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Relationship between DNA methylation in the 5' CpG island of the *SLC47A1* (MATE1) gene and inter-individual variability in MATE1 expression in the human liver

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Running title: DNA methylation effects on hepatic MATE1 expression

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Abbreviations

ABC; ATP-binding casssete

BCRP; breast cancer resistance protein

bp; base pair

cDNA; complementary DNA

CGI; CpG island

DMSO; Dimethyl sulfoxide

GAPDH ; glyceraldehyde 3-phosphate dehydrogenase

Kb; kilobase

MATE1; Multidrug and toxin extrusion protein 1

RT; reverse transcription

SLC; Solute carrier

TSS; transcriptional start site

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Abstract

Multidrug and toxin extrusion protein 1 (MATE1), which is encoded by *SLC47A1*, mediates the excretion of organic cations into bile and urine. Some genetic variants in human MATE1 altered its transport function in *in vitro* experiments; however, differences in the pharmacokinetics of substrate drugs cannot be explained by genetic variations in humans. In the present study, we investigated whether DNA methylation was involved in inter-individual variability in MATE1 expression in the human liver. Approximately 20-fold inter-individual variability in MATE1 mRNA expression levels was observed in liver samples and mRNA expression levels negatively correlated with methylation levels of the CpG island in the 27 kb upstream of *SLC47A1*. DNA demethylation by a treatment with 5-aza-2'-deoxycytidine increased MATE1 mRNA expression in MATE1-negative cell lines. The luciferase reporter assay showed that the CpG island increased the transcriptional activity of the *SLC47A1* promoter. MATE1 mRNA expression levels were significantly lower in CpG island knockout HepG2 cells than in control cells. These results suggest that the 5' CpG island of *SLC47A1* acts as an enhancer for *SLC47A1*, and DNA methylation in the CpG island plays an important role in inter-individual differences in hepatic MATE1 expression.

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Introduction

Inter-individual variability in drug responses is a major clinical issue for effective drug treatment. Since the pharmacokinetics of drugs are related to the functions of drug transporters, variations in the expression of drug transporter genes have been identified as one of the causes of this issue. Previous studies reported that genetic variations in drug transporters altered the responses of some clinical drugs (Niemi *et al.*, 2005; Ieiri *et al.*, 2008); however, the main reasons for inter-individual variability in drug responses currently remain unclear. The molecular mechanisms regulating gene expression need to be elucidated in more detail in order to resolve this issue.

MATE1 was identified as a mammalian ortholog of the bacterial MATE family in 2005 (Otsuka *et al.*, 2005) and has been demonstrated to function as a H^+/organic cation antiporter. Human MATE1 is strongly expressed in the kidney and liver, and is located in the luminal membrane (Otsuka *et al.*, 2005; Masuda *et al.*, 2006). Various drugs including metformin, cimetidine, and topotecan have been identified as the substrates of MATE1. Significantly high hepatic concentrations of metformin have been reported in Mate1 knockout mice (Toyama *et al.*, 2012). This finding suggests that hepatic MATE1 expression levels affect the pharmacokinetics of substrate drugs. Since some genetic variants in human MATE1 have been shown to alter its transport function in *in vitro*

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experiments, several clinical studies were performed to assess the relationship between *SLC47A1* genotypes and the pharmacokinetics of metformin (Tzvetkov *et al.*, 2009; Christensen *et al.*, 2011; Stocker *et al.*, 2013). However, most studies demonstrated that these variants did not affect the pharmacokinetic parameters of metformin. Therefore, other factors may contribute to inter-individual variability in the function of MATE1.

In addition to genetic variations, epigenetic mechanisms are regarded as important factors that alter gene expression. Epigenetics is defined as molecular factors and processes around DNA that regulate gene expression independent of DNA sequences. In epigenetic mechanisms, DNA methylation is one of the most studied epigenetic marks. DNA methylation occurs at the 5-carbon position of cytosine residues located in dinucleotide CpG sites, and is associated with the regulation of embryonic development, transcription, the chromatin structure, X-chromosome inactivation, genome imprinting, and chromosome stability (Bird, 2002). In mammals, regions of high CpG density, called CpG islands (CGIs), have been observed in the promoter regions of 60-70% of genes (Saxonov *et al.*, 2006). Although most CGIs at promoter regions are unmethylated, the methylation of CpG-rich promoters are essentially inactive (Deaton and Bird, 2011).

The role of DNA methylation in gene transcription including that of drug transporters in cancer cells has been studied in detail. For example, previous studies

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reported that the methylation levels of the promoter of the ATP-binding cassette, subfamily G, member2 (ABCG2), which codes breast cancer resistance protein (BCRP), inversely correlated with BCRP mRNA expression levels in some cancer cells (To *et al.*, 2006; Bram *et al.*, 2009). Since cancer-specific changes in DNA methylation were observed in a number of cancer-related genes, researchers have focused on DNA methylation-based biomarkers to diagnose various tumor entities and sensitivity (Warton and Samimi, 2015; Moon *et al.*, 2016). The contributions of DNA methylation to inter-individual variability in expression in normal tissue have also been evaluated (Yasar *et al.*, 2013; Wu *et al.*, 2015). These findings suggest that individualized drug therapy is possible using DNA methylation-based biomarkers. However, there is no reports about the analysis of DNA methylation in the inter-individual difference of MATE1 expression.

In the present study, we examined whether DNA methylation was involved in inter-individual variability in MATE1 expression in the human liver. Since the methylated region located in the 30 kb upstream of the transcriptional start site (TSS) of genes regulated gene expression (Yoon *et al.*, 2005, 2009), we analyzed the methylation levels of CGIs within the 30 kb upstream of *SLC47A1*. We also investigated the effects of CGI on MATE1 expression and the mechanisms by which CGI interact with the *SLC47A1* gene locus.

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Materials and Methods

Tissue samples

Twenty-one human liver samples were obtained from Caucasian donors at the National Disease Research Interchange (Philadelphia, PA, USA). All liver samples were excised from each individual liver. The characteristics of liver samples are shown in Table 1. Tissues were frozen in liquid nitrogen immediately after sampling and stored at -80°C. This study was approved by the Ethical Board of the Faculty of Medicine, Kyushu University.

Cell culture

The HepG2 cell line was obtained from the RIKEN Cell Bank (Tsukuba, Japan). HEK293 and LS174T cell lines were obtained from Dainippon Pharmaceuticals (Osaka, Japan). The HaCaT cell line was obtained from Cell Lines Service (Heidelberg, Germany). All cell lines were grown at 37°C in an incubator with 5% CO_2. The HepG2, HEK293, and HaCaT cell lines were maintained in Dulbecco's modified Eagle's medium (Sigma Aldrich, St Louis, MO). The LS174T cell line was maintained in Eagle's minimum essential medium (Wako, Osaka, Japan). All media were supplemented with 10% fetal bovine serum.

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Nucleic acid extraction and complementary DNA synthesis

Total RNA and genomic DNA from human liver tissues and cell lines were isolated with the RNeasy® Plus Mini Kit (QIAGEN, Valencia, California) or NucleoSpin® Tissue Kit (TaKaRa, Tokyo, Japan). First-strand complementary DNA (cDNA) synthesis was performed using SuperScript II (Invitrogen, Carlsbad, California) and a random primer (Promega, Madison, Wisconsin). Reverse transcription (RT) reactions were always performed in the presence or absence of reverse transcriptase to ensure that genomic DNA did not contaminate subsequent PCR.

Quantification of MATE1 mRNA expression in the human liver

The real-time PCR reaction was performed with SYBR Premix Ex Taq (TakaRa). The specificity of the real-time PCR product was proven by a dissociation curve analysis. In order to compare gene expression levels among different samples, we normalized values using an endogenous reference gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Real-time PCR primers were applied to the quantitative analysis of mRNA expression (Supplemental Table 1) using StepOnePlus^{TM} Real-Time PCR System (Applied Biosystems, Foster City, California).

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DNA methylation analyses

The reference sequence was derived from the NCBI sequence database (NC_000017.10). The CpG Island Searcher Program (Takai and Jones, 2003) was used to identify CGIs located within the 30 kb upstream of *SLC47A1*. A CGI was defined by the following criteria: GC >50%, observed CpG/expected CpG >0.60, and length >200 bp, and Gap between an adjacent island >100 bp. Methylated DNA was enriched with the EpiXplore Methylated DNA Enrichment Kit (TakaRa) according to the manufacturer's protocol. We designed primers within each CGI to perform PCR with methylation-enriched DNA (Supplemental Table 2). The real-time PCR reaction was performed as described above using SYBR Premix Ex Taq GC (TakaRa). We normalized the values of the methylation status in the CGIs using the primer designed within the CGI located in upstream of the TSS of the *CYP3A4* gene, which was previously revealed to be hypermethylated in human liver samples (Hirota *et al.*, 2004). A Combined Bisulfite Restriction Analysis (COBRA) was performed to evaluate the methylation status in some CGIs that were not amplified by real-time PCR. Isolated DNA was treated with sodium bisulfite using the EpiTect Bisulfite Kit (QIAGEN), and was amplified by PCR using bisulfite-conversion-based primers (Supplemental Table 3). DNA amplification was

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performed with Epi Taq HS (Takara) under the following conditions: 30-37 cycles at 98°C for 10 s, 50-60°C for 30 s, 72°C for 30 s. PCR products were digested by specific restriction enzymes and separated by 3.0 % agarose gel electrophoresis, and the intensity of bands was quantified with a fluorescence image analyzer (Hitachi, Tokyo, Japan).

Demethylation by 5-aza-2'-deoxycytidine

All cells were treated with 5 μM 5-aza-2'-deoxycytidine (5-aza-dC; Sigma Aldrich) and dimethyl sulfoxide (DMSO, 0.1%, final concentration) as the vehicle control for 72 h (Saito *et al*., 2013). Cells were dissociated enzymatically using TrypLE Express (Invitrogen), washed, and resuspended in phosphate-buffered saline (PBS).

Semi-quantitative RT-PCR

Total RNA isolation and cDNA synthesis was performed as described above. The amplification of cDNA was conducted using primers specific for MATE1 (NM_018242) and GAPDH (NM_002046). Primers specific for MATE1 cDNA amplification were 5'-ATGCTGTTTCCCACCTCTTTG -3' (forward) and 5'- CCGAGGCACGTTGTTTACTT -3' (reverse). Primers specific for GAPDH amplification were 5'-ATCAAGAAGGTGGTGAAGCAG -3' (forward) and 5'-

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TCGCTGTTGAAGTCAGAGGAG -3' (reverse). The amplification of GAPDH cDNA served as an internal control. PCR amplification for MATE1 and GAPDH was performed with the KOD SYBR qPCR Mix (Toyobo, Osaka, Japan) under the following conditions: 98°C for 2 min; 38 cycles at 98°C for 10 s, 60°C for 10 s, 68°C for 30 s, and with AmpliTaq Gold™ DNA polymerase (Applied Biosystems) under the following conditions: 95°C for 9 min; 30 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 30 s; and 72°C for 5 min, respectively. PCR products were resolved on a 3% agarose gel and stained with ethidium bromide.

Construction of reporter plasmids

We attempted to investigate the role of CGI (-27176 bp to -25203 bp) on the transcriptional activity of the *SLC47A1* promoter, and generated a fragment of the *SLC47A1* promoter and fragments of CGI of various lengths by nested-PCR assays (primer sets were described in Supplemental Table 4). Each amplified fragment of CGI was digested and inserted into the pGL4.10 basic vector (Promega) between the *Kpn*I and *Eco*RV sites. The fragment of the *SLC47A1* promoter was also digested with *Nco*I and *Hind*III and inserted into the pGL4.10 basic vector or joined to the 3' end of CGIs.

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Cell transfection and luciferase assays

HepG2 cells were plated on a 24-well plate at a density of 1.0×10^5 cells/well and transfected with reporter vectors using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Luciferase activity was measured by the Dual-Luciferase Reporter Assay System (Promega).

Chromosome conformation capture assay

A chromosome conformation capture (3C) assay was performed as previously described (Hagège *et al.*, 2007). HepG2 cells were crosslinked with 1% formaldehyde, lysed, and digested overnight with the *Pst*I restriction enzyme (TaKaRa) to obtain the 3C template. Samples were reverse-crosslinked at 65°C overnight, and DNA was purified by phenol-chloroform extraction and ethanol precipitation. Generation of the control 3C template using BAC DNA clones was essentially the same as described above. BAC clone RP11-191D16 (Advanced Geno Techs Co., Tsukuba, Japan) containing the MATE1 gene locus was used for the assay. In order to control differences in the amount of DNA in different samples, an internal control was used within two PstI sites in the *ACTB* locus. The quantification of ligation products was performed using the THUNDERBIRD® Probe qPCR Mix (Toyobo) with appropriate primers and the TaqMan probe designed at an

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anchor fragment including the downstream region of CGI (-25719 bp to -24202 bp) using 3C templates and the control template. The sequence of primers and the TaqMan probe used in the 3C assay are listed in Supplemental Table 5.

Deletion of CGI using the clustered regularly interspaced short palindromic repeats (CRISPR)-associated RNA-guided endonuclease Cas9 (CRISPR/Cas9) system

In order to generate the vector encoding a single guide RNA (sgRNA) and puromycin resistance, the pGL3-U6-sgRNA-PGK-puromycin vector (Addgene ID 51133) was purchased from Addgene (http://www.addgene.org/). The Guide-it™ CRISPR/Cas9 System Kit (TaKaRa) was also used to generate the vector encoding a sgRNA and Cas9. We designed guideRNAs using an online tool (http://crispr.dbcls.jp). DNA oligonucleotides harboring variable 20-nt sequences for Cas9 targeting were annealed to generate short double-strand DNA fragments with 4-bp overhangs compatible with ligation into the BsaI site of the pGL3-U6-sgRNA-PGK-puromycin vector (Addgene) or pGuide-it CRISPR/Cas9 vector (TaKaRa). HepG2 cells were plated on a 6-well plate at a density of 5.0×10^5 cells /well, and transfected using Lipofectamine 3000 (Invitrogen) with 2.5 μg of each vector. In order to select transfected cells, 2 μg/mL puromycin was added to the cell growth medium for 2 days. Cells were cloned by limiting

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dilutions, and DNA was extracted from the cloned cells for PCR amplification to analyze the deleted region by the CRISPR/Cas9 system (Supplemental Fig. 1A). MATE1 mRNA expression in the established clones was evaluated using a qPCR analysis. The sequences of oligos and primers were described in Supplemental Table 6.

Statistical analysis

Data from at least three independent experiments are expressed as the mean ± standard error of the mean. The means for two groups were compared with an unpaired Student's t-test (two-tailed). Comparisons of means for multiple groups against controls were analyzed with Tukey–Kramer's multiple comparison test. A 5% level of probability was considered to be significant. Relationships correlation between variables were assessed using Spearman's rank method.

RESULTS

Quantification of MATE1 mRNA and a DNA methylation analysis of CGIs

Twelve CGIs were detected within the 30 kb upstream of *SLC47A1* (Fig. 1A). CGI-12 contained the TSS of the *SLC47A1* gene. The relationship between hepatic MATE1 expression and the DNA methylation status in CGIs was analyzed in human liver samples.

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The methylation status of CGIs was examined by an affinity-based methylation capture assay or COBRA analysis. Approximately 20-fold inter-individual variability in MATE1 mRNA expression levels were observed in liver samples, and mRNA expression levels were negatively correlated with the methylation levels of CGI-3 (-27176 bp to -25203 bp) (Fig. 1B), while no correlation was found in CGI-12 containing the TSS. These results suggest that the DNA methylation of CGI-3 may play an important role in inter-individual differences in hepatic MATE1 expression.

Evaluation of MATE1 mRNA expression in demethylating agent-treated cells

In order to analyze the contribution of DNA methylation to MATE1 expression in MATE1 expression-positive or -negative cells, four cell lines were exposed to the DNA methyltransferase inhibitor 5-aza-dC. MATE1 mRNA expression levels remained unchanged after the treatment with 5-aza-dC in MATE1-positive HepG2 and HEK293 cells, whereas a marked increase in MATE1 mRNA expression levels was noted in MATE1-negative LS174T and HaCaT cells (Fig. 2).

The effect of CGI on transcriptional activity of *SLC47A1* promoter

CGI-3 showing the DNA methylation status correlated with hepatic MATE1

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expression and was located 27 kb from the TSS. We evaluated the enhancer activity of CGI-3 using luciferase vectors containing the *SLC47A1* promoter region. In order to identify important regions for transcriptional activity, *SLC47A1* promoter vectors containing various segments of CGI-3 were used (Fig. 3). The constructs F1, F2, F3, and F4 had significantly higher transcriptional activities than the *SLC47A1* promoter-only construct. In contrast, the transcriptional activity of the construct F5 was not significantly different from that of the *SLC47A1* promoter-only construct. These results indicate that the deleted region between constructs F4 and F5 (-25670 bp to -25246 bp) played an important role in the enhancer activity of CGI-3.

Analysis of long-range DNA interactions between the *SLC47A1* gene locus and CGI

It is increasingly clear that genomic regulation by distal regulatory regions is associated with direct interactions between distal regulatory regions and target gene promoters by DNA loop formation (Vakoc *et al.*, 2005; Mimura *et al.*, 2012; Lee *et al.*, 2015). We performed a 3C assay to analyze the proximity of the downstream region within CGI-3 (referred to as 3' CGI-3) with the *SLC47A1* gene locus in MATE1-positive HepG2 cells. We used a PCR primer located in the PstI fragment that contains 3' CGI-3 and paired it with primers in restriction fragments throughout a 30-kb region (primer

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sequences available in Supplemental Table 5). We found that the promoter fragment interacted most strongly with nearby restriction fragments and that interaction frequencies markedly decreased for fragments located farther away, while the frequency in the proximal region of the TSS was similar to that in the region immediately adjacent to CGI-3 (Fig. 4). This result suggests long-range DNA looping between the *SLC47A1* gene and CGI-3 in HepG2 cells.

The contribution of CGI to MATE1 expression in HepG2 cells

The results obtained above showed the importance of 3' CGI-3 including -25670 bp to -25246 bp for hepatic MATE1 expression. In order to further understand the role of CGI-3 in MATE1 expression, we deleted the region surrounding 3' CGI-3 in HepG2 cells using the CRISPR/Cas9 system. In brief, the paired synthesized oligonucleotide (Supplemental Table 6) for sgRNA targeting the region were annealed and cloned into the pGL3-U6-sgRNA-PGK-puromycin vector. In accordance with the manufacturer's instructions, HepG2 cells were transfected with sgRNA with Cas9 plasmid by the lipofection method. 3' CGI-3-deleted clones and not deleted clones were obtained (referred to as deletion clones and control clones, respectively) (Supplemental Fig. 1). We confirmed the deletion of 3' CGI-3 by the direct sequencing method (Supplemental Fig.

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2). MATE1 mRNA expression levels were significantly lower in deletion clones than in control clones (Fig. 5). This result indicates that the region surrounding 3' CGI-3 increases *SLC47A1* transcription in HepG2 cells.

DISCUSSION

The expression level of Mate1 in rats has been associated with the tubular secretion and blood levels of the substrate drug (Nishihara *et al*., 2007). The inter-individual difference of MATE1 expression plays an important role for altered function of MATE1. Some genetic variations in the *SLC47A1* promoter region were evaluated and revealed to affect *SLC47A1* promoter activity by altering binding of some transcription factors (Kajiwara *et al.*, 2007; Choi *et al.*, 2009; Kim *et al.*, 2013); however, their contribution was not sufficiently large to explain inter-individual variability in hepatic MATE1 expression. Most studies that focused on the relationship between DNA methylation and gene expression only analyzed the methylation status of CGIs located near target gene promoters. However, CGIs far from the target gene locus have been shown to affect gene regulation (Yoon *et al.*, 2005, 2009). The relationship between epigenetic regulation and *SLC47A1* currently remains unclear; therefore, we performed a comprehensive DNA methylation analysis in the present study in order to precisely evaluate the role of DNA

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methylation in inter-individual differences in hepatic MATE1 expression. Before the methylation analysis, we analyzed the polymorphism in the promoter region of *SLC47A1* gene (rs72466470). The variant was not identified in the promoter region. In the methylation analysis, MATE1 mRNA expression levels negatively correlated with the methylation levels of CGI located in the 27-kb upstream region of the *SLC47A1* gene. A number of genes including drug transporters were revealed to be suppressed by promoter DNA methylation in cancer cell lines (Schaeffeler *et al.*, 2011; Tang *et al.*, 2011; Ikehata *et al.*, 2012). DNA methylation in gene promoters is crucial for gene transcription. However, a relationship was not found between DNA methylation in the *SLC47A1* promoter and MATE1 mRNA expression. This result suggested that the DNA methylation status in the *SLC47A1* promoter is not important for inter-individual variability in MATE1 expression.

In order to analyze the role of DNA methylation in MATE1 expression, the effects of demethylation on MATE1 expression were evaluated using four cell lines. MATE1-negative cell lines showed an increase in MATE1 mRNA expression by demethylation, whereas MATE1-positive cell lines did not. Tissue-specific DNA methylation was previously reported in mammals (Aoki *et al.*, 2008; Imai *et al.*, 2013), and demethylating agents only increased the expression of organic cation/carnitine transporter 2 mRNA in

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the human tissue cell lines showing the methylated DNA status (Qu *et al.*, 2013). These findings suggest that methylated DNA in MATE1-negative cell lines contributes to control the expression of MATE1.

The luciferase reporter assay showed that the region from -25670 bp to -25246 bp in CGI-3 was important for increasing the transcriptional activity of the *SLC47A1* promoter. In addition, HepG2 cells that showed the complete absence of the region surrounding 3' CGI-3 had significantly lower expression levels of MATE1 mRNA. These results suggest the importance of this region for *SLC47A1* gene regulation in HepG2 cells. The relationship between the DNA methylation status in CpG island shores and gene expression has already been described (Ji *et al.*, 2010). CpG island shores are regions of DNA with a low density of CpG dinucleotides that are located near CGIs. Previous studies revealed that tissue-specific DNA methylation and cancer-related methylation changes occurred in CpG island shores (Doi *et al.*, 2009; Irizarry *et al.*, 2009). Since the region from -25670 bp to -25246 bp is located near the 3' end of CGI-3 and shows a low density of CpG, it may fall under the category of a CpG island shore and play an important role in *SLC47A1* gene regulation.

Recent studies revealed that DNA-DNA interactions between enhancer elements and target genes are necessary for the function of enhancers that are distant from their

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target genes (Hwang *et al.*, 2013; Wang *et al.*, 2015). Based on these findings, we analyzed DNA interactions between *SLC47A1* gene locus and CGI. The results of the 3C assay revealed the spatial proximity of 3' CGI-3 to the proximal region of *SLC47A1* TSS caused by DNA looping of the *SLC47A1* locus in HepG2 cells. These results suggest that this region acts as an enhancer and regulates the *SLC47A1* gene in HepG2 cells.

In the present study, we showed that the *SLC47A1* gene was regulated through the CGI located in the 27 kb upstream of the *SLC47A1* gene, and that inter-individual variability in hepatic MATE1 expression correlated with DNA methylation in this CGI. The contribution of altered MATE1 mRNA expression to the protein expression and *in vivo* activity was not elucidated in this study. Further studies are needed in order to clarify whether the DNA methylation status in CGI is involved in the protein expression in the liver and the pharmacokinetics of substrate drugs of MATE1. The mechanisms responsible for variability in the DNA methylation status in CGI and for the regulation of the *SLC47A1* gene via DNA methylation currently remain unclear. It is well known that the environmental factors including diet, long-term diseases and drug exposure can provoke epigenetic responses (Jaenisch *et al*., 2003). In addition to the evaluation of DNA methylation, the analysis of the environmental factors contributing to the inter-individual difference of MATE1 expression is important. It was reported that long-term exposure to

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cisplatin enhanced *SLC22A1* promoter methylation by activating DNA methyltransferase 1 (DNMT1) (Lin *et al*., 2013), but DNMT regulating DNA methylation of *SLC47A1* gene is not identified. The investigation of role of DNMT regulating DNA methylation of *SLC47A1* gene is a key to elucidate the mechanisms causing the inter-individual variability of hepatic MATE1 expression.

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Authorship Contributions

Participated in research design: Toshihiro Tanaka, Takeshi Hirota, and Ichiro Ieiri

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Footnotes

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Figure Legends

Fig. 1 Correlation analysis between MATE1 mRNA levels and methylation frequencies of the CGI in Caucasian liver samples.

(A) Schematic of the upstream region of the *SLC47A1* gene from 30 kb upstream to 1 kb downstream. + 1 indicated the TSS of *SLC47A1*. Grey boxes show CGIs and are CGI-1 to CGI-12. (B) Correlation between the mRNA expression levels of MATE1 and methylation levels of CGI-3. Rs: Spearman's rank-order correlation analysis.

Fig. 2 Effects of 5-aza-dC on MATE1 mRNA expression in four cell lines.

All cell lines were treated with 5-aza-dC for 72 hr before harvesting. MATE1 mRNA expression was assessed using RT-PCR. N: No template control.

Fig. 3 Luciferase activities in the *SLC47A1* promoter and CGI-3.

Luciferase (Luci) reporter gene constructs were transiently transfected into HepG2 cells. Grey and white boxes show CGI-3 (-27176 bp to -25203 bp) and the *SLC47A1* promoter region, respectively. Luciferase values are normalized to the pGL4.70 vector and the mean value obtained with the pGL4.10 vector as 100%. Data represents the mean ± S.D. (n=3) and were analyzed using Dunnett's test. *, $p < 0.05$: significantly

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different from the control vector containing only the *SLC47A1* gene promoter region.

N.S., Not significant.

Fig. 4 3C assay of the *SLC47A1* gene locus in HepG2 cells.

The vertical lines show the positions of PstI restriction sites and the grey box shows CGI-3. The constant primer and TaqMan probe were designed in the PstI fragment containing CGI-3 (anchoring point), and crosslinking frequencies between the anchor fragment and PstI fragment in the *SLC47A1* gene promoter were analyzed by TaqMan real-time PCR. Data represents the mean ± S.D. (n=3).

Fig. 5 Effects of the deletion of the CpG island on MATE1 mRNA expression in HepG2 cells.

MATE1 mRNA expression was measured by quantitative RT-PCR. Results were normalized to the expression of GAPDH. *, $p < 0.05$: statistically analyzed using the Student's *t*-test.

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Table 1: Characteristics of human liver tissues

Gender (M:F)	16:5
Age (years, range)	17-70

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Fig.1

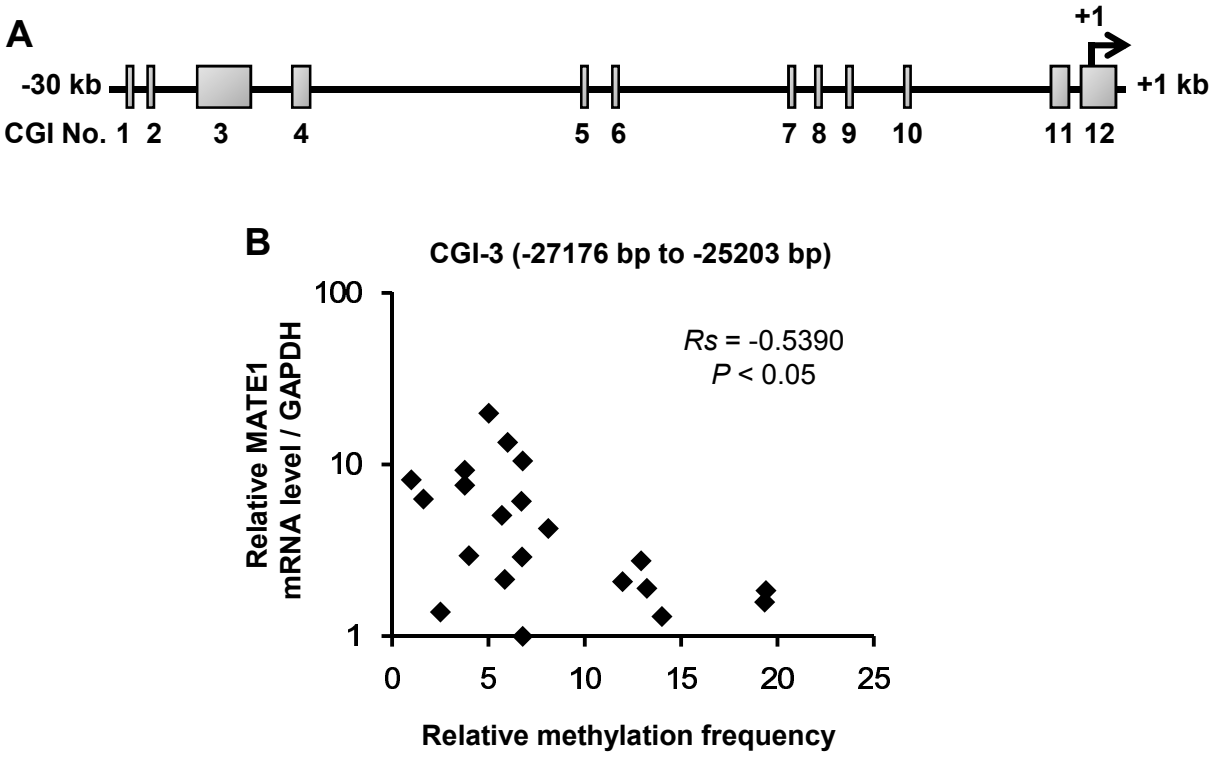

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Fig.2

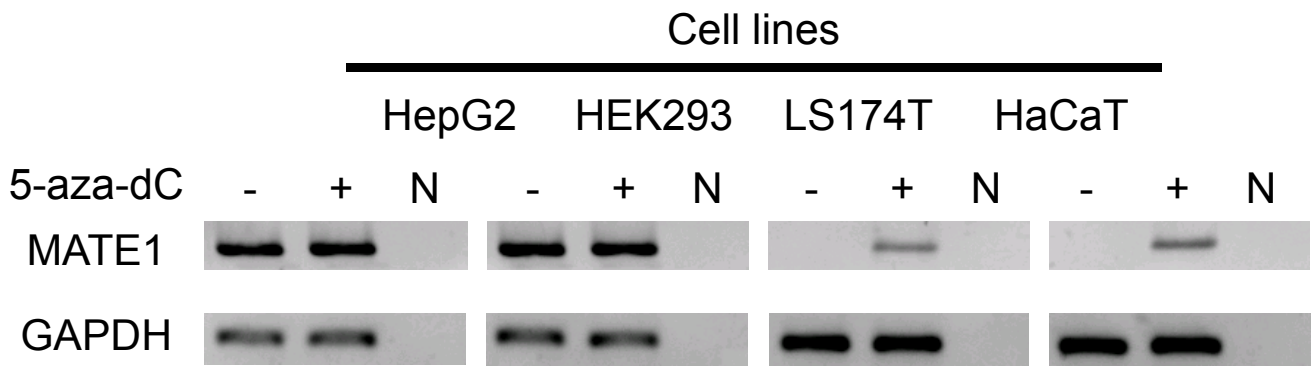

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Fig.3

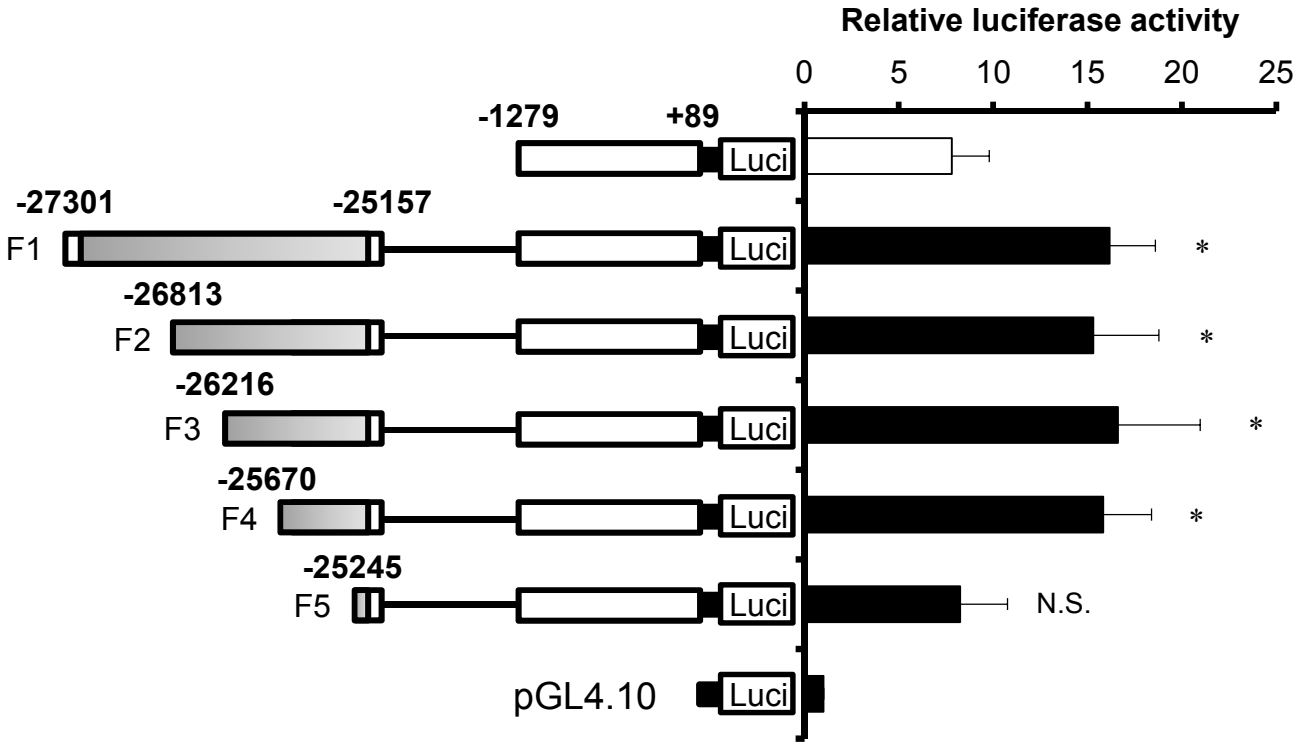

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Fig.4

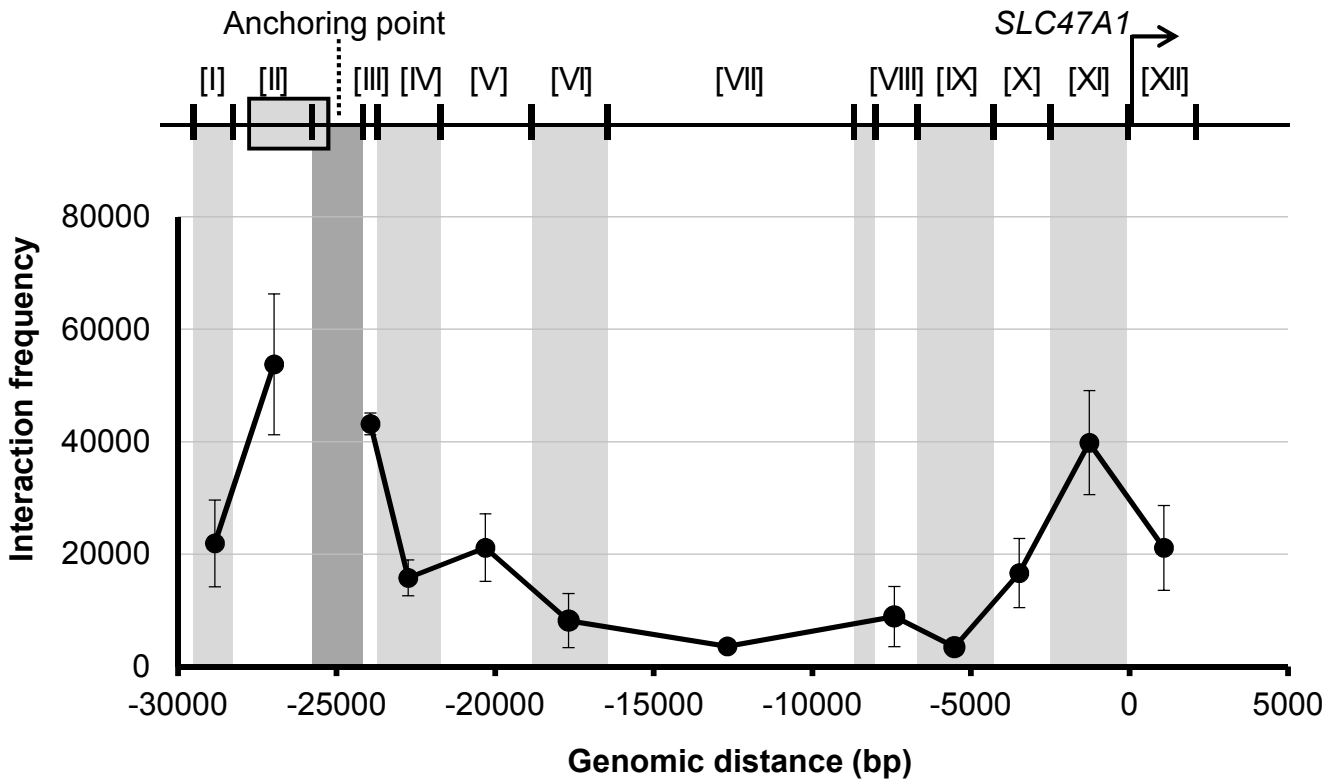

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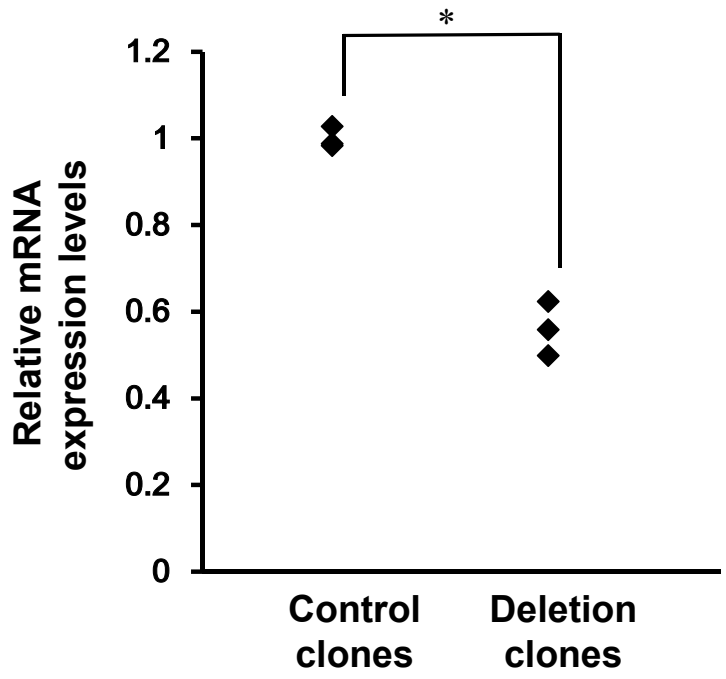
Fig.5



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