers (Roderburg et al., 2011). Recently, it was reported that activation of AhR induces hepatic fibrosis by

directly regulating pro-fibrotic pathways (Pierre et al., 2014). It would be of interest to examine the role of miR-29a-mediated AhR regulation in the development of hepatic fibrosis.

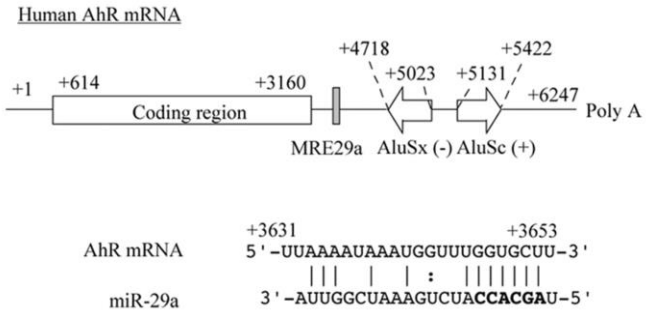

Fig. 13. Schematic representation of the human AhR mRNA, and a predicted MRE of miR-29a in unedited AhR mRNA. The numbering denotes the 5' end of the mRNA as 1. Open arrows indicate Alu elements and directions. Bold letters represent seed sequences.

Finally, it was 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. This study demonstrated that the regulation of AhR expression by RNA editing affects the induction of its downstream target, CYP1A1 (Fig. 8). It is known that CYP1A1 levels are associated with the levels of AhR and its heterodimeic partner, the AhR nuclear translocator (Hayashi et al., 1994). 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 (Hayashi et al., 1994). Accumulating evidence suggests that disrupted RNA editing or abnormal ADAR expression is associated with cancer (Slotkin and Nishikura, 2010). It is possible that the altered ADAR expression in cancer affects AhR expression by changing the levels of AhR mRNA editing.

In summary, it was demonstrated that A-to-I RNA editing regulates the expression of AhR by creating miR-378 binding sites. This mechanism contributes to the inter-individual 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.

CHAPTER 3

Up-regulation of human dihydrofolate reductase expression by RNA editing in human breast cancer

ABSTRACT

DHFR plays a key role in folate metabolism, and is a target of methotrexate. An increase in cellular expression level of DHFR is one of the mechanisms of tumor resistance to methotrexate. The present study investigated a novel possibility that DHFR expression is modulated by A-to-I RNA editing. In human breast adenocarcinoma-derived MCF-7 cells, 26 RNA editing sites were identified in the 3'-UTR of DHFR. Knockdown of ADAR1 decreased the RNA editing levels in the 3'-UTR of DHFR, and resulted in the decrease of DHFR mRNA and protein levels. In the presence of α-amanitin, a transcriptional inhibitor, the edited DHFR mRNA was more slowly degraded than the non-edited mRNA, suggesting that DHFR mRNA are stabilized by RNA editing. Thus, it was found that ADAR1 up-regulates DHFR expression by stabilization of mRNA. miR-25-3p and miR-125a-3p were predicted, by a computational analysis, to bind to the non-edited 3'-UTR of DHFR but not to the edited sequence. The decrease in DHFR expression by the knockdown of ADAR1 was restored by inhibition of these miRNAs, suggesting that RNA editing destroys the miRNA recognition elements to increase DHFR expression. Interestingly, the knockdown of ADAR1 promotes cell viability and increased the sensitivity to methotrexate of MCF-7 cells. ADAR1 expression levels and the RNA editing levels in the 3'-UTR of DHFR in breast cancer tissues were higher than those in adjacent normal tissues. Collectively, ADAR1 positively regulates the expression of DHFR through RNA editing by disrupting the binding of miR-25-3p and miR-125a-3p to the 3'-UTR of DHFR, thus enhancing cellular proliferation and resistance to methotrexate.

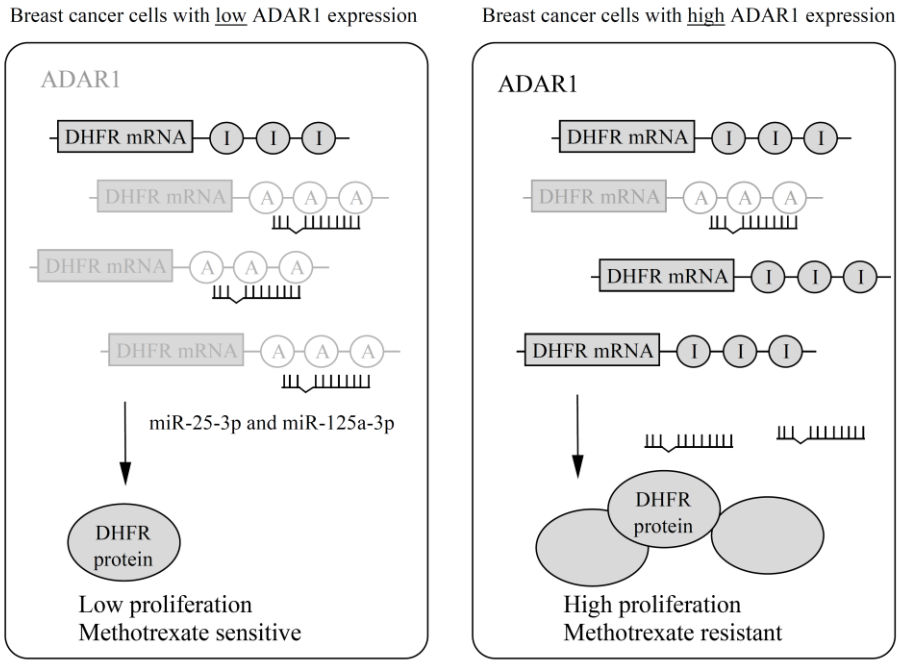

Fig. 14. Graphical abstract of chapter 3. ADAR1 up-regulates DHFR expression by destroying miR-25-3p and miR-125a-3p targeting sites through A-to-I RNA editing, thus enhancing cellular proliferation and resistance to methotrexate in human breast cancer cells with high ADAR1 expression but not in those with low ADAR1 expression.

INTRODUCTION

DHFR is a key enzyme of folate metabolism. It catalyzes the reduction of dihydrofolate to tetrahydrofolate using NADPH as a cofactor. Since tetrahydrofolate is essential for *de novo* purine and thymidylate synthesis required for DNA synthesis, cell growth and proliferation, DHFR is a target for chemotherapeutic agents such as methotrexate and pemetrexed (Schweitzer et al., 1990; Fowler. 2001; Nazki et al., 2014). The clinical efficacy of methotrexate is often limited by the acquisition of resistance in cancer cells. As the mechanisms of methotrexate resistance, mutations in *DHFR* gene leading to decreased affinity of DHFR protein to methotrexate (Albrecht et al., 1972; Jackson et al., 1976; Goldie et al., 1980) and decreased uptake of methotrexate due to impaired transport (Hill et al., 1979; Moscow et al., 1995; Kobayashi et al., 1998) are known. In addition, over-expression of DHFR protein is observed in methotrexate-resistant cells (Assaraf, 2007). DHFR expression is regulated by multiple mechanisms (Abali et al., 2008), including gene amplification (Alt et al., 1978; Dolnick et al., 1979), Sp1 and E2F1-mediated transcriptional regulation (Dynan et al., 1986; Slansky et al., 1993), and miRNA-mediated post-transcriptional regulation (Mishra et al., 2007; Song et al., 2008; Song et al., 2010). This study, it was sought to investigate a possibility that RNA editing might also underlie as the regulation mechanism.

Aberrant ADARs expression has been associated with many human diseases including cancer (Slotkin and Nishikura, 2013). A more recent study indicated that ADAR1 functions as an oncogene in breast cancer (Fumagalli et al., 2015). It was noticed that DHFR has been included in RADAR, and that RNA editing sites were identified at the introns 3, 4, and 5 as well as 3'-UTR (http://rnaedit.com/). However, the effects of these RNA editing on DHFR expression is unknown. In the present study, it was investigated whether RNA editing modulates DHFR expression and subsequently affects the cell proliferation and sensitivity to methotrexate of breast cancer cells.

MATERIALS AND METHODS

Chemicals and Reagents. Methotrexate hydrate and folinic acid calcium salt hydrate were from Tokyo Kasei (Tokyo, Japan) and Sigma-Aldrich (St. Louis, MO), respectively. The pGL3-promoter vector, phRL-TK vector, and Dual-Luciferase Reporter Assay System were purchased from Promega (Madison, WI). Lipofectamine RNAiMAX, Lipofectamine 2000; Silencer Select siRNA for human ADAR1 (s1007) (siADAR1), human ADAR2 (s1010) (siADAR2) and negative control #1 (siControl) and miRNA mimics for miR-25-3p and miR-125a-3p and negative control #1 (miControl) were purchased from Life Technologies (Carlsbad, CA). Antisense locked nucleic acid/DNA oligonucleotides (AsO) for miR-25-3p (5'- TCA GTC CGA GAC AAG TGC AAT G-3'; locked nucleic acids are underlined), miR-125a-3p (5'-GGC TCC CAA GAA CCT CAC CTG T-3') and for negative control (5'-AGA CUA GCG GUA UCU UAA ACC-3') (AsControl) were commercially synthesized by Gene Design (Osaka, Japan). RNAiso, 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). α-Amanitin was purchased from Calbiochem (San Diego, CA). The rabbit anti-human DHFR polyclonal antibody (ab49881) was from Abcam (Cambridge, MA), and mouse anti-human ADAR1 monoclonal antibody (sc-5579) and mouse anti-human ADAR2 monoclonal antibody (sc-73408) were 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). Cell counting kit-8 (CCK-8) was from Dojin Chemical Laboratories (Kumamoto, Japan). Restriction enzymes were from New England Biolabs (Ioswich, MA). All other chemicals and solvents were of the highest grade commercially available.

Cell Cultures. MCF-7, a human breast adenocarcinoma cell line, was obtained from the American Type Culture Collection (Manassas, VA). A549, a human lung carcinoma cell line, was obtained from the Riken Gene Bank (Tsukuba, Japan). The MCF-7 cells were cultured in DMEM supplemented with 0.1 mM nonessential amino acid and 10% FBS. The A549 cells were cultured in DMEM supplemented with 10% FBS. All of the cells were maintained at 37°C under an atmosphere of 5% CO_2 and 95% air.

Transfection of siRNA, miRNA Mimic or AsO into MCF-7 Cells and Preparation of Cell Homogenates and Total RNA. The MCF-7 cells were seeded into 6-well plates and 5 nM siRNA was transfected using Lipofectamine RNAiMAX at the same time. In the case of double transfection, 10 nM miRNA mimic or 10 nM AsO was co-transfected with 10 nM siRNA into the cells. After 72 h, the cells were harvested and suspended in a small amount of TGE buffer and disrupted by freeze-thawing three times. Total RNA was prepared using RNAiso.

SDS-PAGE and Western Blot Analysis. For the analysis of the DHFR, ADAR1, and GAPDH protein levels, cell homogenates from MCF-7 cells were separated by 15%, 7.5% and 10% SDS-PAGE, respectively, and transferred to an Immobilon-P transfer membrane (Millipore, Billerica, MA). For the analysis of the ADAR2 protein levels, the cell homogenates 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 DHFR 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 DHFR, ADAR1 and ADAR2 protein levels were normalized to GAPDH.

Real-time RT-PCR for DHFR Transcript Variants and ADAR1 mRNA.

cDNA was synthesized from total RNA using ReverTra Ace. The sequences of the used primers are shown in Table 1. 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. Real-time PCR was performed using the Mx3000P (Stratagene, La Jolla, CA) with the MxPro QPCR software. The PCR condition for DHFR v1 and v4 was as follows: after an initial denaturation at 95°C for 30 s, amplification was performed by denaturation at 94°C for 20 s, annealing/extension at 62°C for 20 s for 40 cycles. The PCR condition for DHFR v2 and v3 was as follows: after an initial denaturation at 95°C for 30 s, amplification was performed by denaturation at 94°C for 20 s, annealing at 53°C for 20 s, and extension at 72°C for 20 s for 40 cycles. The PCR condition for ADAR1 was as follows: after an initial denaturation at 95°C for 30 s, amplification was performed by denaturation at 94°C for 20 s, annealing/extension at 64°C for 20 s for 40 cycles. The levels of DHFR variant transcripts and were normalized to those of GAPDH mRNA (Tsuchiya et al., 2004). The ADAR1 mRNA levels in human breast cancer tissues described below were normalized to those of 18S rRNA (Komagata et al., 2009).

Table 1. Primers for real time RT-PCR

Target gene	Accession No.	Forward (5'to 3 ')	Reverse (5' to 3')
DHFR v1	NM_000791	TCC AGA GAA TGA CCA CAA CC	ACG TGT CAC TTT CAA AGT CT
DHFR v2	NM_001290354	TGG CCA CCG CTC AGG TAA AC	GTG ATT CAT GGC TTC CTT AT
DHFR v3	NM_001290357	TCC AGA GAA TGA CCA CAA CC	GGG TGA TTC ATG GCT TCT TG
DHFR v4	NR_110936	TGG CCA CCG CTC AGG TAA AC	AGA ACA CCT GGG TAT CTT AT
ADAR1[a]	NM_001025107	GCT TGG GAA CAG GGA ATC G	CTG TAG AGA AAC CTG ATG AAG CC
GAPDH[b]	NM_002046	CCA GGG CTG CTT TTA ACT C	GCT CCC CCC TGC AAA TGA
18S rRNA[c]	NR_003286	GGC CCT GTA ATT GGA ATG AGT C	GAC ACT CAG CTA AGA GCA TCG

[a]Wang et al. (2013)

[b]Tsuchiya et al. (2004)

[c]Komagata et al. (2009)

Evaluation of RNA Editing Levels in the 3'-UTR of DHFR. For the analysis of RNA editing levels, direct sequencing of PCR products was performed. The 3'-UTR of

DHFR was amplified by PCR using genomic DNA or cDNA as a template. The following primer sets for genomic DNA or cDNA were used to amplify the 3'-UTR of DHFR: the forward primer 5'-TTT ATC CAA CTT GAC AGT GG-3' and the reverse primer 5'-CTT CAC CCT TGA TTA TTT GG-3' or the forward primer 5'-AGC TGC TCT ATA GCA AGT CT-3' and the reverse primer 5'-CTT CAC CCT TGA TTA TTT GG-3'. The PCR mixture consists of the genomic DNA or cDNA, 1× PCR buffer, 0.4 μM of each primer and 0.5 U of Gflex polymerase (TAKARA, Shiga, Japan) in a final volume of 25 μL. After an initial denaturation at 94°C for 1 min, amplification was performed with denaturation at 98°C for 10 s, annealing at 54°C for 15 s, and extension at 72°C for 70 s (genomic DNA) or 40 s (cDNA) for 35 cycles, followed by a final extension at 72°C for 5 min. The PCR product was subjected to electrophoresis using a 0.8% 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 purified and subjected to direct sequencing. The extent of editing was 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.

Evaluation of the Stability of DHFR mRNA. siRNA was transfected into MCF-7 cells, as described above. After 36 h, the cells were treated with 10 μg/mL α-amanitin an inhibitor of transcription. Total RNA was prepared 0, 6, 12 and 24 hours later. The DHFR mRNA level was determined by real-time RT-PCR, as described above.

Reporter Plasmid Construction. To construct the luciferase reporter plasmids, fragments from +1901 to +2153 (containing MRE for miR-25-3p (MRE25)) and from +2443 to +2569 (containing MRE for miR-125a-3p (MRE125)) of the non-edited 3'-UTR of DHFR were amplified by PCR using MCF-7 genomic DNA and were inserted into the pGL3p vector at the *Xba*I site downstream of the luciferase gene

(pGL3p/MRE25(A) and pGL3p/MRE125(A), respectively). Reporter plasmids containing these fragment of the edited 3'-UTR of DHFR were constructed by using cDNA from MCF-7 (pGL3p/MRE25(G) and pGL3p/MRE125(G), respectively) as a template. DNA sequencing analyses revealed that pGL3p/MRE25(G) carries edited nucleotides (G) at RNA editing site No. 1, 2, 3, 5, 7, 8, 10 and 11, and that pGL3p/MRE125(G) carries 'G' at No. 16, 17, 18, 19, and 20 (refer to Fig. 16A).

Luciferase Assay. Various pGL3 luciferase reporter plasmids were transfected into MCF-7 cells with the phRL-TK plasmid. Briefly, the cells were seeded into 24-well plates. After 24 h, 190 ng of the pGL3p plasmid, 10 ng of the phRL-TK plasmid and 5 nM of the miR-25-3p, miR-125a-3p mimic or control were transfected using Lipofectamine 2000. After incubation for 48 h, the cells were re-suspended in passive lysis buffer, and luciferase activity was measured on a luminometer using the Dual-Luciferase Reporter Assay System.

Assessment of Cell Viability. To evaluate the cell viability, a WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt) assay, which is a modified MTT assay, was performed. siRNA was transfected into MCF-7 cells as described above and incubated for 72 h. To investigate the effects of folinic acid on ADAR1-induced decrease of cell viability, the siRNA-transfected MCF-7 cells were incubated in the presence or absence of 200 μM folinic acid every 12 h for 72 h. The effects of ADAR1 knockdown on sensitivity of MCF-7 or A549 cells to methotrexate were investigated as follows; MCF-7 or A549 cells were transfected with 0.5 or 1 nM siRNA, respectively. After 24 h, the cells were treated with 50 nM methotrexate for 24, 48 and 72 h. CCK-8 reagent was added and the absorbance of the medium at 450 nm was measured. The percent cell viability was calculated by comparing the absorbance of the treated cells with the control cells.

Human Breast Cancer and Adjacent Normal Tissues. Breast cancer and adjacent normal tissues were obtained as surgical samples from 19 Japanese patients with primary breast carcinoma. The patients (42-77 years old) had not undergone chemotherapy (Tsuchiya et al., 2006). This study was approved by the Ethics Committee of Kanazawa University (Kanazawa, Japan). Written informed consent was obtained from patients. The samples were obtained immediately after resection, divided into breast cancer and adjacent normal tissues, and immediately frozen with liquid nitrogen. The samples were stored at -80°C until use. Total RNA was prepared using RNAiso.

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.

RESULT

Up-regulation of DHFR Expression by ADAR. To investigate whether ADAR1 or ADAR2 regulates human DHFR expression, ADAR1 or ADAR2 expression in MCF-7 cells was knocked down by using siRNA. The siADAR1 used in this study targets both ADAR1 p110 and ADAR1 p150. Following transfection with siADAR1 or siADAR2, the significant decrease in ADAR1 p110, ADAR1 p150 or ADAR2 protein levels were confirmed (Fig. 15A and B). Next, DHFR mRNA and protein expression levels were evaluated. In NCBI database, four different DHFR transcript variants (v1-v4) were registered (Fig. 15C). Taking the effects of RNA editing in introns on splicing into consideration, the expression levels of the four transcripts were determined. The knockdown of ADAR1 resulted in significant decrease in all DHFR variant transcripts levels (Fig. 15D-G). DHFR protein level was also reduced by the transfection of siADAR1 (Fig. 15H). The silencing of ADAR2 significantly decreased the DHFR v2 mRNA level, but not the other DHFR transcript variants mRNA as well as protein levels. These results indicated that ADAR1 positively regulates DHFR expression.

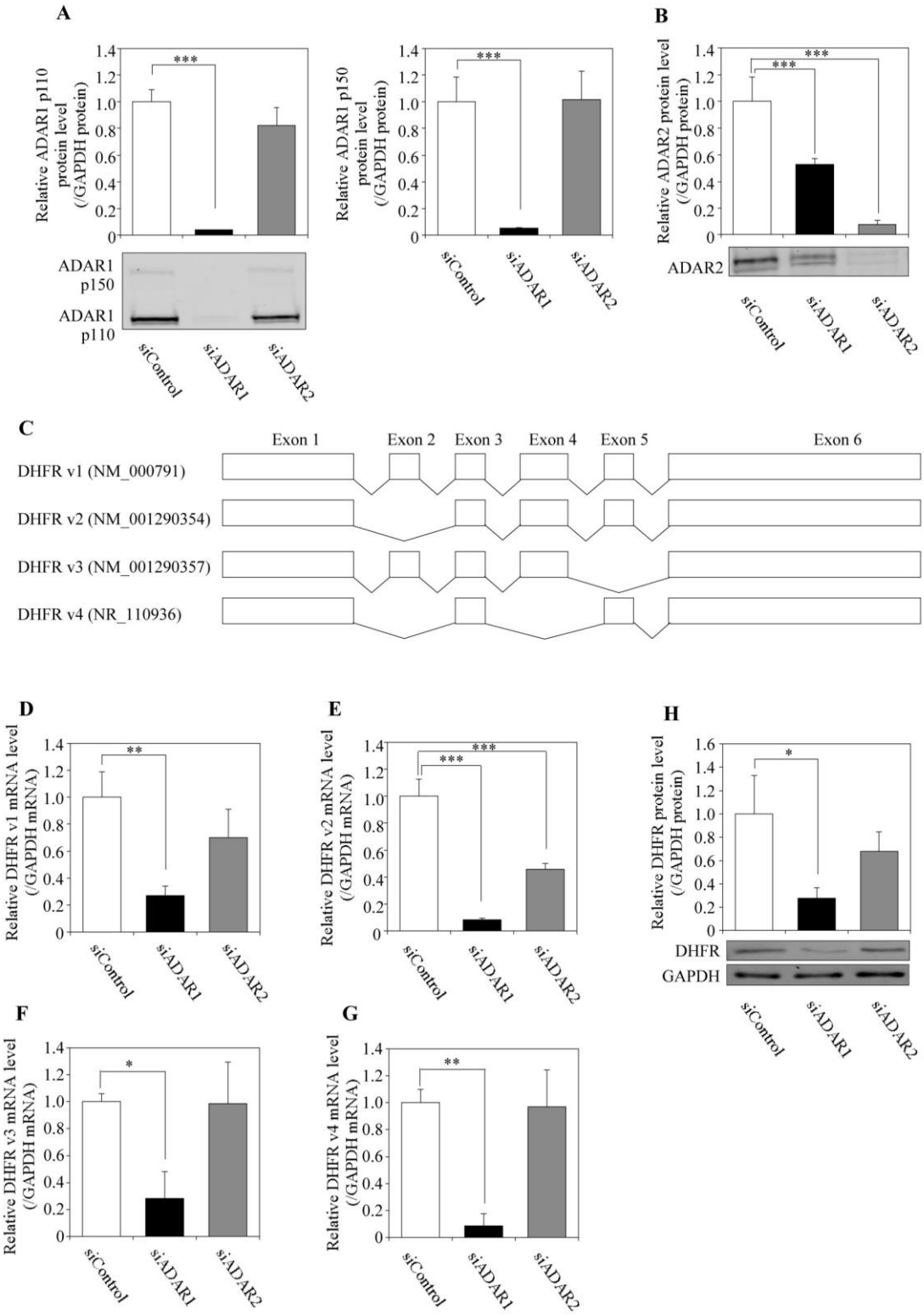

Fig. 15. Effects of ADAR1 or ADAR2 knockdown on DHFR expression levels in MCF-7 cells. ADAR1 p110 and ADAR1 p150 protein (**A**), ADAR2 protein (**B**) in MCF-7 cells 72 h after transfection with 5 nM siADAR1, siADAR2 or siControl. (**C**) Schematic representation of human DHFR transcript variants (v1-v4). Open rectangles indicate exons. DHFR v1 (**D**), v2 (**E**), v3 (**F**), v4 (**G**) mRNA, and DHFR protein (**H**) levels in MCF-7 cells transfected with siRNA were determined by Western blotting or real-time RT-PCR, and were normalized to the GAPDH. The values represent the levels relative to the siControl. Each column represents the mean ± SD of three independent experiments. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001.

ADAR1 Edits the 3'-UTR of DHFR. The RADAR database of RNA editing sites indicates the potential RNA editing in the DHFR intron 3, 4, 5 and 3'-UTR. Given that ADAR1 positively regulates DHFR expression regardless of transcript variants, the effect of RNA editing in the 3'-UTR rather than the intron on DHFR expression was investigated. To investigate whether the 3'-UTR of DHFR is actually subjected to RNA editing in breast cancer cells, direct sequence analyses of DHFR genomic DNA and cDNA from MCF-7 cells were performed. Because 'I' is transcribed as 'G' by reverse transcriptase used in RT-PCR, A-to-I RNA edited sites were detected as overlapping peak of 'A' and 'G'. As the result, 26 edited sites in the 3'-UTR of DHFR were identified (Fig. 16A). They were located within inverted Alu repeats, which can form a dsRNA structure, a typical target of ADAR enzymes. To investigate whether ADAR1 or ADAR2 enzyme is responsible for the RNA editing events, sequence of the DHFR cDNA from MCF-7 cells transfected with siADAR1 or siADAR2 was analyzed. Typical sequencing electropherograms of these RNA edited sites are shown in Fig. 16B. The RNA editing levels of all editing sites were significantly reduced by ADAR1 knockdown, but not by ADAR2 knockdown (Fig. 16C). These results indicated that ADAR1 plays a critical role in editing the 3'-UTR of DHFR.

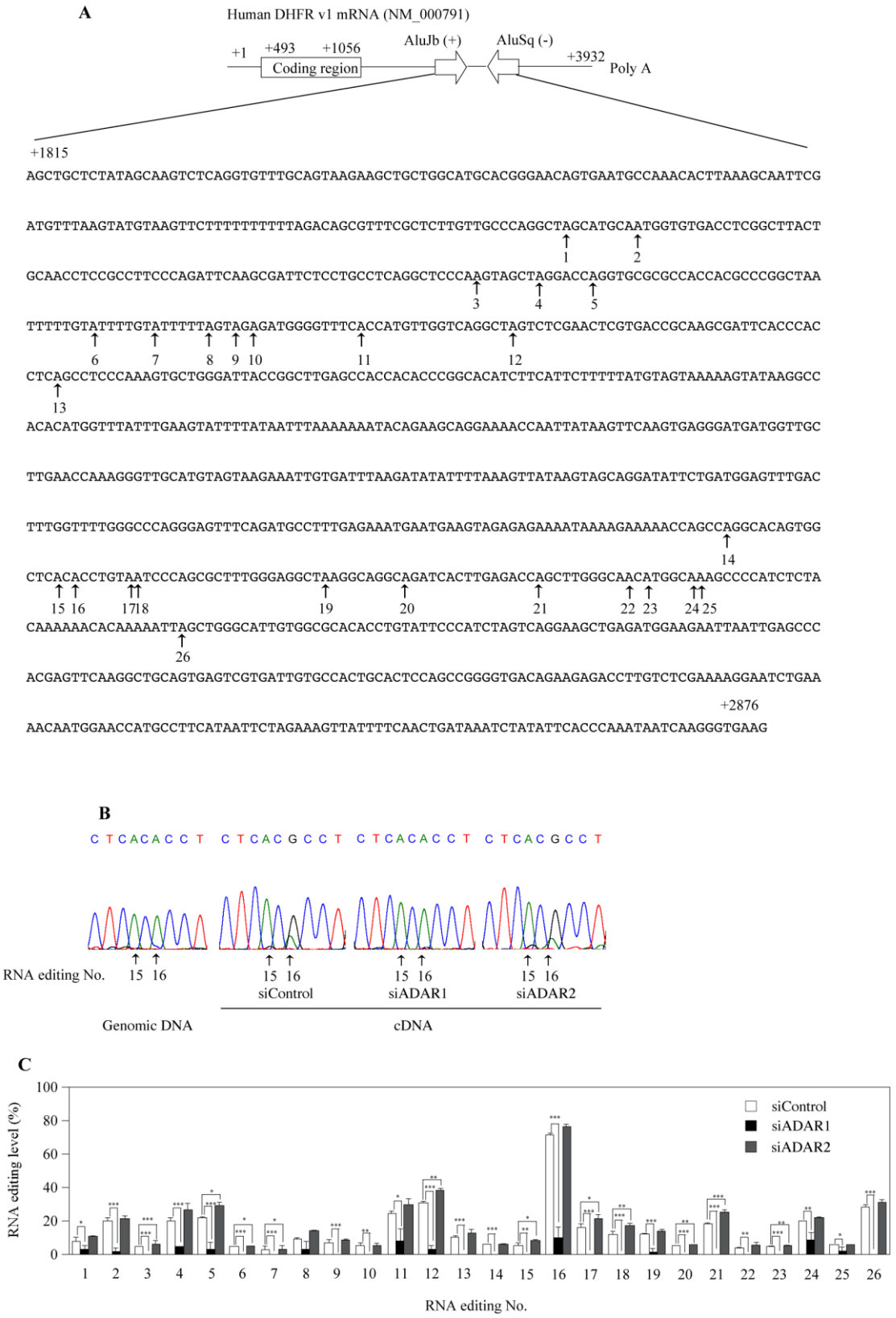

Fig. 16. Effects of ADAR1 or ADAR2 knockdown on RNA editing levels in the 3'-UTR of DHFR. (**A**) Schematic representation of human DHFR v1 mRNA and RNA editing sites in the 3'-UTR of DHFR. Open arrows indicate the direction of the Alu elements. Arrows show the RNA editing sites, numbered as 1-26 from 5' to 3'. (**B**) Representative electropherograms from the direct sequencing that used genomic DNA and cDNA from MCF-7 cells as a template. (**C**) RNA editing levels in the 3'-UTR of DHFR in MCF-7 cells 72 h after transfection with 5 nM siADAR1, siADAR2 or siControl. The editing level was calculated as described in Materials and Methods. Each column represents the mean ± SD of three independent experiments. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

Stabilization of DHFR mRNA by RNA Editing. To determine whether the suppression of DHFR mRNA by ADAR1 knockdown is due to accelerated mRNA degradation, the effects of ADAR1 knockdown on the stability of the DHFR mRNA was examined. In the presence of α-amanitin, the half-life of the DHFR mRNA was 32.1 h, and it was shortened to 13.1 h by the knockdown of ADAR1 (Fig. 17A), suggesting the possibility that a edited DHFR mRNA is more stable than non-edited mRNA. To further investigate the difference of stability between non-edited and edited DHFR mRNA, direct sequence analysis using cDNA derived from siControl-transfected and α-amanitin-treated MCF-7 cells was performed. By treatment of α-amanitin for 24 h, the height of 'G' and that of 'A' were increased and decreased, respectively (Fig. 17B). The levels of DHFR mRNA of which sequence was non-edited or edited in each site were calculated by multiplying DHFR level shown in Fig. 17A by the ratio of the peak height of 'A' or 'G', respectively. (Fig. 17C). As the result, it was demonstrated that the non-edited DHFR mRNA was dramatically decreased by treatment of α-amanitin for 24 h, whereas the edited DHFR was not changed. Collectively, it was found that DHFR mRNA was stabilized by ADAR1-mediated RNA editing.

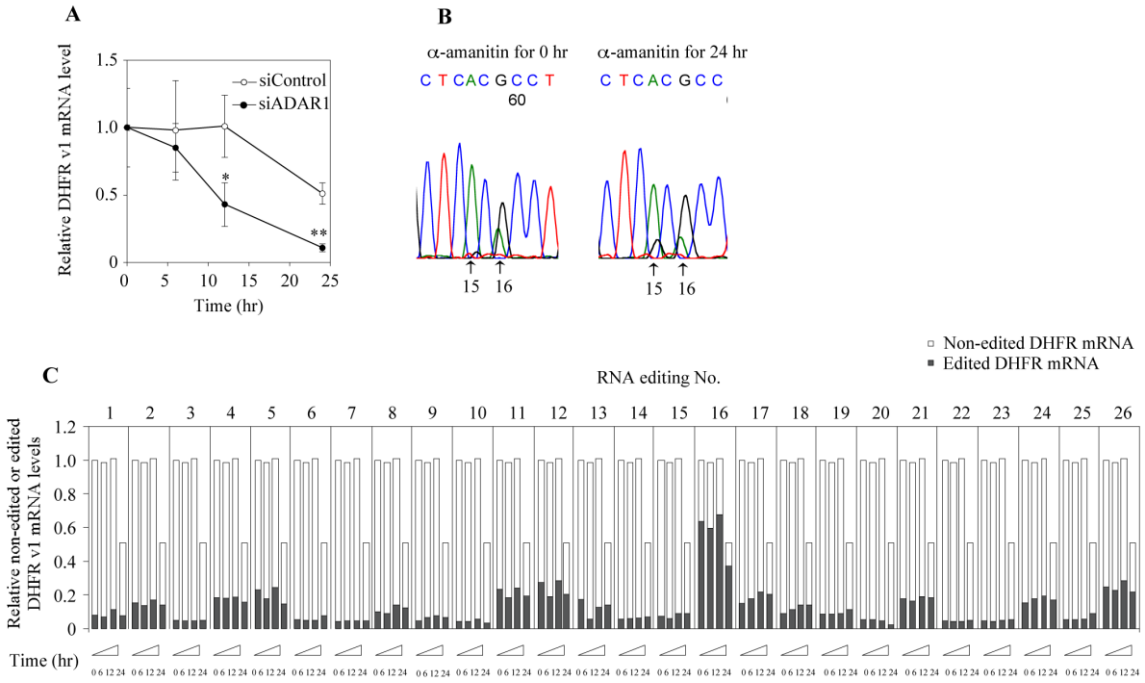

Fig. 17. Effects of RNA editing on DHFR mRNA stability. (**A**) MCF-7 cells 36 h after transfection with 5 nM siADAR1 or siControl were treated with 10 μg/ml of α-amanitin. Total RNA was prepared at 0, 6, 12 and 24 h later. The DHFR mRNA levels were determined using real-time RT-PCR. The values are expressed as relative to the values at 0 h. **P* < 0.05 and ***P* < 0.01 compared with siControl. (**B**) Representative electropherograms from the direct sequencing that used cDNA from MCF-7 cells treated with α-amanitin for 0 or 24 h as a template. (**C**) The expression levels of DHFR mRNA which is non-edited or edited in each editing sites were calculated by multiplying DHFR mRNA expression by the ratio of the peak height of 'A' or 'G', respectively, in the electropherograms resulting from the direct sequencing of PCR amplicons that used cDNA from these cells as a template. The values are expressed as relative to the values of total DHFR mRNA levels at 0 h. Each column represents the mean (n = 3).

RNA Editing of the DHFR Transcript Destroy miRNA Target Sites. It was surmised that the decrease in the extent of RNA editing by ADAR1 knockdown would augment the binding of miRNA(s) to the 3'-UTR of DHFR to decrease its expression, because the 3'-UTR includes binding sites for miRNA, which down-regulates gene expression via translational repression or mRNA degradation (Bartel, 2004). *In silico* analysis using miRediTar (http://microrna.osumc.edu/mireditar) suggested a possibility that miR-25-3p and miR-125a-3p, which are reported to be expressed in MCF-7 (Fix et al., 2010), would bind to the non-edited sequence in the 3'-UTR of DHFR but not to edited sequence (Fig. 18A). To examine whether RNA editing affects the recognition of miR-25-3p and miR-125a-3p, luciferase assay was performed using reporter plasmids

carrying either a non-edited MRE25 (pGL3p/MRE25(A)), an edited MRE25 (pGL3p/MRE25(G), a non-edited MRE125 (pGL3p/MRE125(A)) or an edited MRE125 (pGL3p/MRE125(G) in the 3'-UTR of DHFR. The luciferase activity of the pGL3p/MRE25(A) and pGL3p/MRE125(A) plasmid was significantly reduced (68% and 71% of control, respectively) by the over-expression of miR-25 and miR-125a-3p, respectively, whereas the decrease of luciferase activity of the pGL3p/MRE25(G) and pGL3p/MRE125(G) plasmid were marginal (88% and 91% of control, respectively) (Fig. 18B). The decrease in the activity of these plasmids containing edited sequence would be irrelevant with the inserted fragment of the 3'-UTR of DHFR, because slightly reduction of activities of empty plasmid was observed. This result indicated that miR-25-3p and miR-125a-3p recognizes the non-edited but not edited sequence within the 3'-UTR of DHFR. To examine the effects of the non-edited DHFR-specific regulation by these miRNAs on ADAR1-mediated regulation of DHFR expression, over-expression or inhibition experiment was performed by using miRNA mimic or AsO, respectively. When miR-25-3p or miR-125a-3p mimic was co-transfected with siADAR1, the decrease of DHFR mRNA (Fig. 18C) and protein (Fig. 18D) levels by knockdown of ADAR1 was enhanced. On the other hand, inhibition of these miRNA function attenuated the knockdown of ADAR1-induced decrease of DHFR expression (Fig. 18E and F). These results indicated that the non-edited DHFR-specific down-regulation by miR-25-3p and miR-125a-3p would be attribute to reduction of DHFR expression by ADAR1 knockdown.

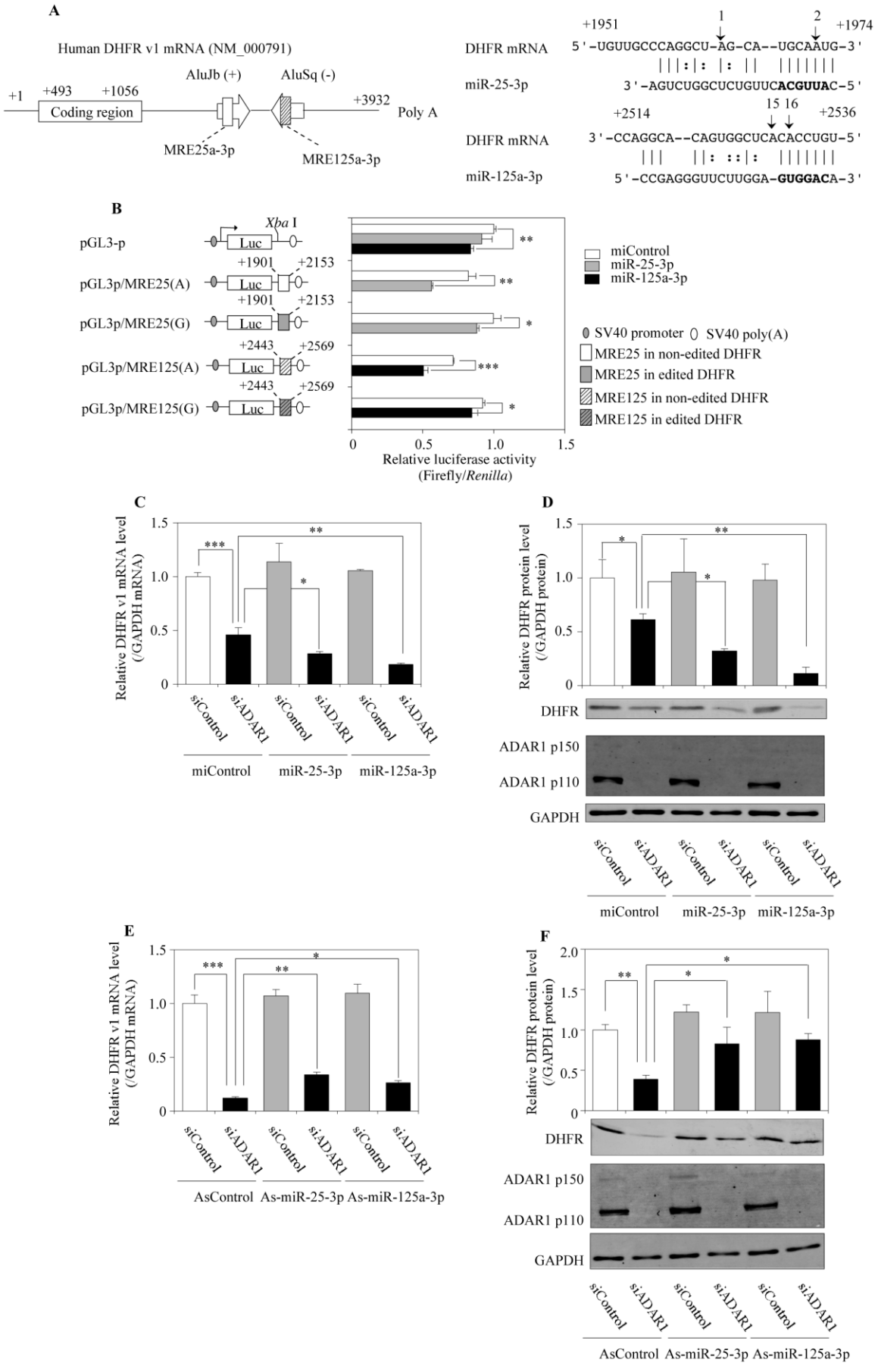

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Fig. 18. Roles of miR-25-3p and miR-125a-3p on ADAR1-dependent regulation of DHFR in MCF-7 cells. (**A**) Schematic representation of human DHFR mRNA, and the predicted MREs of miR-25-3p, miR-125a-3p (named as MRE25 and MRE125, respectively) in non-edited DHFR mRNA. Open arrows indicate the direction of the Alu elements. Bold letters represent seed sequences. RNA editing sites are shown by numbered (refer to fig. 16A) arrows. (**B**) Luciferase assay using plasmids containing a fragment of the 3'-UTR of DHFR. MCF-7 cells were transfected with the reporter plasmids (190 ng) along with the phRL-TK plasmid (10 ng) and the miR-25-3p, miR-125a-3p mimic or miControl. Firefly luciferase activity was normalized to *Renilla* luciferase activity. The values are expressed relative to the pGL3-p plasmid. DHFR mRNA (**C** and **E**) and protein (**D** and **F**) levels in MCF-7 cells 72 h after co-transfection of 10 nM siRNA (siADAR1 or siControl) and 10 nM miRNA mimic (miR-25-3p, miR-125a-3p mimic, or miControl) (**C** and **D**) or co-transfection of 10 nM siRNA and 10 nM AsO (As-miR-25a-3p, As-miR-125a-3p, or AsControl) (**E** and **F**) were determined by real time PCR or Western blot analysis, and were normalized to GAPDH. The values are expressed relative to the levels observed following the co-transfection of MCF-7 cells with control. Each column represents the mean ± SD of three independent experiments. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

Decrease in Cell Viability and Resistance to Methotrexate of MCF-7 Cells by ADAR1 knockdown. It was demonstrated that the viability of MCF-7 cells was significantly decreased by knockdown of ADAR1 (Fig. 19A). Because DHFR has a key role in regulating the DNA synthesis by converting dihydrofolate into tetrahydrofolate, it was postulated that the decrease in the cell viability would be due to the decreased in DHFR protein level. To determine whether this hypothesis is true, folinic acid, a molecule that is readily converted into tetrahydrofolate, was added to the siADAR1-transfected MCF-7 cells (Lladó et al., 2009). As shown in Fig. 19B, the decreased cell viability by ADAR1 knockdown was partially restored by treatment of folinic acid. This result suggests that reduction of DHFR protein level is one of the causes of the decreased cell viability by ADAR1 knockdown.

Next, it was investigated whether ADAR1 knockdown enhances sensitivity to methotrexate of MCF-7 cells, because it is known that tumors with lower expression of DHFR are more sensitive to methotrexate treatment (Salonga et al., 2000; Banerjee et al., 2002). In this experiment, the concentration of siADAR1 was decreased to 0.5 nM to determine the effects of methotrexate on the cell viability. As the result, after 48 and 72 h treatment of methotrexate, the cell number was lower in siADAR1-transfected cells than in that siControl-transfected cells (Fig. 19C), suggesting that the anti-proliferative effects of methotrexate was enhanced by ADAR1 knockdown.

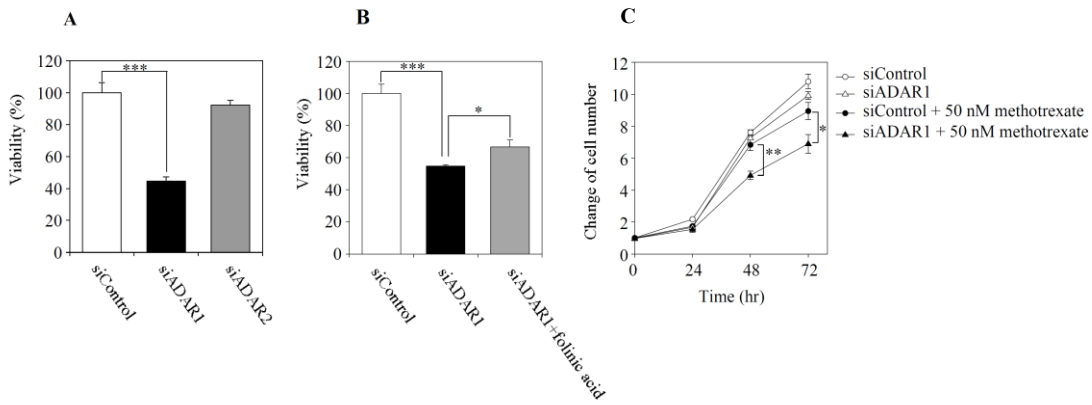

Fig. 19. Effects of ADAR1-dependent regulation of DHFR expression on viability and sensitivity to methotrexate of MCF-7 cells. (**A**) The viability of MCF-7 cells 72 h after transfection with 5 nM siADAR1, siADAR2 and siControl. The values are expressed as relative to the values of siControl. (**B**) The viability of siADAR1-transfected MCF-7 cells incubated for 72 h in the presence of 200 μM folinic acid. The values are expressed as relative to the values of siControl. (**C**) MCF-7 cells 24 h after transfection with 0.5 nM siADAR1 or siControl were treated with 50 nM methotrexate. After 0, 24, 48 and 72 h, the cell number was evaluated by WST-8 assay. The values are expressed as relative to the values of 0 h. Each column and data represents the mean ± SD of three independent experiments. **P* < 0.05 ***P* < 0.01 and ****P* < 0.001.

Higher ADAR1 Expression and RNA Editing Levels in DHFR mRNA in Breast Cancer Tissues than in Normal Tissues. To examine the significance of ADAR1 expression in RNA editing levels in the 3'-UTR of DHFR *in vivo*, RNA editing levels in DHFR transcript were determined using breast cancer tissues and adjacent normal tissues from 19 patients. The expression levels of ADAR1 mRNA tended to be higher in breast cancer tissues than in adjacent normal tissues (Fig. 20A) in consistent with a previous report (Fumagalli et al., 2015). Next, the RNA editing levels in the 3'-UTR of DHFR were evaluated using samples with higher (n = 3) or lower (n = 3) ADAR1 levels in cancer tissue than normal tissues. In the former samples, the RNA editing levels in DHFR transcript were higher in cancer tissues than in adjacent normal tissues (Fig. 20B), but not in the latter samples (Fig. 20C). These results suggest that the over-expression of ADAR1 in breast cancer contributes to hyper-editing of DHFR transcript, which may cause increase in DHFR expression levels.

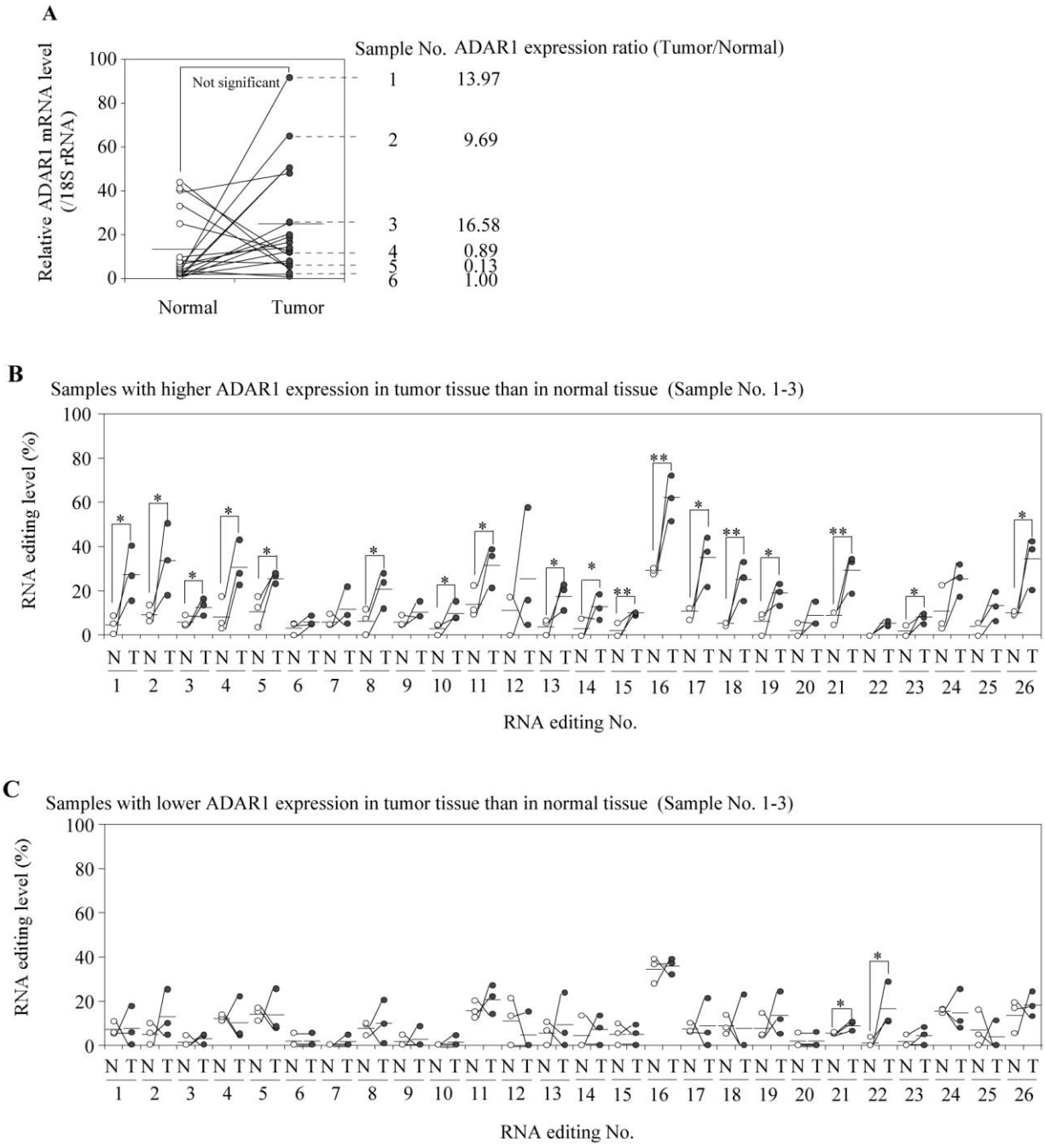

Fig. 20. Expression levels of ADAR1 mRNA and RNA editing levels in the 3'-UTR of DHFR in human breast cancer tissues. (**A**) Expression levels of ADAR1 mRNA in breast cancer tissues and adjacent normal tissues obtained from 19 patients were determined by real-time RT-PCR. The expression levels were normalized with the 18S rRNA level. Data represent the mean of two independent experiments. The values are expressed relative to the sample with the lowest levels. Horizontal bars represent the mean. The RNA editing levels in the 3'-UTR of DHFR in breast cancer tissues with higher (No. 1-3) (**B**) and lower (No. 4-6) (**C**) than adjacent normal tissues. The editing level was calculated as described in Materials and Methods. N: normal tissue, T: tumor tissue. Each column represents the mean of two independent experiments. *$P < 0.05$ and **$P < 0.01$.

Discussion

The possibility that RNA editing may alter miRNA recognition has been proposed (Liang and Landweber, 2007; Peng et al., 2012), but the evidence is very limited (Borchert et al., 2009; Wang et al., 2013; Nakano et al., 2016). In this study, it was examined whether ADAR1 regulates DHFR expression by editing 'A's within the miRNA binding sites in the 3'-UTR. This finding provides further experimental evidence for the role of RNA editing in target recognition by miRNA.

It was found that ADAR1-mediated RNA editing events occur in the 3'-UTR of DHFR in MCF-7 cells and breast cancer tissues (Fig. 16B, C, Fig. 20B and C). The RNA editing sites were identified in inverted Alu repeats, which can form perfectly matched dsRNA structure. These results were consistent with a recent report showing that ADAR1, but not ADAR2, is the main RNA editing enzyme for reversely oriented Alu repeats (Stellos et al., 2016). In this study, knockdown of ADAR2 marginally increased RNA editing levels in the 3'-UTR of DHFR. It is known that homodimerization is important for functions of both human ADAR1 and ADAR2, whereas heterodimerization between ADAR1 and ADAR2 has been shown to decrease editing activity (Deffit and Hundley, 2016). The increased RNA editing levels by ADAR2 knockdown may be due to the increase in functional ADAR1 homodimerization along with decrease in the ratio of heterodimeraization of ADAR1 and ADAR2.

It was found that the degradation of DHFR mRNA was facilitated by ADAR1 knockdown in MCF-7 cells (Fig. 17A). Supporting this result, the higher stability of edited DHFR mRNA than non-edited mRNA was observed (Fig. 17C), suggesting that RNA editing is the main factor to determine DHFR mRNA degradation rate. The underlying mechanism of RNA editing-dependent regulation of DHFR expression was investigated focusing on miRNA. By a computer analysis, miR-25-3p and miR-125a-3p were predicted to regulate non-edited but not edited DHFR expression (Fig. 18A). The non-edited sequence-specific recognition by these miRNAs were experimentally

demonstrated by luciferase assay (Fig. 18B). Through co-transfection of siADAR1 with miRNA mimics or AsO, it was demonstrated that ADAR1-mediated regulation of DHFR requires function of these miRNAs (Fig. 18C-F). miR-25-3p and miR-125a-3p have been reported to regulate the expression of Smad7 and breast cancer early onset gene 1 in MCF-7 cells, respectively (Smith et al., 2012; Xu et al., 2016), suggesting the functional expression of these miRNAs in this cell lines. The present study indicated that RNA editing in the 3'-UTR of DHFR would allow DHFR mRNA to escape from repression by these miRNAs, leading to elevation of DHFR expression in breast cancer cells. To examine whether the decrease in DHFR expression by ADAR1 knockdown is fully owing to the function of miR-25-3p and miR-125a-3p, double transfection of AsOs for these miRNAs with siADAR1 was performed. As the result, double inhibition of these miRNAs could not completely restore the decreased DHFR expression level by knockdown of ADAR1 (data not shown), suggesting a possibility that the other factors may be also involved in this regulatory mechanism. It is highly possible that miR-92a-3p and miR-92b-3p have the similar role as miR-25-3p, because these miRNAs shares the seed sequence. Beside miRNAs, a recent study revealed that RNA editing within reversely oriented Alu repeats in the 3'-UTR of cathepsin S enables to recuit RNA-binding protein human antigen R to this region, thereby increasing its mRNA stability (Stellos et al., 2016). Further studies are needed to uncover the significance of these factors on DHFR expression.

Another possible mechanism for the decrease in DHFR expression by ADAR1 knockdown is induction of negative regulators such as miRNA for DHFR expression, because A-to-I editing events can alter the processing of miRNA, thereby affecting miRNA expression (Yang et al., 2006; Kawahara et al., 2008). It would be of interest to investigate the effects of RNA editing on expression levels of miRNAs including miR-24, miR-192 and miR-215, which were reported to regulate DHFR expression (Mishra et al., 2007; Song et al., 2008; Song et al., 2010).

By ADAR1 knockdown, the viability of MCF-7 cells were decreased (Fig. 19A) in

consistent with a previous report (Fumagalli et al., 2015), suggesting the oncogenic ability of ADAR1 in breast cancer cells. The decrease was partially restored by treatment of folinic acid, which can supply folic acid derivatives (Fig. 19B). These results suggest that DHFR is likely to be one of the downstream editing targets that are responsible for the oncogenic function of ADAR1. In addition, as shown in Fig. 19C, ADAR1 knockdown enhanced methotrexate sensitivity of MCF-7 cells although the extent was small. A previous report indicated that MCF-7 cells are more resistant to methotrexate (IC_{50} value: 114 nM) than other cell lines (IC_{50} value: 6-38 nM) because MCF-7 cells have mutations in *reduced folate career* gene, leading to decrease in methotrexate uptake (Yoon et al., 2010). Thus, we tested the effects of ADAR1 knockdown on sensitivity to methotrexate of A549 cells which have relatively lower IC_{50} value of methotrexate: 38 nM. In accordance with the report by Yoon et al. (2010), the anti-proliferative effect of methotrexate on A549 cells (52.9% of control at 72 hr) (Fig. 21) was stronger than that of MCF-7 cells (82.8% of control at 72 hr) (Fig. 19C). However, the effect of ADAR1 knockdown on sensitivity to methotrexate in A549 cells (73.0% of control at 72 h) was as small as that in MCF-7 cells (77.1% of control at 72 h) (Fig. 21), indicating that the contribution of ADAR1 to resistance is not different between MCF-7 and A549 cells. It has been reported that dihydrofolate reductase like 1, whose gene is known as pseudogene *DHFRP4*, is expressed and has same function as DHFR in folate metabolism (McEntee et al., 2011). It is possible that this enzyme may complement the decreased expression of DHFR by ADAR1 knockdown, leading to residual resistance to methotrexate in these cell lines.

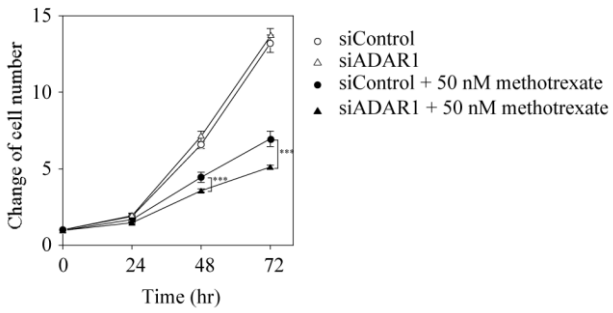

Fig. 21. Effects of ADAR1-dependent regulation of DHFR expression on sensitivity to methotrexate of A549 cells. A549 cells 24 h after transfection with 1 nM siADAR1 or siControl were treated with 50 nM methotrexate. After 0, 24, 48 and 72 h, the cell number was evaluated by WST-8 assay. The values are expressed as relative to the values of 0 h. Each column and data represents the mean ± SD of three independent experiments. ***$P < 0.001$.

Accumulating evidence suggests a role of ADAR-mediated A-to-I RNA editing in cancer development and progression (Slotkin and Nishikura, 2013). Interestingly, the role of RNA editing differs among cancer types. In brain tumors, ADAR1 and ADAR2 are down-regulated, leading to a global reduction in A-to-I editing, which is functionally required for brain tumor development (Paz et al., 2007). In contrast, ADAR1 has been reported to be up-regulated in hepatocellular carcinoma (Chan et al., 2014) and esophageal squamous cell carcinoma (Qin et al., 2014) and cell-based assay revealed that ADAR1 can induce tumorigenic phenotypes in these cancer cells. More recently, it was revealed that ADAR1 expression and global editing frequency were higher in breast tumors compared to normal tissues (Fumagalli et al., 2015). In consistent with the report, the higher expression levels of ADAR1 in breast cancer tissues than in adjacent normal tissues was observed in the present study, although the difference was not significant owing to the large inter-individual variability in the expression (Fig. 20A). *ADAR1* gene is located on chromosome 1q, of which amplification is often observed in breast cancer tissues (Chen et al., 1989). Fumagalli et al. (2015) reported that ADAR1 expression levels in breast cancer are correlated with the extent of gene amplification as well as the expression of signal transducer and activator of transcription 1 (as a proxy for interferon response). The inter-individual difference in ADAR1 expression in human breast cancer tissues would be explained in part by these factors.

In conclusion, it was clarified that DHFR is post-transcriptionally regulated through ADAR1-mediated RNA editing, affecting cell proliferation and sensitivity to methotrexate of breast cancer cells. This study could provide new insights into the regulatory mechanism of DHFR expression and the role of RNA editing in methotrexate response. ADAR1 may be a potential anti-tumor target for use with anti-folate agents such as methotrexate.

CHAPTER 4

Conclusion

RNA editing is an important post-transcriptional modulation to affect diverse gene expression and function. A-to-I RNA editing is the most frequent type of RNA editing in mammals, and is catalyzed by ADAR enzymes. The biological significance of RNA editing in drug response remains poorly understood. The purpose of this study was to clarify the impact of the RNA editing on expression of pharmacokinetics and pharmacodynamics-related genes, focusing on AhR and DHFR.

In **chapter 2**, the effects of RNA editing in the 3'-UTR of AhR on its expression in human liver cells was examined. AhR is a ligand-dependent transcription factor that regulates the expression of xenobiotic-metabolizing enzymes, including CYP1A1, CYP1A2 and CYP1B1. In 32 human liver samples, significant positive correlation was not observed between AhR mRNA and AhR protein levels, suggesting that post-transcriptional regulation plays an important in AhR expression. Cell-based experiments demonstrated that AhR is negatively regulated by ADAR1-mediated RNA editing. The induction of CYP1A1, a downstream gene of AhR, by TCDD was augmented by ADAR1 knockdown, suggesting that RNA editing affected the expression of P450 isoform. The underlying mechanism for regulation of AhR expression by RNA editing was investigated, focusing on miRNA. The miR-378-dependent downregulation of AhR was abolished by knockdown of ADAR1, indicating that the mechanism of the ADAR1-mediated downregulation of AhR would be attributed to the creation of the miR-378 recognition site in the 3'-UTR of AhR. The inter-individual differences in the RNA editing levels within the 3'-UTR of AhR 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. This study uncovered that A-to-I RNA editing modulates the AhR and its downstream P450 expression in human liver.

In **chapter 3**, potential role of RNA in the regulation of DHFR expression in human breast cancer cells was investigated. It was found that the 3'-UTR of DHFR was subjected to RNA editing in a breast cancer-derived MCF-7 cells. Experiments using MCF-7 cells demonstrated that DHFR is positively regulated by ADAR1-mediated RNA editing which stabilizes DHFR mRNA. Luciferase assay revealed that miR-25-3p and miR-125a-3p can bind to the 3'-UTR of non-edited DHFR but not to the edited sequence. The decrease in DHFR expression by knockdown of ADAR1 was restored by inhibition of these microRNAs, suggesting that RNA editing by ADAR1 destroys these miRNA recognition elements to increase DHFR expression. The knockdown of ADAR1 decreased cell viability and increased the sensitivity to methotrexate of MCF-7 cells. These results demonstrated that ADAR1 positively regulates the expression of DHFR through RNA editing by disrupting the binding of miRNAs to the 3'-UTR of DHFR, thus enhancing cellular proliferation and resistance to methotrexate. The RNA editing levels in breast cancer tissues were higher than in normal tissues, suggesting that over-expression of ADAR1 in breast cancer contributes to hyper-editing of DHFR transcript, which may increase the DHFR expression levels.

In conclusion, it was clarified that RNA editing modulates AhR and DHFR expression by creating or destroying miRNA recognition sites in their 3'-UTRs, respectively. These studies are the first to demonstrate the biological significance of RNA editing of pharmacokinetics- and pharmacodynamics-associated genes.

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LIST OF PUBLICATIONS

Main publications

1. **Nakano M**, Fukami T, Gotoh S, Takamiya M, Aoki Y and Nakajima M (2016) RNA editing modulates human hepatic aryl hydrocarbon receptor expression by creating microRNA recognition sequence. *J Biol Chem* **291**: 894-903.
2. **Nakano M**, Fukami T, Gotoh S and Nakajima M A-to-I RNA editing up-regulates human dihydrofolate reductase in breast cancer. *J Biol Chem* in press.

Supplemental publications

1. Asakura M, **Nakano M**, Hayashida K, Fujii H, Nakajima M, Atsuda K, Itoh T and Fujiwara R (2014) Human nitrilase-like protein does not catalyze the hydrolysis of vildagliptin. *Drug Metab Pharmacokinet*. **29**: 463-469.
2. **Nakano M**, Fukushima Y, Yokota S, Fukami T, Takamiya M, Aoki Y, Yokoi T and Nakajima M (2015) CYP2A7 pseudogene transcript affects CYP2A6 expression in human liver by acting as a decoy for miR-126*. *Drug Metab Dispos* **43**: 703-712.
3. **Nakano M**, Mohri T, Fukami T, Takamiya M, Aoki Y, McLeod HL and Nakajima M (2015) Single-nucleotide polymorphisms in cytochrome P450 2E1 (CYP2E1) 3'-untranslated region affect the regulation of CYP2E1 by miR-570 *Drug Metab Dispos* **43**: 1450-1457.

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