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**Epigenetic regulation of the tissue-specific expression of human  
UDP-glucuronosyltransferase (UGT) 1A10**

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## Abstract

Human UDP-glucuronosyltransferase (UGT) 1A10 is not expressed in the liver; however, UGT1A10 is exclusively expressed in the intestine, contributing to presystemic first-pass metabolism. Earlier studies revealed that hepatocyte nuclear factor (HNF) 1 $\alpha$  and Sp1, as well as an intestine-specific transcription factor, caudal type homeobox (Cdx) 2, are involved in the constitutive expression of UGT1A10. However, why UGT1A10 is not expressed in the liver, where HNF1 $\alpha$  and Sp1 are abundantly expressed, is unknown. In this study, we sought to elucidate the mechanism, focusing on epigenetic regulation. Bisulfite sequence analysis revealed that the CpG-rich region (-264 to +117) around the *UGT1A10* promoter was hypermethylated (89%) in hepatocytes, whereas the *UGT1A10* promoter was hypomethylated (11%) in the epithelium of the small intestine. A luciferase assay revealed that the methylation of the *UGT1A10* promoter by *SssI* methylase abrogated transactivity even with overexpressed Cdx2 and HNF1 $\alpha$ . The *UGT1A10* promoter was highly methylated (86%) in liver-derived HuH-7 cells, where UGT1A10 is not expressed. In contrast, the *UGT1A10* promoter was hardly methylated (19%) in colon-derived LS180 cells, where UGT1A10 is expressed. Treatment with 5-aza-2'-deoxycytidine (5-Aza-dC), an inhibitor of DNA methylation, resulted in an increase in UGT1A10 expression only in HuH-7 cells. Moreover, overexpression of HNF1 $\alpha$  and Cdx2 further increased UGT1A10 expression only in the presence of 5-Aza-dC. Collectively, we found that DNA hypermethylation would interfere with the binding of HNF1 $\alpha$  and Cdx2, resulting in the defective expression of UGT1A10 in human liver. Thus, epigenetic regulation is one of the mechanisms that determine the tissue-specific expression of UGT1A10.

Key words: epigenetics; DNA methylation; tissue-specific regulation;  
UDP-glucuronosyltransferase

## 1. Introduction

UDP-Glucuronosyltransferases (UGTs) catalyze the glucuronidation of a variety of endogenous and exogenous compounds. In humans, there are 19 functional UGT enzymes, which are classified into three subfamilies: UGT1A, UGT2A, and UGT2B [1]. The *UGT1A* genes, which are located on chromosome 2q37, contain multiple unique first exons and common exons 2 to 5 and encode nine kinds of functional UGT1A enzymes [2]. The *UGT2* genes, which are located on chromosome 4q13, encode three UGT2A and seven UGT2B functional enzymes.

Human UGT enzymes are expressed in a tissue-specific manner. Most UGTs, including UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, UGT2B4, UGT2B10 and UGT2B7, are predominantly expressed in the liver [3, 4] and expressed to a lesser extent in extra-hepatic tissues. Several UGTs are preferentially expressed in extra-hepatic tissues, including the kidney, small intestine, colon, stomach, lungs, epithelium, ovaries, testis, mammary glands and prostate. In particular, UGT1A7, UGT1A8, and UGT1A10 are exclusively expressed in the gastrointestinal tract, excluding the liver. This expression limits the bioavailability of orally administered drugs, such as raloxifene, naloxon, and mycophenolic acid, as well as xenobiotics, such as resveratrol and quercetin [5, 6]. The intestine-specific expression of UGT1A8 and UGT1A10 was explained by transcriptional regulation through an intestine-specific transcription factor, caudal type homeobox 2 (Cdx2), as well as Sp1 and hepatocyte nuclear factor (HNF) 1 $\alpha$  [7, 8, 9, 10]. However, why UGT1A8 and UGT1A10 are not expressed in the liver, where Sp1 and HNF1 $\alpha$  are abundantly expressed, remains unsolved.

The purpose of this study was to clarify the underlying mechanisms of the defective expression of UGT1A10 in the liver, focusing on epigenetic regulation. Although UGT1A8 mRNA is substantially detected in intestine, the expression of UGT1A8 protein has never

been proven. In contrast, UGT1A10 protein could be clearly detected in the intestine by Western blot analysis using an anti-UGT1A10 specific antibody that we prepared (unpublished data). This observation is the reason we focused on UGT1A10 in this study. It is generally accepted that epigenetics, including DNA methylation and histone modification, are key regulators of tissue-dependent gene expression [11, 12]. We investigated whether DNA methylation and histone modification might be determinants of the tissue-specific expression of human UGT1A10.

## **2. Materials and Methods**

### *2.1. Chemicals and reagents*

5-Aza-2'-deoxycytidine (5-Aza-dC) and trichostatin A (TSA) were purchased from Sigma-Aldrich (St. Louis, MO). Goat anti-human HNF1 $\alpha$  polyclonal antibody (C-19), goat anti-human Cdx2 polyclonal antibody (C-20), and control rabbit and goat IgGs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Primers were commercially synthesized at the Hokkaido System Science (Sapporo, Japan). All other chemicals and solvents were of the highest grade commercially available.

### *2.2. Human tissues*

Human liver (a 39-year-old Japanese female) and small intestine (a 49-year-old Caucasian female) were obtained from autopsy materials that were discarded after pathological investigation. The use of the human liver and small intestine was approved by the ethics committees of Kanazawa University (Kanazawa, Japan) and Iwate Medical University (Morioka, Japan).

### *2.3. Cell culture*

Colorectal adenocarcinoma cell lines LS180, Caco-2, HT-29, and SW480 and the hepatocellular carcinoma cell line HepG2 were obtained from the American Type Culture Collection (Manassas, VA). A hepatocellular carcinoma cell lines HuH-7 were obtained from the RIKEN BioResource Center (Ibaraki, Japan). HT-29 and SW480 cells were cultured in RPMI1640 (Nissui Pharmaceutical, Tokyo, Japan) that was supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). The other cells were cultured as previously described [13].

#### 2.4. RNA isolation and real-time reverse transcription (RT)-polymerase chain reaction (PCR)

Total RNA was isolated from cell lines using RNAiso (Takara, Otsu, Japan) according to the manufacturer's protocol. The cDNA was synthesized from the total RNA using Rever Tra Ace® (Toyobo, Osaka, Japan). The UGT1A10 mRNA levels were determined by real-time RT-PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels as described previously [3].

#### 2.5. Genomic DNA extraction and bisulfite reaction

Genomic DNA samples were prepared from human hepatocytes (HH268, a 54-year-old Caucasian female, Tissue Transformation Technologies), whole small intestine or epithelium of the small intestine, and cell lines using a Genra Puregene Tissue kit (Qiagen, Valencia, CA). Five hundred nanograms of genomic DNA digested with *EcoR* I was treated with bisulfite using an EZ DNA Methylation kit (Zymo Research, Orange, CA) according to the manufacturer's protocol. The DNA fragments spanning the transcription start site (TSS) of the *UGT1A10* or *UGT1A8* genes and the 5'-flanking region of *UGT1A9* were amplified by PCR using the primer pairs that are shown in Table 1. The PCR products were cloned into the pT7Blue T-Vector (Novagen, Madison, WI). Because the primer pair for the bisulfite analysis of UGT1A8 and UGT1A10 amplifies the corresponding regions of not only UGT1A8 and UGT1A10 but also UGT1A9, clones containing UGT1A9 sequence were precluded by digestion with *Mbo* II, and clones containing UGT1A8 or UGT1A10 sequences were subjected to sequence analysis. The DNA methylation status of the sequence was analyzed using the web-based tool QUMA [14].

#### 2.6. Construction of expression plasmids and luciferase reporter plasmids

A luciferase reporter plasmid, pCpGL-basic, which completely lacks CpG dinucleotides,

was kindly provided by Dr. Rehli [15]. The 5'-flanking regions of *UGT1A9* (-955 to +29) or the promoter region of *UGT1A10* (-365 to +140), which was amplified by PCR using the human liver genome as a template, was cloned into the pCpGL-basic plasmid. The products were termed UGT1A9/pCpGL and UGT1A10/pCpGL, respectively. HNF4 $\alpha$  and HNF1 $\alpha$  expression plasmids were constructed previously [16, 17]. For the construction of the Cdx2 expression plasmid, human Cdx2 cDNA was amplified by PCR using the primer pair that is shown in Table 1 and human small intestine cDNA as a template. The PCR product was subcloned into the pTARGET vector (Promega, Madison, MI). DNA sequencing analysis confirmed the nucleotide sequence.

### *2.7. Luciferase reporter assays*

The pCpGL-basic, UGT1A9/pCpGL, and UGT1A10/pCpGL plasmids were treated with a CpG methylase *SssI* (New England Biolabs, Beverly, MA). For the luciferase assays, HuH-7 cells were seeded onto a 24-well plate at  $1 \times 10^5$  cells/well. After 24 h, 200 ng of pCpGL-basic plasmid and 300 ng each of human Cdx2, HNF1 $\alpha$ , and HNF4 $\alpha$  expression plasmids or pTARGET empty plasmid were transfected into the cells using Lipofectamine 2000 (Invitrogen). The cells were harvested 48 h after the transfection and lysed to measure the luciferase activity using a Dual Luciferase Reporter Assay System (Promega). The protein concentration was determined using Bradford protein assay reagent (Bio-Rad Laboratories, Hercules, CA) using  $\gamma$ -globulin as a standard. The relative luciferase activities were normalized to the protein content.

### *2.8. Chemical treatment and transfection of expression plasmid into the cells*

HuH-7 or LS180 cells were seeded onto a 12-well plate at  $0.5 \times 10^5$  cells/well and incubated for 24 h. For dose response experiments, the cells were treated with 0.01 to 10  $\mu$ M



5-Aza-dC for 120 h or treated with 50 to 300 nM TSA for 24 h, and then subjected to RNA isolation. For the overexpression of HNF1 $\alpha$  and Cdx2, the cells were treated with 0.1  $\mu$ M 5-Aza-dC for 120 h. Sixty hours before harvesting, the cells were transiently transfected with 0.5  $\mu$ g of an HNF1 $\alpha$  and/or Cdx2 expression plasmids using the X-tremeGENE HP DNA transfection reagent (Roche Applied Science, Indianapolis, IN). The UGT1A10 mRNA levels were determined as described above.

### *2.9. Immunoblot analysis of HNF1 $\alpha$ and Cdx2*

Total cell homogenates (40  $\mu$ g) from HuH-7 and LS180 cells that were transfected with HNF1 $\alpha$  and Cdx2 expression plasmids were separated by 10% SDS-PAGE and transferred to an Immobilon-P transfer membrane (Millipore). The membranes were blocked with Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) for 1 h and were probed with goat anti-human HNF1 $\alpha$  or rabbit anti-human Cdx2 antibodies diluted 1:500 for 3 h followed by IRDye 680LT-labeled donkey anti-goat or goat anti-rabbit secondary antibodies diluted 1:5,000 (LI-COR Biosciences) for 1 h. The membranes were then scanned using the Odyssey Infrared Imaging system (LI-COR Biosciences).

### *2.10. Statistical analyses*

For the DNA methylation status, the statistical significance was evaluated by the Mann-Whitney *U*-test or Fisher's exact test using the web-based tool QUMA. For the mRNA expression and luciferase assay, the statistical significance was determined using unpaired, two-tailed Student's *t*-test or one-way analysis of variance (ANOVA) followed by Dunnett's test. Correlation analyses were performed by Spearman's rank method. When the *p* value was less than 0.05, the differences were considered statistically significant.

### 3. Results

#### 3.1. DNA methylation status of the 5'-flanking regions of *UGT1A8*, *UGT1A9*, and *UGT1A10* in human hepatocytes and small intestine

We searched the CpG dinucleotides between 300 bp upstream and 200 bp downstream of the TSS of human *UGT1A8*, *UGT1A9*, and *UGT1A10* genes (Fig. 1A). Five and 12 CpG dinucleotides were found around TSS (-365 to +140 bp) for *UGT1A8* and *UGT1A10*, whereas only two CpG dinucleotides were observed around TSS for *UGT1A9*. In the case of *UGT1A9*, there were multiple CpG dinucleotides spanning 800 to 600 bp upstream of the TSS. The DNA methylation status of the promoter regions of *UGT1A10* spanning -365 to +140 in the small intestine and liver was determined by bisulfite sequence analysis (Fig. 1B). Previously, when we used a whole liver tissue sample for the analysis of DNA methylation of *UGT1A1*, the results indicated a mixed methylation pattern derived from parenchymal and non-parenchymal cells [17]. Accordingly, we used hepatocytes in this study. As shown in Fig. 1B, 89% of CpG sites (128 of 144 CpG sites) in the promoter region of the *UGT1A10* gene were methylated in hepatocytes, whereas 51% of CpG sites (86 of 168 CpGs) were methylated in the whole small intestine. Notably, in the whole small intestine, the methylated CpG sites were biased in specific clones. It was surmised that these clones showing hypermethylation might be from the submucosa of the small intestine, where UGT enzymes are not expressed [18]. Therefore, epithelium cells that were prepared from the small intestine were used to determine the DNA methylation status of the *UGT1A10* promoter. The methylation status was found to be only 11% (18 of 168 CpG sites). Collectively, we found that the DNA methylation status of the *UGT1A10* promoter region was quite lower in the small intestine epithelium than hepatocytes ( $p < 0.0001$ , Mann-Whitney *U*-test).

Next, the DNA methylation status of the promoter of *UGT1A8*, which shows high sequence similarity with *UGT1A10*, was investigated (Fig. 1B). In the promoter of *UGT1A8*

from hepatocytes, 79% of CpG sites (51 of 65 CpG sites) were methylated, whereas in the small intestine epithelium, 16% of CpG sites (9 of 55 CpGs) were methylated ( $p = 0.0004$ , Mann-Whitney  $U$ -test). The difference in the DNA methylation pattern of *UGT1A8* between two tissues was almost identical to that of *UGT1A10*. Because there is only one CpG site in the promoter of *UGT1A9*, we investigated the farther upstream CpG-rich region (-765 to -639 bp) for its DNA methylation status. In the 5'-flanking region of *UGT1A9* from hepatocytes, 93% of CpG sites (65 of 70 CpG sites) were methylated, and in the small intestine epithelium, 96% of CpG sites (67 of 70 CpGs) were methylated ( $p = 0.34$ , Mann-Whitney  $U$ -test). Thus, the DNA methylation status of this region would not be associated with the tissue-specific expression of *UGT1A9*.

### *3.2. Effects of DNA methylation on the transactivity of UGT1A10 and UGT1A9*

To determine the effects of DNA methylation on promoter activity, luciferase assays were performed using methylated and unmethylated luciferase constructs (Fig. 2). In the case of the unmethylated *UGT1A10/pCpGL* construct, the overexpression of either *Cdx2* ( $p < 0.05$ ) or *HNF1 $\alpha$*  ( $p < 0.01$ ) highly increased the activity of luciferase, and the synergistic increase of the activity was observed by the coexpression of these factors ( $p < 0.01$ ), supporting the previous study [8]. In the case of the methylated *UGT1A10/pCpGL* construct, the overexpression of *Cdx2* and/or *HNF1 $\alpha$*  did not significantly increase luciferase activities. Luciferase activities of unmethylated *UGT1A10/pCpGL* constructs were significantly higher than methylated constructs, indicating that DNA methylation statuses have a great impact on the transcriptional activity of *UGT1A10*. The overexpression of *HNF4 $\alpha$*  did not increase the luciferase activity of *UGT1A10*. This observation may be because the sequence of the *HNF4 $\alpha$*  recognition element in the *UGT1A10* gene was different by one nucleotide from the consensus sequence of the *HNF4 $\alpha$*  response element [19]. In the case of the *UGT1A9/pCpGL*

construct, the overexpressed HNF4 $\alpha$  increased ( $p < 0.01$ ) the activity regardless of methylation status, although the overexpressed Cdx2 and HNF1 $\alpha$  did not significantly increase the activity of luciferase. These results suggest that the DNA methylation of the 5'-flanking region was not associated with the transcriptional activity of UGT1A9.

### *3.3. DNA methylation status of the UGT1A10 promoter region in colon- and liver-derived cell lines*

We next investigated the DNA methylation status of the UGT1A10 promoter region in six kinds of human cell lines: colon adenocarcinoma cell lines, LS180, Caco2, HT29, and SW480 and hepatocellular carcinoma cell lines, HepG2 and HuH-7. The degree of DNA methylation in the *UGT1A10* promoter was, in ascending order, HT-29 < LS180 < HepG2 < SW480 < HuH-7 < Caco-2 (Fig. 3). The expression level of UGT1A10 mRNA in these cells was measured, and the relation with the DNA methylation status was analyzed. DNA methylation levels tended to be inversely correlated with UGT1A10 mRNA expression levels (Spearman's  $r = -0.54$ ,  $p = 0.29$ ). These results suggest that the DNA methylation status would determine the basal expression level of UGT1A10 in cell lines. In the subsequent experiments, two cell lines, human colon adenocarcinoma LS-180 and hepatocellular carcinoma HuH-7 cells, were selected as representatives of UGT1A10-positive and -negative cells, respectively.

### *3.4. Effects of 5-Aza-dC and TSA on the expression of UGT1A10 mRNA*

To investigate the significance of the DNA methylation at the promoter region in UGT1A10 expression, experiments using epigenetic modulatory agents were performed. When these cells were treated for 5 days with 5-Aza-dC, which is an inhibitor of DNA methylation, UGT1A10 mRNA expression was dramatically increased in HuH-7 cells

( $\approx$ 16-fold at maximum), whereas UGT1A10 mRNA was marginally increased in LS180 cells ( $\approx$ 1.6-fold at maximum) (Fig. 4A). It was confirmed that 5-Aza-dC treatment decreased the methylation status in HuH-7 cells from 86% to 60% ( $p = 0.15$ ). These results demonstrated that UGT1A10 expression is silenced by DNA methylation. Unexpectedly, the methylation status in LS180 cells was slightly increased from 19% to 41% ( $p = 0.09$ ) by 5-Aza-dC treatment, although the reason is unknown (Fig. 4B). Next, the involvement of histone acetylation for the expression of UGT1A10 was investigated. When these cells were treated for 1 day with TSA, which is an inhibitor of histone deacetylase, the expression of UGT1A10 mRNA in HuH-7 cells was not changed (Fig. 4A). The expression of UGT1A10 mRNA in LS180 cells was decreased in a dose-dependent manner (Fig. 4A), but it may be due to the cytotoxicity of TSA. These results suggest that the impact of histone acetylation on the UGT1A10 regulation would be limited. The differential expression levels ( $\approx$ 3-fold) of UGT1A10 in LS180 cells between the controls for 5-Aza-dC and TSA treatment may be due to the difference in culture time.

### *3.5. Effects of 5-Aza-dC and the overexpression of HNF1 $\alpha$ and Cdx2 on the expression of UGT1A10*

We investigated whether the demethylation of DNA allows transcription factors to bind to the promoter of *UGT1A10* and thereby to activate transcription (Fig. 5). In intact HuH-7 and LS180 cells, HNF1 $\alpha$  was marginally expressed, and Cdx2 was not expressed (Fig. 5). The transfection of HNF1 $\alpha$  and Cdx2 expression plasmids into HuH-7 cells resulted in a dramatic increase in HNF1 $\alpha$  and Cdx2 proteins (Fig. 5) but did not increase UGT1A10 expression (Fig. 5). However, under 5-Aza-dC treatment, the overexpression of HNF1 $\alpha$  and Cdx2 resulted in a significant increase in UGT1A10 mRNA expression (11-fold) in HuH-7 cells. These results suggested that DNA methylation inhibits the binding of HNF1 $\alpha$  and Cdx2

to the promoter of *UGT1A10*. The overexpression of HNF1 $\alpha$  and Cdx2 under 5-Aza-dC treatment did not result in the upregulation of UGT1A10 in LS180 cells most likely because the extent of DNA methylation was originally low, and the endogenous HNF1 $\alpha$  expression levels might be sufficient for the interaction with unknown components that might be essential for UGT1A10 expression in LS180 cells (Fig. 5).

#### 4. Discussion

In this study, we investigated the underlying mechanism of defective expression of UGT1A10 in the liver focusing on epigenetics. We found that the CpG-rich region at the promoter of the *UGT1A10* gene was hypermethylated in the hepatocytes, whereas the CpG-rich region was hypomethylated in the small intestine epithelium (Fig. 1B). Reporter gene assays revealed that the methylation of the *UGT1A10* promoter leads to an almost complete loss of transactivity even under the overexpression of Cdx2 and HNF1 $\alpha$  (Fig. 2). Cell line-based studies clearly demonstrated the significance of DNA methylation in the regulation of UGT1A10 as follows: 1) the substantial expression of UGT1A10 mRNA was observed in LS180 cells with the DNA hypomethylation status, 2) 5-Aza-dC treatment resulted in the increase of UGT1A10 expression, reflecting the change in the DNA methylation status, and 3) exogenously expressed HNF1 $\alpha$  and Cdx2 could increase UGT1A10 expression only under 5-Aza-dC treatment in HuH-7 cells. These findings clearly illustrated that DNA methylation inhibits the expression of UGT1A10, and the unmethylated DNA status is a prerequisite for the transcriptional activation of UGT1A10. Concerning UGT1A8, which is also expressed in the gastrointestinal tract but not the liver, like UGT1A10, the promoter was hypomethylated in the small intestine epithelium and hypermethylated in the hepatocytes (Fig. 1B). The expression of UGT1A8 may also be regulated by DNA methylation, although this study did not examine it.

In general, gene silencing by DNA methylation is explained by following two mechanisms: 1) the methyl group physically interrupts the binding of transcription factors to their recognition sequences, and 2) methyl-CpG-binding proteins bind to the methylated DNA followed by the recruitment of corepressor molecules, including histone deacetylase, to induce chromatin structure condensation [11]. In the case of UGT1A10, absence of CpG dinucleotides in a Cdx2 or an HNF1 $\alpha$  recognition element unlikely to support the former

mechanism although the possibility that methylated CpGs outside the elements affect the binding of these factors could not be denied. The presence of CpGs (at positions -51 and -11) in two Sp1 sites on the *UGT1A10* promoter may explain the former mechanism. The latter mechanism is also unlikely to be involved because TSA treatment to inhibit histone deacetylation did not result in the activation of UGT1A10 expression. Although we have no exclusive explanation, the other mechanisms may be involved in the DNA methylation-dependent repression of UGT1A10.

The inhibition of DNA methylation and concomitant overexpression of HNF1 $\alpha$  and Cdx2 tremendously increased UGT1A10 mRNA levels in HuH-7 cells (Fig. 5). However, even under this condition, UGT1A10 levels remained lower than the level in intact LS180 cells (Fig. 5). This might be because the DNA methylation status in *UGT1A10* promoter of 5-Aza-dC-treated HuH-7 cells was still higher (60%) than that in intact LS180 cells (19%) (Fig. 4). Another possible reason may relate to differences in histone modifications or unidentified transcription factors that regulate UGT1A10 expression between the two cells. Sp1, which has been proven to enhance UGT1A10 promoter activity [7], was substantially expressed in both cell lines. To investigate the role of Sp1 on high expression of UGT1A10 in LS180 cells, we performed knockdown experiments using siRNA. When a siRNA for Sp1 was transfected into LS180 cells, severe cytotoxicity was observed (data not shown). The severe cytotoxicity was also observed with a siRNA for HNF1 $\alpha$  (data not shown). This may be caused because these transcription factors are indispensable for cell proliferation. Thus, the knockdown experiments unfortunately could not provide additional information. Further analysis is required to uncover the mechanism of the differential expression level of UGT1A10 in HuH-7 and LS180 cells.

In contrast with UGT1A10 and UGT1A8, UGT1A9, of which the promoter sequence (-1 kb from TSS) shares 80% and 79% similarity with the promoter sequences of the *UGT1A10*



and *UGT1A8* genes, respectively, is expressed in the liver but not in intestines. As supported by the present study (Fig. 2), it has been reported that *UGT1A9* is not transactivated by *Cdx2* but is transactivated by *HNF1 $\alpha$*  and *HNF4 $\alpha$*  [8, 19]. The present study found that the DNA methylation status at the 5'-flanking region of *UGT1A9* was almost the same between the small intestine and liver (Fig. 1) and that the methylation status did not affect the transcriptional activity (Fig. 2). The results suggest that the DNA methylation in the 5'-flanking region of *UGT1A9* is not associated with the tissue-specific expression of *UGT1A9*. Although the reason for the defective expression of *UGT1A9* in the small intestine remains to be studied, the involvement of histone modification or repressive transcription factors may be possible.

Our previous study demonstrated that DNA hypermethylation and histone H3 hypoacetylation results in the defective expression of *UGT1A1* in the kidney, revealing the impact of epigenetic modification in the tissues-specific expression of *UGT1A1* [17]. In this study, we found that *UGT1A10* expression is distinctly regulated by DNA methylation. Previous studies have revealed that the expression of *UGT1A6*, *UGT2B15*, and *UGT2B28* [20] as well as *UGT2B7* and *UGT2B11* [21], in cancer cell lines were increased by treatment with 5-Aza-dC or valproate, which is also a DNA methylation inhibitor. Although the DNA methylation status of these five *UGT* isoforms has not been investigated, the tissue- or cell-specific expression of most *UGTs* may be epigenetically regulated.

Interestingly, accumulating evidence reveals that inverse correlation was observed between the extent of DNA methylation and expression levels among individuals for some gene such as *CYP1A2*, monoamine oxidase A, ATP-binding cassette A1, *SLC6A4*, and *SLC22A2* [22-26]. This raises a possibility that interindividual variability of DNA methylation contributes to the variability in the *UGT1A10* expression, although the present study did not investigate it because we focused on the tissue specific expression. Yasar et al. [27] reported

for UGT1A1 that there was no clear inverse correlation between the DNA methylation and interindividual variability of gene expression in 46 individual livers. This may occur because they used whole liver sample, which contains non-parenchymal cells not expressing UGT1A1. It would be of interest to investigate the association between the interindividual variability of UGT expression and the extent of DNA methylation with careful selection of tissues or cell types.

In summary, we found that DNA methylation in the *UGT1A10* gene promoter limits the binding of transcription factors to repress the expression of UGT1A10 in the liver. The finding provides novel mechanisms of the tissue-specific expression of UGT1A10.

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## Figure legends

**Fig. 1.** DNA methylation status of *UGT1A8*, *UGT1A9*, and *UGT1A10* in human hepatocytes and small intestine. **(A)** A schematic diagram of the 5'-flanking region of *UGT1A8*, *UGT1A9*, and *UGT1A10*. The TSS of each UGT isoform is designated as +1. The vertical lines and rectangles represent the CpG sites and the binding sites of each transcription factor, respectively. Bisulfite sequencing was performed in the regions that are outlined with a dashed line. The homologies of -1000 bp upstream to TSS of each UGT1A isoform are shown on the right. **(B)** DNA methylation status of CpG sites in the 5'-flanking region of *UGT1A8*, *UGT1A9*, and *UGT1A10* genes. Bisulfite sequencing analysis was performed using genomic DNA that was extracted from human hepatocytes (HH268) or small intestine epithelium (a 49-year-old Caucasian female). For *UGT1A10*, the DNA methylation status in the genomic DNA that was extracted from whole small intestine was also investigated. At least ten clones from each sample were sequenced. Open and closed circles represent unmethylated and methylated cytosines, respectively.

**Fig. 2.** Effects of DNA methylation on the transactivity of *UGT1A10* and *UGT1A9*. pCpGL-basic plasmids containing either -365 to +140 of *UGT1A10* or -955 to +29 of *UGT1A9*, as well as pCpGL-basic plasmids, were treated with *Sss* I DNA methylase. Both the treated or untreated reporter construct and *Cdx2*, *HNF1 $\alpha$*  or *HNF4 $\alpha$*  expression plasmids were transiently transfected into HuH-7 cells. After 48 h, the cells were harvested, and the luciferase activities were measured. Each column represents the mean  $\pm$  SD of relative activities (firefly/ $\mu$ g protein) of triplicate determinations. \*  $p < 0.05$  and \*\*  $p < 0.01$ , compared with no transfection by one way ANOVA followed by Dunnett's test. ##  $p < 0.01$  and ###  $p < 0.001$ , compared with the unmethylated construct by two-tailed Student's *t*-test.

**Fig. 3.** Relation between the DNA methylation status of the promoter region of *UGT1A10* and mRNA expression levels of *UGT1A10* in colon- (circle) and liver-derived (triangle) carcinoma cells lines. The DNA methylation status of *UGT1A10* was analyzed by bisulfite sequence analysis of at least five clones for each cell. The methylation status was expressed as the percentage of methylated cytosines per total CpG dinucleotides among all of the sequenced clones. The expression levels of *UGT1A10* mRNA were expressed relative to those levels in HepG2 cells.

**Fig. 4.** Effects of 5-Aza-dC and/or TSA treatment on the *UGT1A10* expression in HuH-7 and LS180 cells. **(A)** *UGT1A10* mRNA levels in HuH-7 and LS180 cells that were treated with 5-Aza-dC or TSA, which were normalized to the GAPDH mRNA levels. Each column represents the mean  $\pm$  SD of triplicate determinations. \*\*  $p < 0.01$ , compared with non-treated cells by one way ANOVA followed by Dunnett's test. **(B)** DNA methylation status of the *UGT1A10* promoter region in HuH-7 and LS180 cells before and after treatment with 0.1  $\mu$ M 5-Aza-dC. Bisulfite sequencing analysis of at least eight clones for each cell was performed.

**Fig. 5.** Effects of 5-Aza-dC treatment and the overexpression of HNF1 $\alpha$  and Cdx2 on *UGT1A10* expression in HuH-7 and LS180 cells. The cells were treated with 5-Aza-dC followed by the transient transfection of HNF1 $\alpha$  and/or Cdx2 expression plasmids (+) or empty plasmid (-). The expression level of *UGT1A10* mRNA was determined by real-time RT-PCR and was normalized to GAPDH mRNA levels. The data were expressed relative to *UGT1A10* expression compared with non-treated HuH-7 cells. The HNF1 $\alpha$  and Cdx2 protein levels were analyzed by Western blot of total cell homogenates. Each column represents the mean  $\pm$  SD of triplicate determinations. \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ , compared with non-treated cells by one way ANOVA followed by Dunnett's test.



## **Footnotes**

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**Table 1.** Sequences of oligonucleotides that were used in the present study.

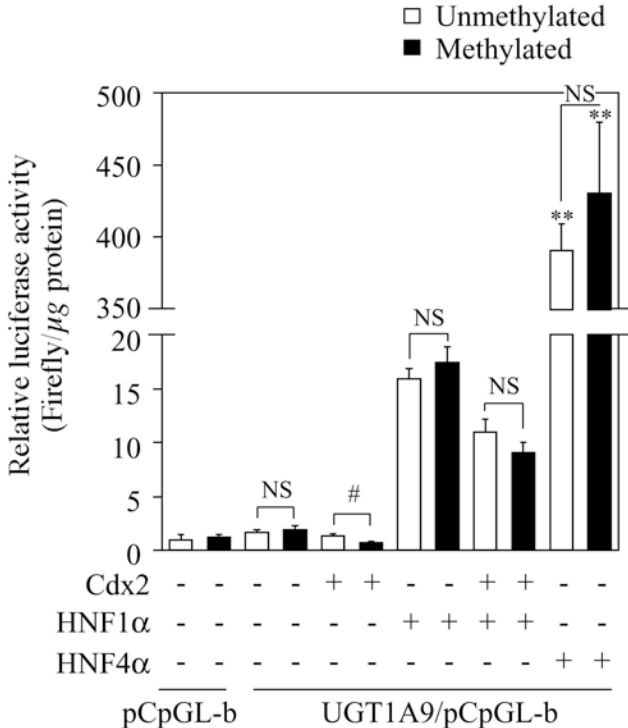
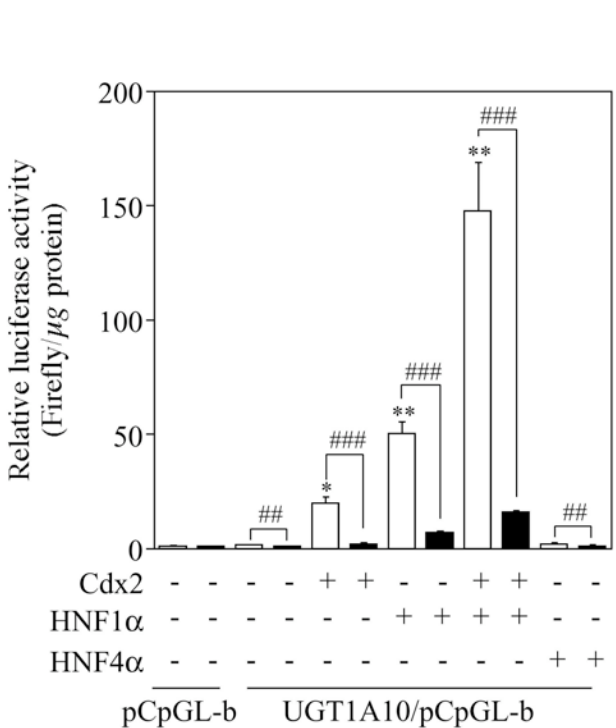
Oligonucleotides	5' to 3' sequence	Position
Bisulfite analysis of UGT1A8 and UGT1A10		
Forward	AGAGAGTATTTGGTTGGTTAAAG	-365 to -343 <sup>a</sup>
Reverse	ACACTACCAACAACCTCCCTACC	+118 to +140 <sup>a</sup>
Bisulfite analysis of UGT1A9		
Forward	TTGAAGGAGGGTATTGGAGTGATG	-754 to -730
Reverse	CCAAACCCTAAAAACCTCTAAAATAC	-540 to -514
Cloning of promoter region of UGT1A10		
Forward	CTTTGGATCCAGAGAGTATTTGGTTGGC	-365 to -347
Reverse	CCATAGATCTGCACTACCAGCAGCTTCCC	+122 to +140
Cloning of promoter region of UGT1A9		
Forward	GGCAGCTGCAGTTGATCTTTCCCTTTAAG	-955 to -937
Reverse	CAGAGATCTGCAGCTGAGAG	+17 to +29
ChIP assay of UGT1A10		
Forward	AATGATACTCGTGTGTTATC	-135 to -116
Reverse	AGACACACACATAAAGGAAC	+76 to +95
Cloning of Cdx2		
Forward	CCGGACCCTCGCCACCATGTA	-16 to +5
Reverse	GTGGGTCCTGGGTGACGGT	+927 to +947

Nucleotides are numbered with the TSS designated as +1 in the genomic DNA sequence of UGTs and with base A in the initiation codon ATG designated as +1 in the Cdx2 cDNA sequence. The restriction sites that were used for cloning are underlined.

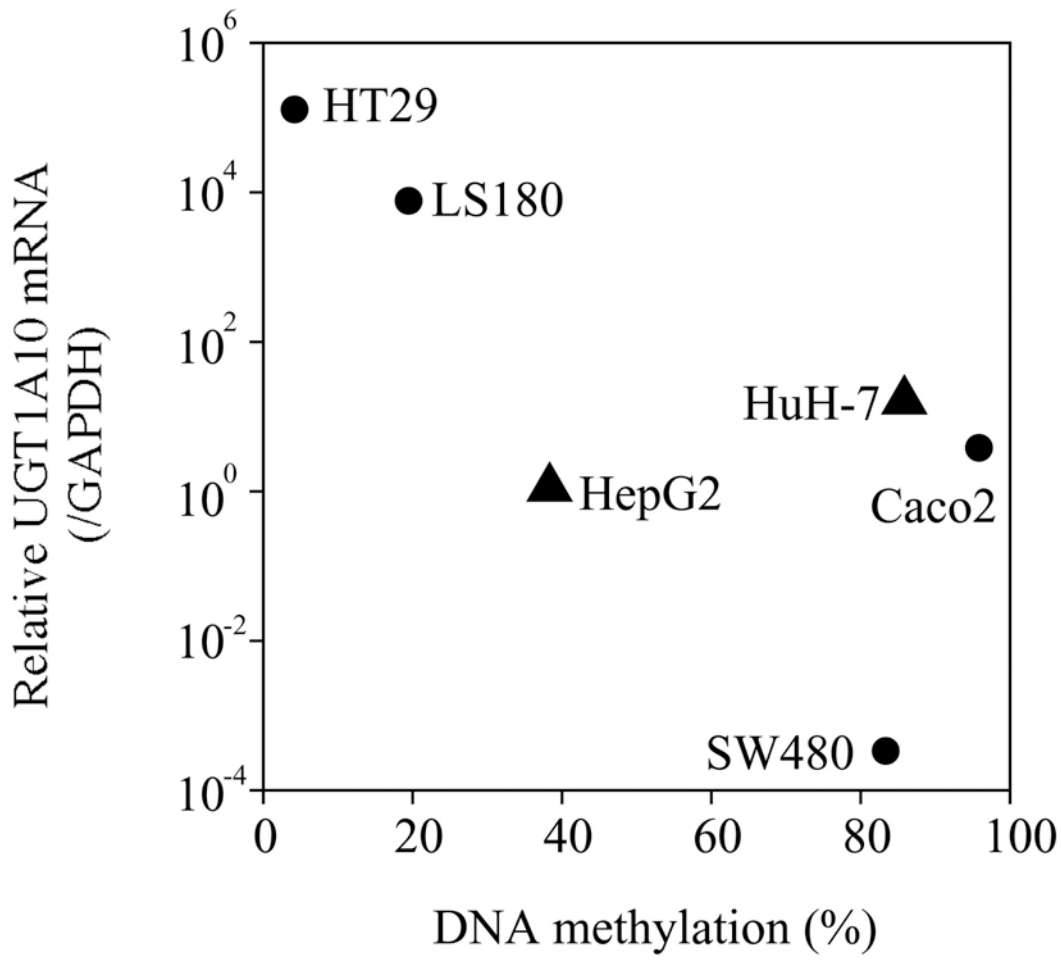
<sup>a</sup> The numbers refer to the nucleotide position of *UGT1A10*.



**Figure 2**

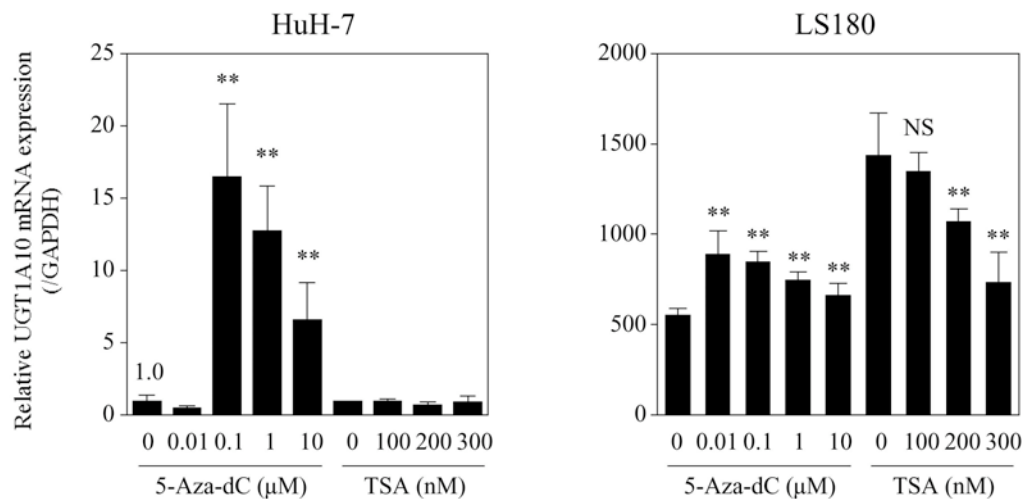


**Figure 3**

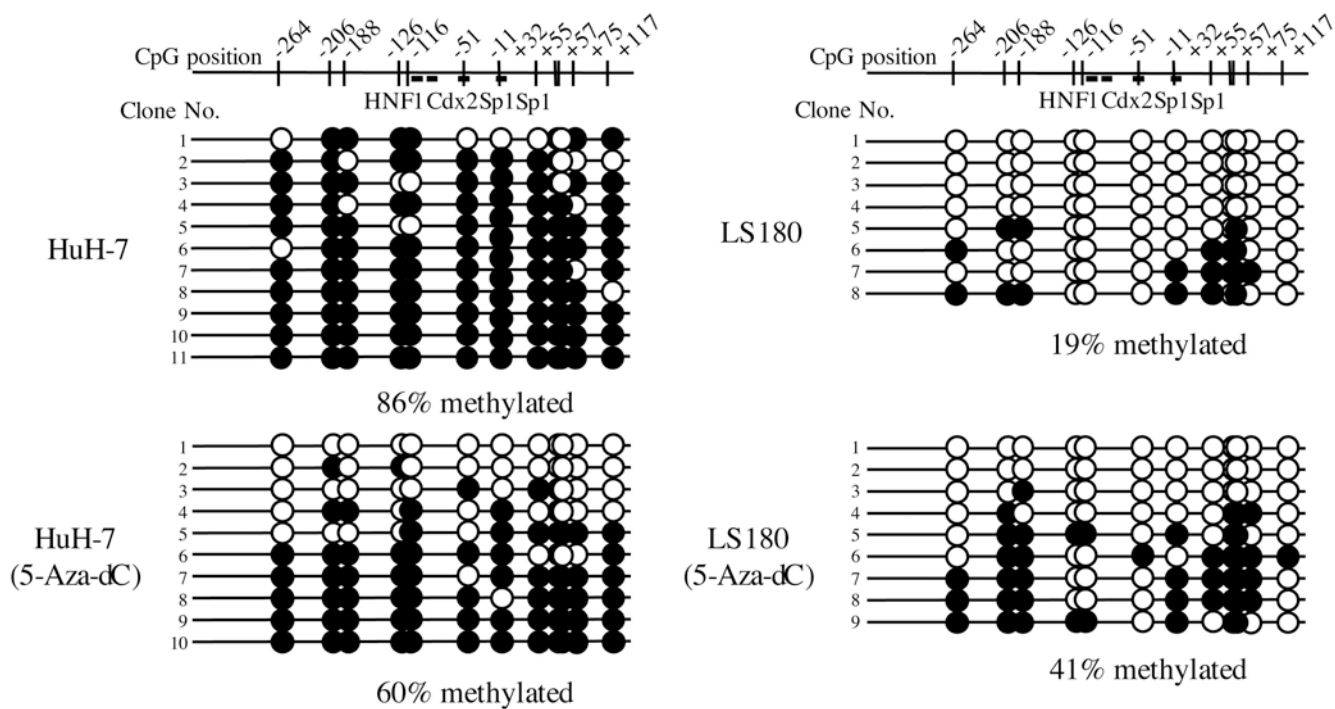


**Figure 4**

**A**



**B**



**Figure 5**

