



## Human CYP2E1 is regulated by miR-378

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### ABSTRACT

Human CYP2E1 is one of the pharmacologically and toxicologically important cytochrome P450 isoforms. Earlier studies have reported that the CYP2E1 expression is extensively regulated by post-transcriptional and post-translational mechanisms, but the molecular basis remains unclear. In the present study, we examined the possibility that microRNA may be involved in the post-transcriptional regulation of human CYP2E1. In silico analysis identified a potential recognition element of miR-378 (MRE378) in the 3'-untranslated region (UTR) of human CYP2E1 mRNA. Luciferase assays using HEK293 cells revealed that the reporter activity of the plasmid containing the MRE378 was decreased by co-transfection of precursor miR-378, indicating that miR-378 functionally recognized the MRE378. We established two HEK293 cell lines stably expressing human CYP2E1 including or excluding 3'-UTR. When the precursor miR-378 was transfected into the cells expressing human CYP2E1 including 3'-UTR, the CYP2E1 protein level and chlorzoxazone 6-hydroxylase activity were significantly decreased, but were not in the cells expressing CYP2E1 excluding 3'-UTR. In both cell lines, the CYP2E1 mRNA levels were decreased by overexpression of miR-378, but miR-378 did not affect the stability of CYP2E1 mRNA. In a panel of 25 human livers, no positive correlation was observed between the CYP2E1 protein and CYP2E1 mRNA levels, supporting the post-transcriptional regulation. Interestingly, the miR-378 levels were inversely correlated with the CYP2E1 protein levels and the translational efficiency of CYP2E1. In conclusion, we found that human CYP2E1 expression is regulated by miR-378, mainly via translational repression. This study could provide new insight into the unsolved mechanism of the post-transcriptional regulation of CYP2E1.

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### 1. Introduction

Human cytochrome P450 (CYP) 2E1 catalyzes the metabolism of numerous low molecular-weight xenobiotics including drugs (e.g., acetaminophen, isoniazid), organic solvents (e.g., ethanol, acetone, carbon tetrachloride), and procarcinogens (e.g., *N*-nitrosodimethylamine) [1]. CYP2E1 is induced by its own substrates such as isoniazid, ethanol, and acetone, resulting in the enhancement of their metabolism [2]. It should be noted that the induction of CYP2E1 protein by these chemicals was not necessarily accompanied by an increase of CYP2E1 mRNA level [3]. The proposed mechanisms for the induction of CYP2E1 are the stabilization of mRNA [4] or protein [5]. Previously, Sumida et al. [6] reported that the CYP2E1 mRNA levels in 15 human liver samples were not positively correlated with the chlorzoxazone 6-hydroxylase activities, which is a probe activity of CYP2E1. In

addition, special attention should be paid to the fact that CYP2E1 is the most abundant isoform among all P450s in human liver (56% of total P450) at the mRNA level, followed by CYP2C9, CYP2C8 and CYP3A4 (8–11% of total P450) [7], whereas it is the fourth most abundant isoform (about 7% of total P450) at the protein level after CYP3A (30% of total P450), CYP2C (20% of total P450), and CYP1A2 (about 13% of total P450) [8]. Collectively, the post-transcriptional regulation would be responsible for not only the inducible but also the constitutive expression of CYP2E1 in liver. However, the molecular basis of the human CYP2E1 regulation largely remains unknown, in contrast to the other human P450s for which much progress has been made in understanding the regulation mechanisms at the transcriptional level.

To uncover the molecular mechanism of the post-transcriptional regulation of CYP2E1, we sought to determine whether microRNA (miRNA) might be involved in the regulation of CYP2E1. MiRNAs, an evolutionarily conserved class of endogenous ~22-nucleotide non-coding RNAs, recognize the 3'-untranslated region (3'-UTR) of the target mRNA and cause translational repression or mRNA degradation [9]. The regulation by miRNAs is involved in

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diverse biological processes, including development, cell proliferation, differentiation, apoptosis, and cancer initiation and progression [10–12]. The human genome may contain up to 1000 miRNAs and 30% of human mRNAs are predicted to be targets of miRNAs [13]. However, the targets of miRNAs largely remain to be identified. Based on the evidence of the post-transcriptional regulation, we investigated whether miRNAs might be involved in the regulation of human CYP2E1.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Chlorzoxazone, 6-hydroxychlorzoxazone, and coumarin were from Sigma–Aldrich (St. Louis, MO). NADP<sup>+</sup>, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Oriental Yeast (Tokyo, Japan). The pGL3-promoter vector, phRL-TK plasmid, pTARGET vector, and a dual-luciferase reporter assay system were purchased from Promega (Madison, WI). LipofectAMINE2000 and LipofectAMINE RNAiMAX were from Invitrogen (Carlsbad, CA). Pre-miR miRNA Precursors for miR-378 and negative control #1 were from Ambion (Austin, TX). RNAiso, random hexamer, and SYBR Premix Ex Taq were from Takara (Shiga, Japan). ROX was purchased from Stratagene (La Jolla, CA). ReverTra Ace was obtained from Toyobo (Osaka, Japan). All primers and oligonucleotides were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). G418 was obtained from Wako Pure Chemicals (Osaka, Japan).  $\alpha$ -Amanitin was purchased from Calbiochem (San Diego, CA). Goat anti-human CYP2E1 polyclonal antibodies and rabbit anti-human GAPDH polyclonal antibodies were from Daiichi Pure Chemicals (Tokyo, Japan) and IMGENEX (San Diego, CA), respectively. Restriction enzymes were from Takara, Toyobo, and New England Biolabs (Ipswich, MA). All other chemicals and solvents were of the highest grade commercially available.

### 2.2. Construction of reporter plasmids

Various fragments were inserted into the *Xba* I site, downstream of the luciferase gene in the pGL3-promoter vector. The sequence from +1559 to +1580 of the human CYP2E1 mRNA (5'-TCA AAT TGT TTG AGG TCA GGA T-3') was termed the miR-378 recognition element (MRE378). A fragment containing three copies of the MRE378, 5'-CTA GAG TTT TCA AAT TGT TTG AGG TCA GGA TTT CTC GTT TTC AAA TTG TTT GAG GTC AGG ATT TCT CGT TTT CAA ATT GTT TGA GGT CAG GAT TTC TCT-3' (MRE378 is italicized), was cloned into the pGL3-promoter vector (pGL3/3xMRE). The complementary sequence of three copies of the MRE378 was also cloned into the pGL3-promoter plasmid (pGL3/3xMRE-Rev). A fragment containing the perfectly matching sequence with the mature miR-378, 5'-CTA GAA CAC AGG ACC TGG AGT CAG GAG T-3' (the matching sequence of miR-378 is italicized), was cloned into the pGL3-promoter vector (pGL3/c-378). A fragment from +1549 to +1627 of the human CYP2E1 mRNA containing the MRE378 was cloned into the pGL3-promoter vector, resulting in single (pGL3/UTR1) and double (pGL3/2xUTR1) insertion. A fragment +1627 to +1657 excluding the MRE378 was also cloned into the pGL3-promoter vector, resulting in single (pGL3/UTR2) insertion. DNA sequencing analyses using Long-Read Tower DNA sequencer (GE Healthcare Bio-Sciences, Piscataway, NJ) confirmed the nucleotide sequences of these constructed plasmids.

### 2.3. Cell culture and luciferase assay

Human embryonic kidney cell line HEK293 was obtained from the American Type Culture Collection (Manassas, VA). HEK293

cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 4.5 g/l glucose, 10 mM HEPES, and 10% fetal bovine serum (Invitrogen). These cells were maintained at 37 °C under an atmosphere of 5% CO<sub>2</sub>–95% air.

Various luciferase reporter plasmids (pGL3) were transiently transfected with phRL-TK plasmid into the HEK293 cells. Briefly, the day before transfection, the cells were seeded into 24-well plates. After 24 h, 170 ng of pGL3 plasmid, 30 ng of phRL-TK plasmid and the precursors for miR-378 or control were co-transfected using LipofectAMINE 2000. After incubation for 48 h, the cells were lysed with a passive lysis buffer and then the luciferase activity was measured with a luminometer (Wallac, Turku, Finland) using the dual-luciferase reporter assay system.

### 2.4. Establishment of two HEK293 cell lines stably expressing human CYP2E1 including or excluding 3'-UTR

A fragment containing the full-length coding region as well as 3'-UTR of the human CYP2E1 cDNA (from +34 to +1667) was amplified by PCR using the primers of 5'-ATG TCT GCC CTC GGA GTC AC-3' and 5'-AAA ATA ATC ATG TGA TGA TTT ATT TAT ATT CTG GG-3'. A fragment containing only the full-length coding region of the human CYP2E1 cDNA (from +34 to +1516) was also amplified using the primers of 5'-ATG TCT GCC CTC GGA GTC AC-3' and 5'-CTC ATG AGC GGG GAA TGA CA-3'. These fragments were subcloned into the pTARGET vector and the resultants were termed pTARGET/CYP2E1 + UTR and pTARGET/CYP2E1. The nucleotide sequences of the plasmids were confirmed by DNA sequencing analyses. HEK293 cells were seeded into 24-well plates, and 500 ng of pTARGET/CYP2E1 + UTR or pTARGET/CYP2E1 plasmids were transfected using LipofectAMINE 2000 according to the manufacturer's protocols. At 48 h post-transfection, the cells were passaged and subsequently grown in medium containing 400  $\mu$ g/ml G418, and diluted from 1:10 to 1:1000. The selective medium was changed every 3–4 days and individual G418-tolerable colonies were selected after 2 weeks in culture. Tolerable clones were screened by immunoblotting and measurement of enzyme activity. We confirmed that the CYP2E1 protein level and the enzyme activity were sustained regardless of the repeated subculture.

### 2.5. Transfection of precursor for miR-378 into HEK293/2E1 + UTR cells and HEK293/2E1 cells and preparation of cell homogenates and total RNA

The precursor for miR-378 was transfected into the HEK293/2E1 + UTR cells and HEK293/2E1 cells as follows: the day before transfection, the cells were seeded into 6- or 12-well plates. After 24 h, 10 nM precursors for miR-378 or control were transfected into HEK293/2E1 + UTR and HEK293/2E1 cells using LipofectAMINE RNAiMAX. After incubation for 48 h, the cells were harvested and suspended in a small amount of TGE buffer [10 mM Tris–HCl, 20% glycerol, 1 mM EDTA (pH 7.4)] and disrupted by freeze-thawing three times. Total RNA was prepared using RNAiso according to the manufacturer's protocols.

### 2.6. Determination of the half-life of CYP2E1 mRNA

The HEK293/2E1 + UTR cells and HEK293/2E1 cells transfected with the precursor for miR-378 or control as described above were simultaneously treated with 2  $\mu$ g/ml  $\alpha$ -amanitin. Total RNA was prepared at 1, 2, 3, 4, 5, 6 and 9 h later. The CYP2E1 mRNA levels were determined by real-time RT-PCR as described below.

## 2.7. Human livers and preparation of microsomes and total RNA

Human liver samples from 15 donors were obtained from Human and Animal Bridging (HAB) Research Organization (Chiba, Japan) which is in partnership with the National Disease Research Interchange (NDRI, Philadelphia, PA), and those from 10 donors were obtained from autopsy materials that were discarded after pathological investigation [14]. The use of the human livers was approved by the Ethics Committees of Kanazawa University (Kanazawa, Japan) and Iwate Medical University (Morioka, Japan). Microsomes fractions were prepared from 25 human livers according to the method described previously [15]. The protein concentration was determined using Bradford protein assay reagent (Bio-Rad, Hercules, CA) with  $\gamma$ -globulin as a standard. Total RNA was prepared using RNAiso and the integrity of the RNA was assessed by estimating the ratio of the band density of 28S and 18S rRNA.

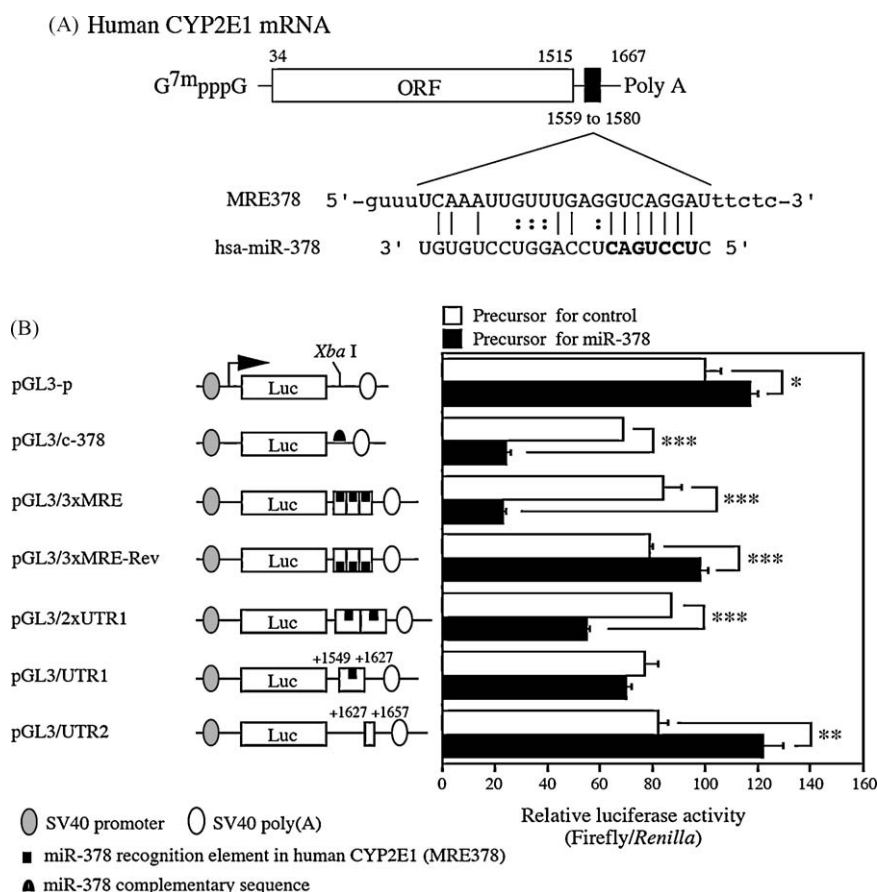
## 2.8. SDS-PAGE and Western blot analyses of CYP2E1

Cell homogenates from HEK293/2E1 + UTR cells or HEK293/2E1 cells (10–40  $\mu$ g) and human liver microsomes (3  $\mu$ g) were separated on 10% SDS-PAGE and transferred to Immobilon-P transfer membrane (Millipore, Billerica, MA). The membrane was probed with goat anti-human CYP2E1 antibody. Biotinylated anti-goat IgG and a Vectastain ABC kit (Vector Laboratories, Burlingame,

CA) were used for diaminobenzidine staining. As for cell homogenates from the HEK293 expression systems, the CYP2E1 protein level was normalized with the GAPDH protein level. As for human liver microsomes, a standard curve using recombinant human CYP2E1 expressed in baculovirus-infected insect cells (BD Gentest, Woburn, MA) was used to determine the absolute expression level of CYP2E1 protein. The quantitative analysis was performed using ImageQuant TL Image Analysis software (GE Healthcare Bio-Sciences).

## 2.9. Enzyme activity

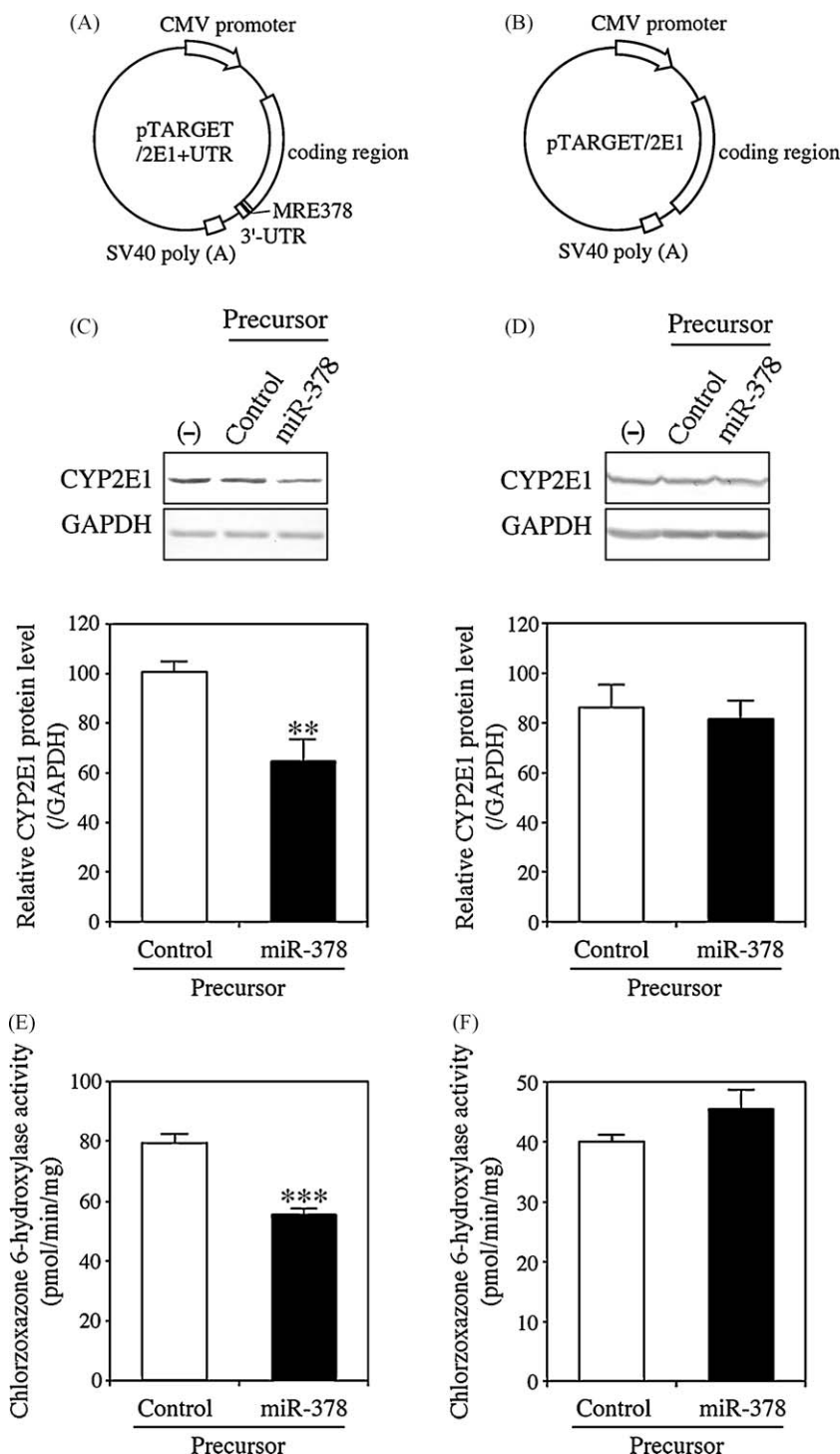
Chlorzoxazone 6-hydroxylase activity was determined as follows: a typical incubation mixture (final volume of 0.2 ml) contained 50 mM potassium phosphate buffer (pH 7.4), 500  $\mu$ M chlorzoxazone, and 0.5 mg/ml cell homogenates from HEK293/2E1 + UTR cells or HEK293/2E1 cells or human liver microsomes. The reaction was initiated by the addition of the NADPH-generating system (0.5 mM NADP<sup>+</sup>, 5 mM glucose-6-phosphate, 5 mM MgCl<sub>2</sub>, and 1 U/ml glucose-6-phosphate dehydrogenase) after 2 min preincubation at 37 °C. After the 10–30 min incubation at 37 °C, the reaction was terminated by the addition of 10  $\mu$ l of ice-cold 10% perchloric acid. Coumarin (0.25–1 nmol) was added as an internal standard. After removal of the protein by centrifugation at 10,000 rpm for 5 min, a portion of the supernatant was subjected to high-performance liquid chromatography



**Fig. 1.** Luciferase assay using the plasmids containing the MRE378 in the 3'-UTR of human CYP2E1 mRNA. Schematic representation of human CYP2E1 mRNA and the predicted target sequence of miR-378 (A). The numbering refers to the 5' end of mRNA as 1, and the coding region is from +34 to +1515. MRE378 (from +1559 to +1580) is located on the 3'-UTR of human CYP2E1 mRNA. **bold letters**, seed sequence. Luciferase assays using the reporter plasmids containing various fragments downstream of the firefly luciferase gene (B). The reporter plasmids (170 ng) were transiently transfected with pRL-TK plasmid (30 ng) and 20 nM precursors for miR-378 or negative control #1 (control) into HEK293 cells. The firefly luciferase activity for each construct was normalized with the *Renilla* luciferase activities. Values are expressed as percentages of the relative luciferase activity of pGL3-p plasmid. Each column represents the mean  $\pm$  SD of three independent experiments. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001, compared with the precursor for control.

(HPLC). The HPLC analyses were performed using an L-7100 pump (Hitachi, Tokyo, Japan), an L-7200 autosampler (Hitachi), an L-7405 UV detector (Hitachi), and a D-2500 chromato-integrator (Hitachi) equipped with a Mightysil RP-18 C18 GP (4.6 mm × 150 mm, 5  $\mu$ m) column (Kanto Chemical, Tokyo, Japan). The eluent was monitored at 295 nm with a noise-base

clean Uni-3 (Union, Gunma, Japan). The mobile phase was 28% methanol containing 50 mM potassium phosphate (pH 4.2). The flow rate was 1.0 ml/min. The column temperature was 35 °C. The quantification of the metabolites was performed by comparing the HPLC peak height with that of authentic standards with reference to an internal standard.



**Fig. 2.** Effects of overexpression of miR-378 on protein level and enzyme activity of CYP2E1. Schematic representation of the CYP2E1 expression plasmids including (A) or excluding (B) 3'-UTR of CYP2E1 (pTARGET/2E1-UTR or pTARGET/2E1). The CYP2E1 protein levels in HEK293/2E1 + UTR (C) and HEK293/2E1 (D) cells 48 h after the transfection of 10 nM precursors for miR-378 or negative control #1 (control). The CYP2E1 protein levels were determined by Western blot analysis and were normalized with the GAPDH protein level. Values are expressed as percentages relative to no transfection (-). Each column represents the mean  $\pm$  SD of three independent experiments. \*\* $P$  < 0.01, compared with the precursor for control. Enzyme activity of CYP2E1 in HEK293/2E1 + UTR (E) and HEK293/2E1 (F) cells 48 h after the transfection of 10 nM precursors for miR-378 or control. Chlorzoxazone 6-hydroxylase activity was measured using the cell homogenate at a substrate concentration of 500  $\mu$ M. The control activities in homogenates from non-treated HEK293/2E1 + UTR (E) and HEK293/2E1 (F) cells were  $70.5 \pm 8.5$  and  $41.9 \pm 1.0$  pmol/min/mg, respectively. Each column represents the mean  $\pm$  SD of three independent experiments. \*\*\* $P$  < 0.001, compared with the precursor for control.

### 2.10. Real-time RT-PCR for CYP2E1

The cDNA was synthesized from total RNA using ReverTra Ace. The CYP2E1 mRNA levels were quantified by real-time RT-PCR using the Mx3000P™ (Stratagene). The forward and reverse primers for CYP2E1 mRNA were 5'-ACG GTA TCA CCG TGA CTG TGG-3' and 5'-GCA TCT CTT GCC TAT CCT TGA-3', respectively. A 1 µl portion of the reverse-transcribed mixture was added to a PCR mixture containing 10 pmol of each primer, 12.5 µl of SYBR Premix Ex Taq solution and 75 nM ROX in a final volume of 25 µl. The PCR condition was as follows: after an initial denaturation at 95 °C for 30 s, the amplification was performed by denaturation at 94 °C for 4 s, annealing and extension at 58 °C for 20 s for 45 cycles. The PCR product was digested with appropriate restriction enzymes to confirm that the amplicon was indeed CYP2E1. The CYP2E1 mRNA levels were normalized with GAPDH mRNA as described previously [16].

### 2.11. Real-time RT-PCR for mature miR-378

The expression levels of mature miR-378 in human livers were determined by TaqMan quantitative real-time PCR using the TaqMan microRNA assay (Applied Biosystems, Foster City, CA). The cDNA templates were prepared with the TaqMan microRNA Reverse Transcription kit which utilized the stem-loop reverse primers according to the manufacturer's protocols. After the reverse transcription reaction, the product was mixed with TaqMan Universal PCR Master Mix and TaqMan MicroRNA assay containing the forward and reverse primers as well as the TaqMan probe for miR-378. The PCR condition was as follows: after an initial denaturation at 95 °C for 10 min, the amplification was performed by denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 60 s for 40 cycles. The expression levels of

U6 small nuclear RNA (U6 snRNA) were also determined by TaqMan quantitative real-time PCR and were used to normalize the miR-378 levels.

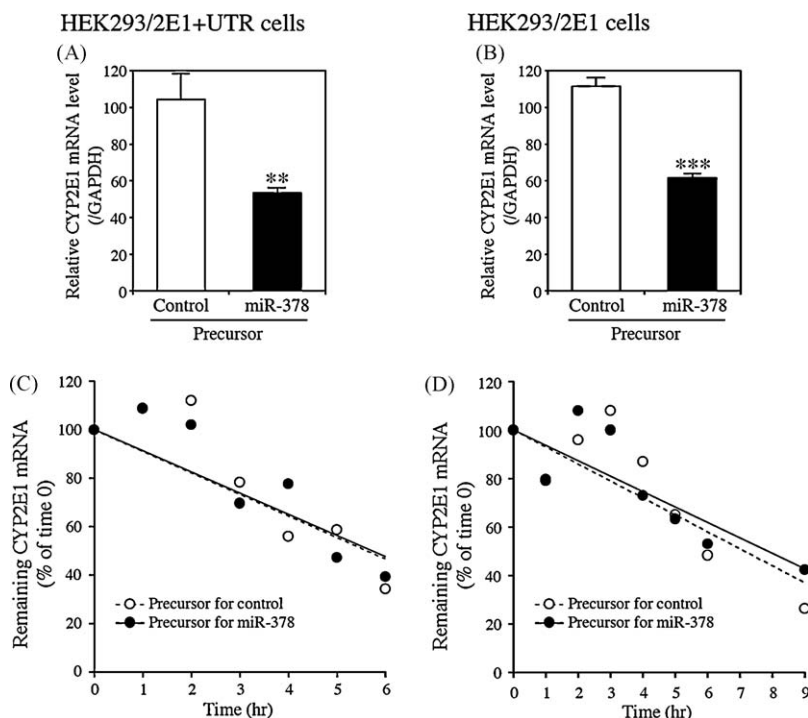
### 2.12. Statistical analyses

Statistical significance was determined by unpaired, two-tailed student's *t*-test. Correlation analyses were performed by Pearson's product-moment method. A value of  $P < 0.05$  was considered statistically significant.

## 3. Results

### 3.1. A miR-378 complementary sequence on the 3'-UTR of human CYP2E1 mRNA

The length of the 3'-UTR of human CYP2E1 is 152 bp. Computational prediction using miRBase Target database (<http://microrna.sanger.ac.uk/>) [17] indicated that 24 miRNAs including miR-378, miR-607, miR-223, and miR-105 share complementarity with sequences in the 3'-UTR. Meanwhile, when a Targetscan (<http://www.targetscan.org/>) was used, 6 miRNAs were found to share complementarity. The common miRNAs predicted in both web sites were only miR-378 and miR-607. We focused on miR-378 because it showed higher complementarity with the CYP2E1 mRNA (score 18.31 and energy  $-16.95$ ) than the others. Fig. 1A shows the alignment of hsa-miR-378 with 3'-UTR of human CYP2E1 mRNA drawn using RNAhybrid (<http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/>) [18]. We termed the sequence from +1559 to +1580 of the human CYP2E1 mRNA miR-378 recognition element (MRE378). We investigated whether the miR-378 might be involved in the regulation of CYP2E1 via the MRE378.



**Fig. 3.** Effects of overexpression of miR-378 on the CYP2E1 mRNA level and its stability. The CYP2E1 mRNA levels in HEK293/2E1 + UTR (A) and HEK293/2E1 (B) cells 48 h after the transfection of 10 nM precursors for miR-378 or negative control #1 (control). The CYP2E1 mRNA levels were determined by real-time RT-PCR and normalized with GAPDH mRNA. Values are expressed as percentages relative to no transfection. Each column represents the mean  $\pm$  SD of three independent experiments. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , compared with the precursor for control. Stability of the CYP2E1 mRNA in the HEK293/2E1 + UTR (C) and HEK293/2E1 (D) cells. The cells transfected with 10 nM precursors for miR-378 or negative control #1 (control) were simultaneously treated with 2 µg/ml  $\alpha$ -amanitin. Total RNA was prepared at 1, 2, 3, 4, 5, 6 and 9 h later. The CYP2E1 mRNA levels were determined by real-time RT-PCR and normalized with GAPDH mRNA. The amounts of mRNA at time 0 (the time of addition of  $\alpha$ -amanitin) in each group (miR-378 or control treated) were assigned a value of 100%, and all other values at different time points were expressed as percentages of the time 0 value. Data are the mean of two independent experiments.

### 3.2. Luciferase assay to investigate whether the MRE378 is functional

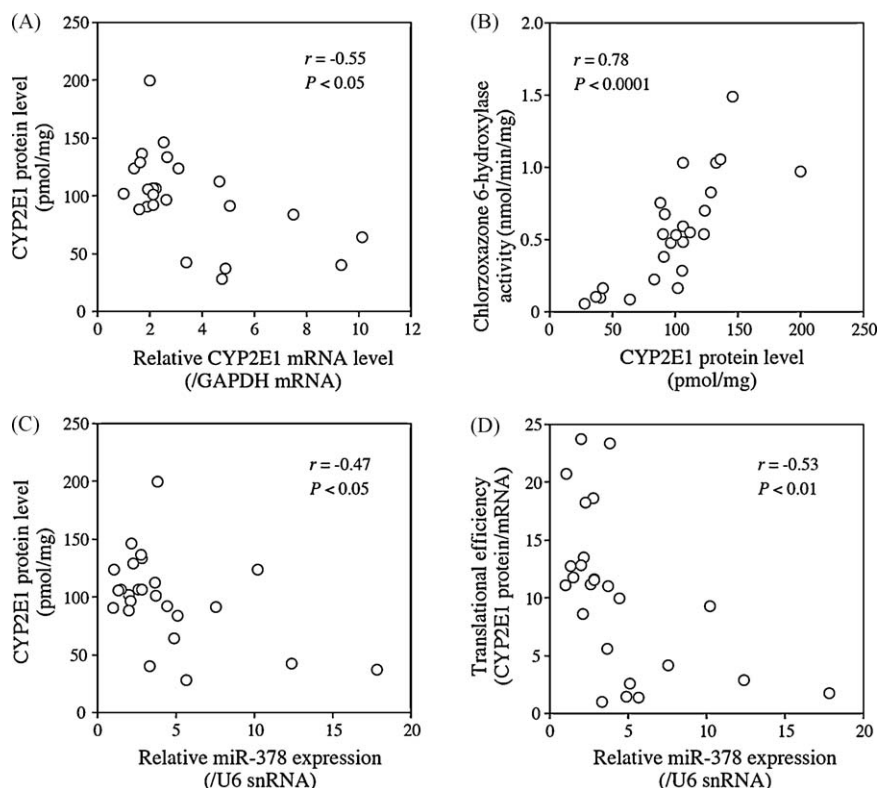
To investigate whether MRE378 is functional in the regulation by miR-378, luciferase assays were performed using HEK293 cells (Fig. 1B) that barely express miR-378 (data not shown). We first confirmed that the luciferase activity of the pGL3/c-378 plasmid, which contains the miR-378 complementary sequence, was significantly ( $P < 0.001$ ) decreased (35% of control) by the co-transfection of the precursor for miR-378. The luciferase activity of the pGL3/3xMRE plasmid containing three copies of the MRE378 was significantly ( $P < 0.001$ ) decreased (27% of control) by the overexpression of miR-378, whereas that of the pGL3/3xMRE-Rev plasmid with the inverted MRE378 was not. The luciferase activity of pGL3/UTR1 plasmid containing the single insertion of MRE378 was decreased by the overexpression of miR-378 (91% of control), although the difference was statistically insignificant. The luciferase activity of the pGL3/2xUTR1 plasmid containing the double insertion of the MRE378 was significantly ( $P < 0.001$ ) decreased (63% of control) by the overexpression of miR-378. In contrast, the luciferase activity of the pGL3/UTR2 plasmid containing the 3'-UTR sequence excluding MRE378 was increased by the overexpression of miR-378, although the reason is not clear. The luciferase activity of the pGL3-p plasmid was also increased by the overexpression of miR-378. Possibly, miR-378 might affect some factors for the SV40 promoter. This may suggest that the repressive effects of miR-378 on 3'-UTR of the reporter gene were underestimated. The results presented here suggest that miR-378 functionally recognized the MRE378 on the human CYP2E1 mRNA.

### 3.3. Effects of overexpression of miR-378 on protein level and enzyme activity of CYP2E1

Since the CYP2E1 expression levels in cell lines derived from human cancers are too low to be detected by Western blot analysis, we sought to establish cell lines expressing CYP2E1. To examine the role of MRE378 on the 3'-UTR of CYP2E1 gene, two HEK293 transfectants with pTARGET/CYP2E1 + UTR (Fig. 2A) and pTARGET/CYP2E1 (Fig. 2B) plasmids were established. When the miR-378 was overexpressed in HEK293/2E1 + UTR cells, the CYP2E1 protein level was significantly ( $P < 0.01$ ) decreased (60% of control) (Fig. 2C). The chlorzoxazone 6-hydroxylase activity was also significantly ( $P < 0.01$ ) decreased (70% of control) by the overexpression of miR-378 (Fig. 2E). In contrast, the overexpression of miR-378 did not affect the CYP2E1 protein level (Fig. 2D) and enzyme activity (Fig. 2F) in the HEK293/2E1 cells. These results clearly indicated that 3'-UTR including MRE378 plays an important role in the miR-378-dependent down-regulation of CYP2E1. In silico searches raised other candidates including miR-223 (score 17.51, energy -16.47) and miR-105 (score 16.97, energy -15.39) for human CYP2E1. However, the co-transfection of precursor for miR-223 or miR-105 into the HEK293/2E1 + UTR cells did not cause a decrease of the CYP2E1 protein (data not shown). Thus, we found that human CYP2E1 is specifically regulated by miR-378.

### 3.4. Effects of overexpression of miR-378 on the CYP2E1 mRNA level and its degradation

To investigate if the down-regulation of CYP2E1 by miR-378 involves mRNA degradation, we determined the CYP2E1 mRNA



**Fig. 4.** Relationship between the miR-378, CYP2E1 mRNA, and CYP2E1 protein levels and enzyme activity in human livers. Relationship between the CYP2E1 mRNA and protein levels (A), the CYP2E1 protein levels and chlorzoxazone 6-hydroxylase activities (B), the miR-378 and CYP2E1 protein levels (C), and the miR-378 levels and translational efficiency of CYP2E1 (CYP2E1 protein/mRNA ratio) (D). The expression levels of miR-378 and CYP2E1 mRNA in a panel of 25 human livers were determined by real-time RT-PCR and normalized with U6 snRNA levels and GAPDH mRNA levels, respectively. The values represent the levels relative to that of the lowest sample. The absolute CYP2E1 protein levels were determined by Western blot analysis using a standard curve with recombinant human CYP2E1 protein. The chlorzoxazone 6-hydroxylase activity was measured using human liver microsomes at a substrate concentration of 500  $\mu$ M. Data are the mean of two independent experiments.

levels. When the miR-378 was overexpressed in HEK293/2E1 + UTR cells, the CYP2E1 mRNA level was significantly ( $P < 0.01$ ) decreased (51% of control) (Fig. 3A). Similarly, the CYP2E1 mRNA level was also significantly ( $P < 0.001$ ) decreased (55% of control) in HEK293/2E1 cells (Fig. 3B). Next, we investigated whether miR-378 affects the stability of the CYP2E1 mRNA. When the HEK293/2E1 + UTR cells were treated with  $\alpha$ -amanitin, an inhibitor of transcription, the half-life of the CYP2E1 mRNA was estimated to be 5.6 h (Fig. 3C). The overexpression of miR-378 did not affect the half-life. The half-life of the CYP2E1 mRNA in the HEK293/2E1 cells was 7.1 h (Fig. 3D), and it was not affected by the overexpression of miR-378. These results suggest that miR-378 did not affect the degradation of CYP2E1 mRNA.

### 3.5. Relationship between the expression levels of miR-378, CYP2E1 mRNA, and CYP2E1 protein in human livers

To investigate the impact of the miR-378 on the CYP2E1 regulation in human livers, we examined the relationship between the expression levels of miR-378, CYP2E1 mRNA and protein as well as enzyme activity using a panel of 25 human livers. The CYP2E1 mRNA levels showed 10-fold interindividual variability. The CYP2E1 protein levels (27.7–199.7 pmol/mg, 7-fold variability) were significantly ( $r = 0.78$ ,  $P < 0.0001$ ) correlated with the chlorzoxazone 6-hydroxylase activities (0.05–1.49 nmol/min/mg, 30-fold variability) (Fig. 4B), but were inversely correlated with the CYP2E1 mRNA levels ( $r = -0.55$ ,  $P < 0.05$ ) (Fig. 4A), supporting the involvement of post-transcriptional regulation. Interestingly, the miR-378 levels (18-fold variability) showed a significant inverse correlation with the CYP2E1 protein levels ( $r = -0.47$ ,  $P < 0.05$ ) (Fig. 4C) and the translational efficiency of CYP2E1 (CYP2E1 protein/mRNA ratio) ( $r = -0.53$ ,  $P < 0.01$ ) (Fig. 4D). These results suggest that miR-378-dependent regulation has a great impact on the CYP2E1 expression in human livers.

## 4. Discussion

Earlier studies have reported that the induction of CYP2E1 seems to be regulated at the post-transcriptional or post-translational levels by the stabilization of mRNA [4] or protection against the rapid degradation of protein [5,19]. To obtain a clue towards understanding the mechanisms, we investigated the possibility that miRNAs may be involved in the regulation of human CYP2E1. As the results, we found that the miR-378 is involved in the post-transcriptional regulation of CYP2E1.

The overexpression of miR-378 significantly decreased the CYP2E1 protein level and enzyme activity in the cells expressing CYP2E1 including 3'-UTR, but not in the cells expressing CYP2E1 excluding 3'-UTR indicating that the 3'-UTR plays a role in the miR-378-dependent repression. Unexpectedly, the CYP2E1 mRNA levels in both cell lines were decreased by the overexpression of miR-378. However, the miR-378 did not facilitate the degradation of the CYP2E1 mRNA. Therefore, the down-regulation of CYP2E1 by miR-378 would mainly be due to the translational repression, not the mRNA degradation. The decrease of the CYP2E1 mRNA levels in the absence of  $\alpha$ -amanitin (Fig. 3A and B) suggests the possibility that miR-378 affects the transcription of CYP2E1. To examine the possibility that the miR-378 might affect the CMV promoter activity, we utilized other heterologous expression systems with the pTARGET vector (i.e., HEK/CYP2A6, HEK/UGT1A3, and HEK/UGT1A4). These heterologously expressed mRNA levels were not affected by the overexpression of miR-378 (data not shown). Therefore, it was concluded that the decrease of CYP2E1 mRNA level by miR-378 was not due to the effects on the CMV promoter. Although the cause of the decrease of CYP2E1 mRNA by miR-378 remains to be clarified, a major mechanism of the down-regulation

of CYP2E1 by miR-378 would be the translational repression, supported by the inverse correlation between the miR-378 levels and translational efficiency of CYP2E1 (Fig. 4C).

The sequences of mature miR-378 are completely conserved among human, rat, and mouse, but the sequence of the 3'-UTR of CYP2E1 is poorly conserved. Therefore, the regulation of CYP2E1 by miR-378 would be specific in human. Further study is needed to determine whether other miRNAs except miR-378 might be involved in the regulation of the CYP2E1 in other species.

As for miR-378, it has been reported that it promotes cell survival, tumor growth, and angiogenesis by repressing the expression of Sufu (suppressor of fused) and Fus-1, which are tumor suppressors [20]. Hua et al. [21] have reported that miR-378 binds to the 3'-UTR of vascular endothelial growth factor (VEGF) competing with other miRNAs and promotes the expression of VEGF. In addition to these studies, we provide new information concerning the role of miR-378 from pharmacological and toxicological aspects.

The gene coding miR-378 is within the intron 1 of the *peroxisome-proliferator-activated receptor- $\gamma$  co-activator 1 $\beta$*  (*PGC1 $\beta$* ) gene on human chromosome 5q33.1 (<http://microrna.sanger.ac.uk/sequences/>). This means that the expression of miR-378 would be in parallel with that of PGC1 $\beta$ . PGC1 $\beta$  is known as a regulator of hepatic lipid synthesis and lipoprotein production [22]. It has been reported that the expression of PGC1 $\beta$  is down-regulated in diabetes or obesity, but up-regulated by insulin treatment [23,24]. In contrast, the expression of CYP2E1 is up-regulated in diabetes or obesity, but down-regulated by insulin treatment [25–27]. It would be of interest to investigate the expression of miR-378 in these pathophysiological conditions with reference to the changes in the CYP2E1 expression. In addition, the changes in the expression of miR-378 under the treatment with typical chemical inducers of CYP2E1 *in vivo* or *in vitro* are worth pursuing in the future.

In conclusion, we found that human CYP2E1 expression is regulated by miR-378, mainly via translational repression. This study should provide new insight into the unsolved mechanism of the post-transcriptional regulation of CYP2E1.

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