

CYP1B1, but not CYP1A1, is downregulated by promoter methylation in colorectal cancers

WATARU HABANO¹, TOSHIE GAMO¹, TAMOTSU SUGAI², KOKI OTSUKA³,
GO WAKABAYASHI³ and SHOGO OZAWA¹

¹Department of Pharmacodynamics and Molecular Genetics, School of Pharmacy, Iwate Medical University, 2-1-1 Nishitokuta, Yahaba-Cho, Shiwa-Gun, Iwate 028-3694; ²Division of Molecular Diagnostic Pathology, Department of Pathology, ³Department of Surgery, School of Medicine, Iwate Medical University, Iwate Medical University, 19-1 Uchimarui, Morioka, Iwate 020-8505, Japan

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Abstract. Cytochrome P450 (CYP) 1A1 (CYP1A1) and CYP1B1, dioxin-inducible CYP1s, are associated with carcinogenesis in extrahepatic tissues. CYP1B1 is featured in carcinogenesis of hormone-responsive tissues, where the CYP1B1 level is considerably high. Although aberrant expression of these enzymes is also observed in cancers that are not related to hormone response, their roles in carcinogenesis are not yet fully understood. We examined DNA methylation status of the CpG islands within the 5'-flanking region of the *CYP1B1* and *CYP1A1* genes in 7 colorectal cancer cell lines and 40 primary colorectal cancers. By bisulfite-modified direct sequencing, *CYP1B1* gene methylation was detected in 2 cell lines (SW48 and Caco-2) and 2 (5%) cancers, but not in corresponding normal tissues. Treatment of the cells with 5-aza-2'-deoxycytidine revealed a clear increase in the CYP1B1 mRNA levels in SW48 and Caco-2 cells, while the amount of methylated alleles decreased. Only HT29 cells showed a clear increase in CYP1A1 mRNA, although there were no apparent differences in methylation status among these cell lines. None of these cell lines showed significant change in mRNA levels of aryl hydrocarbon receptor (AhR) and AhR nuclear translocator (ARNT), which are known to directly activate *CYP1* transcription. This observation suggested that expression of CYP1B1, but not CYP1A1, was downregulated by promoter methylation rather than decreased expression of AhR/ARNT. In conclusion, CpG methylation of the *CYP1B1* promoter region epigenetically regulates CYP1B1 expression during

development of some colorectal cancers. Moreover, cancers with aberrant CYP1B1 expression might show altered response to procarcinogen metabolism and chemotherapy.

Introduction

Human cytochrome P450 (CYP) 1A1 (CYP1A1) and CYP1B1, dioxin-inducible members of the CYP family, activate procarcinogens, including benzo[*a*]pyrene and 7,12-dimethylbenz[*a*]anthracene (1-3). The expression of CYP1 family is controlled by binding of several transcription factors, such as aryl hydrocarbon receptor (AhR) and AhR nuclear translocator (ARNT) heterodimer, with a particular tissue specificity (4-7). CYP1B1 is also known to catalyze the hydroxylation of 17 β -estradiol (8), and is associated with carcinogenesis in hormone-mediated cancers, including breast and prostate cancers (9,10). On the other hand, aberrant expression of the CYP1B1 is observed in wide variety of cancers, independent of hormone response (11). Colorectal cancers generally overexpress CYP1B1, but with a large heterogeneity (12,13). In addition, certain genetic polymorphisms of the CYP1B1 were also associated with incidences of colorectal cancers (14). These observations prompt us to speculate that CYP1B1 plays a possible role in colorectal carcinogenesis. However, the mechanisms regulating tissue-specific and cancer-specific expression of CYP1B1 and their roles in carcinogenesis are not yet clarified.

Aberrant expression of cancer-related genes (oncogenes and tumor suppressor genes) is caused by epigenetic mechanisms during carcinogenesis. One of the well-characterized mechanisms is DNA methylation of the promoter CpG islands, which leads to a transcriptional downregulation of tumor suppressor genes (15). Recent studies demonstrated that some drug-metabolizing enzyme genes also have CpG islands in their promoter region and can be downregulated by DNA methylation in cancers (16). CYP1B1 in prostate cancers was reported to be overexpressed through the *CYP1B1* promoter hypomethylation (17). These observation prompted us to examine whether *CYP1B1* promoter methylation in normal colon mucosa is lost during colon carcinogenesis to overexpress CYP1B1.

Correspondence to: Dr Wataru Habano, Department of Pharmacodynamics and Molecular Genetics, School of Pharmaceutical Sciences, Iwate Medical University, 2-1-1 Nishitokuta, Yahaba-Cho, Shiwa-Gun, Iwate 028-3694, Japan
E-mail: whabano@iwate-med.ac.jp

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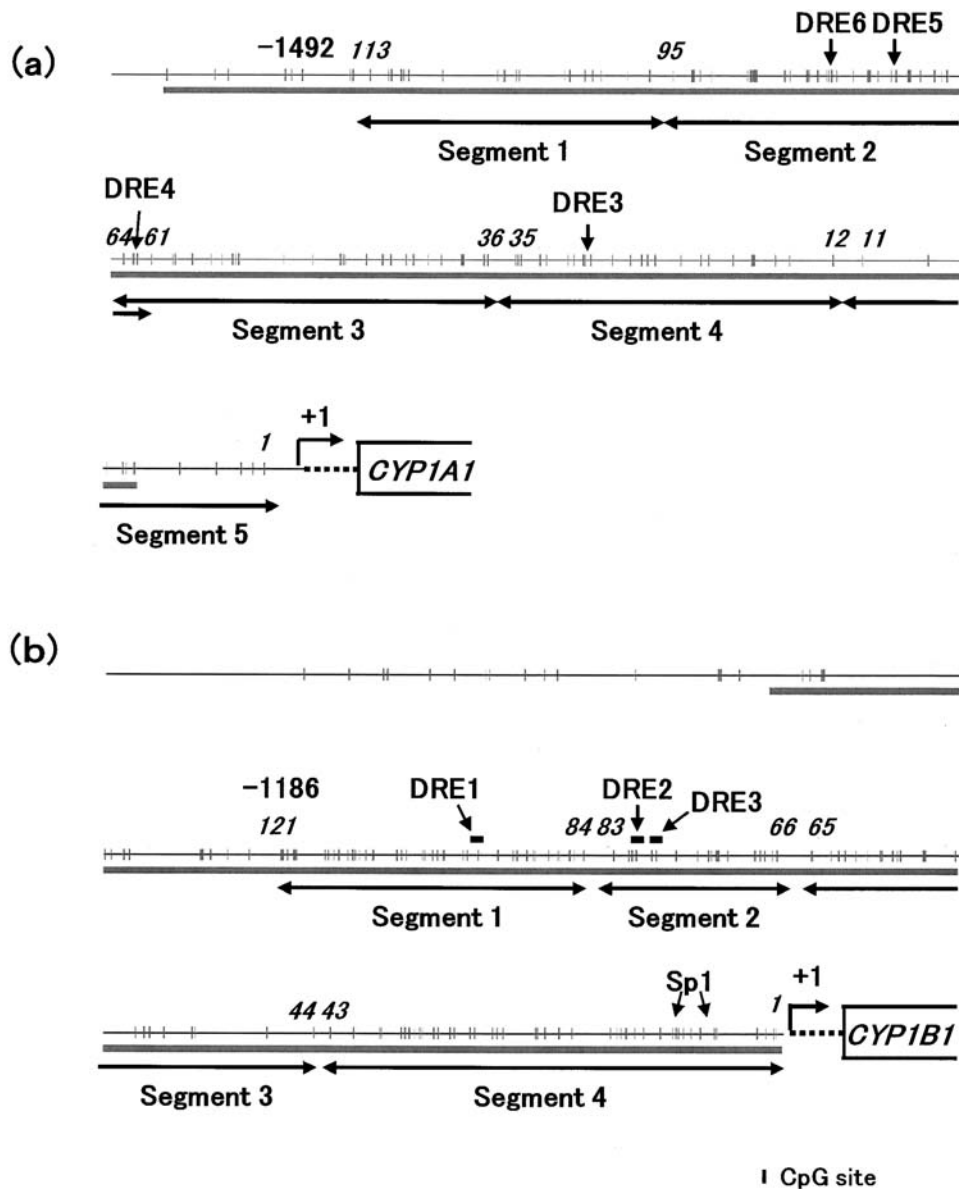


Figure 1. Schematic diagram of locations of the CpG sites (vertical bars), transcription start site (curved arrow) and potential binding sites of transcription factors (Sp1, DREs) within the 5' prime region of: (a), *CYP1A1*; and (b), *CYP1B1* gene. CpG sites are numbered in *italics* sequentially starting from the transcription start site with an increasing order to the upstream direction. For bisulfite sequencing analysis, 1.5 and 1.2 kb upstream regions (including 113 and 121 CpG sites) of the *CYP1A1* and *CYP1B1* gene were divided into five and four segments, respectively.

In order to clarify the mechanisms by which cancers can acquire altered expression of the *CYP1A1* and *CYP1B1*, we focused on epigenetic mechanisms such as DNA methylation. We examined DNA methylation status in the 5' upstream region of the *CYP1B1* and *CYP1A1* genes in colorectal cancer cell lines and primary cancers. If DNA methylation is involved in these cancers, these enzymes would be associated with cancer development and affects the chemosensitivity of cancer cells through epigenetic mechanisms.

Materials and methods

Cell lines and tissue samples. Human colorectal cancer cell lines used in this study (HT29, HCT116, DLD-1, LoVo, SW48, SW620 and Caco-2) were purchased from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). HT29, HCT116 and SW48 cells were cultured in McCoy's 5A medium (Invitrogen

Corp., Carlsbad, CA). DLD-1 and LoVo cells were cultured in Ham's F12 medium (Invitrogen). SW620 cells were cultured in Leibovitz L-15 medium (Invitrogen). Caco-2 cells were cultured in E-MEM medium (Invitrogen). All mediums were supplemented with 10% fetal bovine serum (FBS, HyClone Laboratories Inc., Logan, UT). These cells were cultured at 37°C under an atmosphere of 5% CO₂.

All clinical tissue samples were obtained from patients following provision of informed consent according to the Ethics Committee guidelines of Iwate Medical University, Japan. Forty pairs of cancer and adjacent normal mucosa were excised from surgical specimens of colorectal cancers and then subjected to crypt isolation (18) to obtain cancer and normal epithelia without stromal cells.

Treatment with 5-aza-2'-deoxycytidine. HT29, HCT116, SW48 and Caco-2 cells were seeded at a concentration of

Table I. Primers used for DNA methylation analysis.

Gene	Segment	Primer sequence (5'-3')	Annealing temperature (°C)	Product size (bp)
<i>CYP1B1</i>	1F	GGTTTTTTTATAAAGGGAGGGTTT	55	310
	1R	ACTCCAATCATATCCCTAAAC		
	2F	GTTTAGGGATATGATTGGAGT	50	189
	2R	ACTAAAAAACCTAAAAAACTAAC		
	3F	GTTAGTTTTTTTAGGTTTTTTTAGT	55	343
	3R	CCAATATAATAACAACRATTTACCT		
	4F	ATTAGGGGYGTTGTGAGGTAAA	60	432
	4R	TCCCACTCCCACTCCAAAAT		
<i>CYP1A1</i>	1F	GGGATTATTTTTTGGTTTGGATTAG	50	303
	1R	TCTAAATCTACRTATAACTTCTAACCT		
	2F	AGGTTAGAAGTTATAYGTAGATTTAGA	55	310
	2R	CATCCCTCTAAAAAACAAAAATC		
	3F	GATTTTTGTTTTTTAGAGGGATG	60	369
	3R	ACTAAACCTATCCCCCAAACC		
	4F	GGTTTTGGGGGATAGGTTTAGT	60	329
	4R	TCCCAAAAACAATACAAAACCCAAC		
	5F	GTTGGGTTTTGTATTGTTTTTGGGA	55	270
	5R	TAAAAACTACCACCTTTATAAAC		

F, forward primer; R, reverse primer.

1x10⁵ cells on a 100 mm dish. The next day, treatment of cells with 0, 0.5 or 5 μ M 5-aza-2'-deoxycytidine (5-aza-dC) (Sigma Chemical, St. Louis, MO) was started, and 5-aza-dC was removed by changing medium 24 h later. The cells were harvested 4 days after removal of 5-aza-dC for DNA and RNA extraction.

DNA methylation analysis. CpG islands within the 5'-flanking region of the *CYP1B1* and *CYP1A1* genes were screened by CpG Island Searcher program (<http://cpgislands.usc.edu/>). The CpG islands encompassed 1.2 and 1.5 kb upstream region of the transcription start sites of the *CYP1A1* and *CYP1B1* genes, respectively. For PCR amplifications, these regions of the *CYP1A1* and *CYP1B1* genes were divided into five and four segments, respectively (Fig. 1). All primer sequences and PCR conditions used for methylation analysis are shown in Table I. Genomic DNAs obtained from colorectal cancer cell lines and crypt isolation samples were modified by sodium bisulfite and subjected to direct sequencing (19). A combined bisulfite restriction analysis (COBRA) (20) using an *HpyCH4IV* (New England Biolabs, Inc., Ipswich, MA) restriction site was also designed to determine *CYP1B1* methylation status with a high degree of quantitative accuracy. A relative methylation level in each sample was visually determined by the density of each *HpyCH4IV*-digested band. The validity of the COBRA assay was con-

firmed by bisulfite sequencing on at least 8 individual DNA strands after subcloning of PCR products into pCR4-TOPO vector using TOPO TA Cloning Kit for Sequencing (Invitrogen).

Quantitative real-time PCR analysis. Total RNA was isolated from cultured cells with RNeasy Mini Kit and DNase Set (Qiagen, Valencia, CA) following the manufacturer's protocol. mRNA levels of *CYP1A1*, *CYP1B1*, AhR, ARNT and β -actin were evaluated by real-time PCR system 7500 (Applied Biosystems, Foster City, CA) using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and FastStart Universal SYBR Green Master (ROX) (Roche, Basel, Switzerland). The primer sequences were described previously (21). Quantitative values were obtained from the threshold cycle (C_t) number. The mRNA level of the target gene was normalized to its β -actin mRNA content for each sample. All samples were analyzed in duplicate and average quantities of the gene transcripts were used for calculation. Deviation of the mRNA level of each sample was within 7% of the average.

Results

DNA methylation of the *CYP1B1* and *CYP1A1* genes in colorectal cancer cell lines. Using CpG Island Searcher program, we found CpG islands within the 5' prime region of

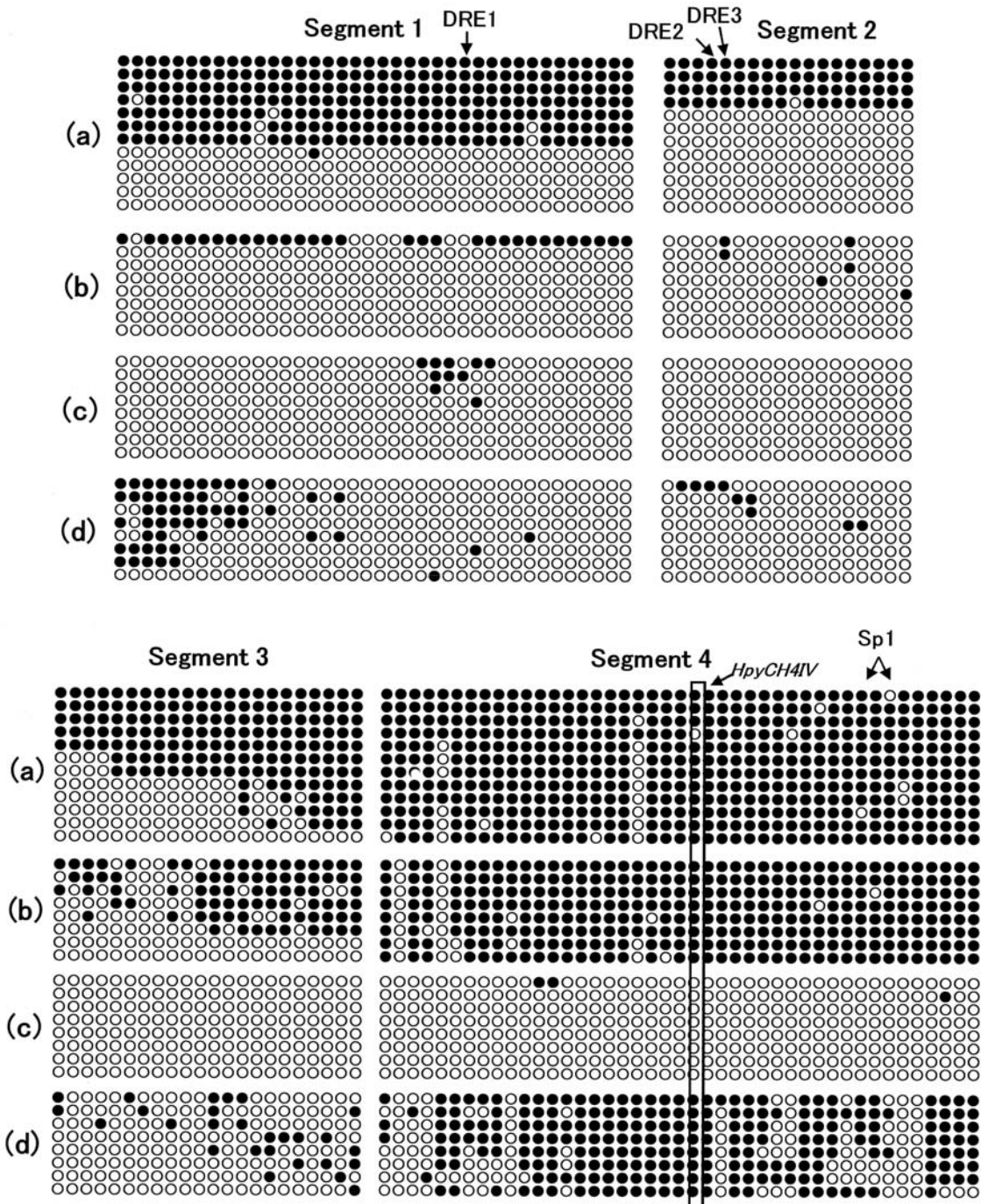


Figure 2. Summary of the results of bisulfite sequencing analysis of the four segments within the *CYP1B1* gene. Methylation status of: (a), SW48 cells; (b), Caco-2 cells; (c), a normal tissue; and (d), a paired cancer tissue (showing methylation with the highest magnitude) are shown. Open and closed circles represent unmethylated and methylated CpG dinucleotides, respectively. The arrow with '*HpyCH4IV*' indicates restriction site using COBRA assay.

the *CYP1A1* and *CYP1B1* genes (Fig. 1). DNA methylation status of these CpG islands was prescreened on a limited set of pilot samples (7 cancer cell lines and 3 pairs of cancer and normal tissues of colorectum). Bisulfite direct sequencing demonstrated that *CYP1A1* gene methylation was exclusively detected in segment 1, which is upstream of functional enhancer dioxin response elements (DREs 3-6) and promoter regions, and was consistent with a previous report (22). In addition, there were no apparent differences in the CpG methylation pattern among these samples. Therefore, CpG methylation of the *CYP1A1* gene might be tightly controlled and highly stable during colorectal carcinogenesis.

On the other hand, we found *CYP1B1* gene methylation in two cell lines (SW48 and Caco-2) and one of the three cancer tissues, but none in the paired normal tissues. Bisulfite sequencing on each DNA strand revealed that methylation occurs throughout the CpG islands, including three DREs (enhancer) and two Sp1-binding sites (promoter) (23-25). In particular, CpG sites within the segment 4, which encompasses these Sp1-binding sites, were the most densely methylated in these cell lines and the cancer tissue (Fig. 2a, b and d). Consequently, we determined *CYP1B1* gene methylation status by COBRA assay using an *HpyCH4IV* restriction site proximal to these Sp1-binding sites (see below).

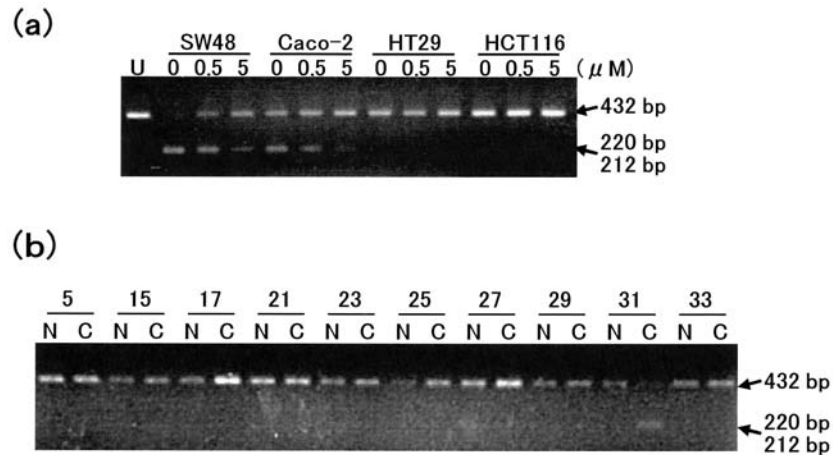


Figure 3. Methylation status of the *CYP1B1* gene (segment 4, COBRA assay). (a) SW48, Caco-2, HT29 and HCT116 cells were treated with 5-aza-2'-deoxycytidine (0, 0.5 and 5 μ M). A 432-bp band shows uncut (U) or unmethylated DNA, while 220/212-bp bands show methylated DNA. (b) Methylation status in ten primary colorectal cancers (numbers are those for particular cases). DNA samples from normal and cancer tissue are shown as N and C, respectively. Note that a high degree of methylation was detected in cancer, but not in normal tissue of case 31.

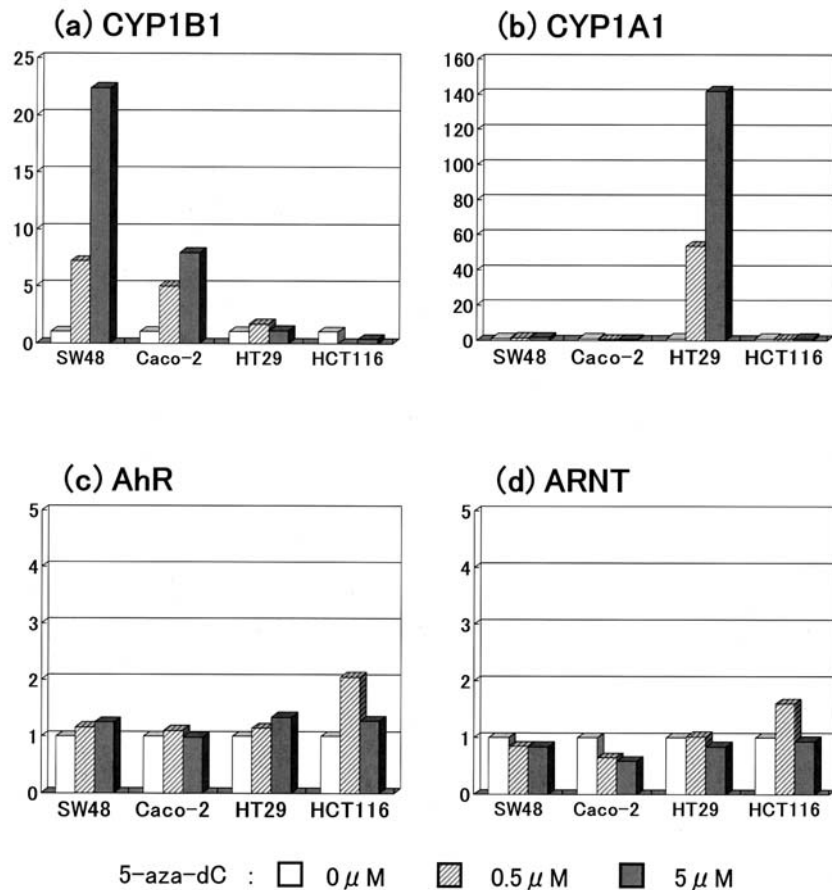


Figure 4. Levels of: (a), *CYP1B1*; (b), *CYP1A1*; (c), *AhR*; and (d), *ARNT* transcripts (real-time PCR analysis). SW48, Caco-2, HT29 and HCT116 cells were treated with 5-aza-2'-deoxycytidine (0, 0.5 and 5 μ M). The vertical axis indicates a relative transcript level (ratio to cells without 5-aza-dC treatment).

Inhibition of DNA methylation and re-expression of the CYP1B1 gene by 5-aza-dC. In order to determine whether demethylation of the CpG sites resulted in re-expression of the *CYP1B1* gene, cultured cells were treated with 0.5 or 5 μ M of 5-aza-dC. The COBRA assay revealed decreased amounts of methylated *CYP1B1* alleles in SW48 and Caco-2 cells, and the decreased levels were dependent on the dose of 5-aza-dC

(Fig. 3a). Moreover, real-time PCR analysis showed an increase (recovery) in *CYP1B1* transcripts in SW48 (7-22-fold increase) and Caco-2 (5-8-fold increase) cells in a dose-dependent manner, but not in HCT116 and HT29 cells (Fig. 4a). Thus, the magnitude of methylation was associated with decreased levels of *CYP1B1* gene expression. This also implied that methylation level of the *HpyCH4IV* sites could

reflect the level of reduced expression of *CYP1B1* gene. In contrast, 5-aza-dC had little effect on the constitutive levels of AhR and ARNT mRNAs in all cells examined (Fig. 4c and d). This result was supported by our findings that there was no CpG island in the 5' proximal upstream region of the AhR and ARNT genes. These results suggested that the *CYP1B1* gene was transcriptionally downregulated in SW48 and Caco-2 cells by direct methylation of their promoter/enhancer CpG sites rather than AhR and/or ARNT downregulation.

On the other hand, HT29 cells showed a clear increase in CYP1A1 mRNA (more than 140-fold increase) after 5-aza-dC treatment (Fig. 4b). However, methylation status of the *CYP1A1* gene exhibited no differences among the four cell lines examined, suggesting that CYP1A1 expression was downregulated in HT29 cells by mechanisms other than its promoter methylation.

DNA methylation of the CYP1B1 gene in primary colorectal cancers. The methylation status of *CYP1B1* gene was examined in 40 pairs of colorectal cancers and adjacent normal tissues by COBRA assay. Two cancer tissues (5%) exhibited methylation in the promoter region, while none of the normal tissues showed methylation (Fig. 3b). Therefore, the *CYP1B1* promoter was somatically hypermethylated in some colorectal cancers, and this likely led to transcriptional downregulation of *CYP1B1* gene. There were no differences in clinicopathological findings between colorectal cancers with *CYP1B1* methylation and those without methylation.

Discussion

In the present study, we found DNA hypermethylation of the 5' upstream region of the *CYP1B1* gene in colorectal cancer cell lines and primary cancers. *In vitro* studies using demethylating agent (5-aza-dC) revealed that the *CYP1B1* gene was transcriptionally downregulated in cancer cells by direct methylation of their promoter/enhancer CpG sites. Previous studies demonstrated that CYP1B1 overexpression observed in lung carcinomas was associated with elevated expression of AhR (26). However, we found that CYP1B1 was not associated with relevant changes of AhR and ARNT expression in colorectal cancer cells. Therefore, DNA methylation likely interfered with the binding of AhR/ARNT heterodimer, or affected histone modifications and chromatin structure, which indirectly prevented transcription factor binding (15) in colorectal cancer cells. The previous study by others (26) and our study may suggest possible differences in tissue specific mechanisms of CYP1B1 expression between lung and colon cancers. The CpG sites around two Sp1-binding sites were most densely methylated in these cell lines and cancer tissue, suggesting that promoter methylation might play more critical roles in loss of CYP1B1 functions than enhancer methylation.

It is interesting that only HT29 cells showed a clear increase in *CYP1A1* mRNA after 5-aza-dC treatment. There were no apparent differences in CYP1A1 methylation status among these cell lines. Moreover, DNA methylation could not be identified in segments 2 and 3, which were known regulatory region and corresponded to cancer-specific

methylation sites of prostate (27). Therefore, CpG methylation status of the *CYP1A1* gene might be tightly controlled and highly stable, even during colorectal carcinogenesis. Consequently, downregulation of CYP1A1 in colorectal cancers resulted from promoter methylation of certain CYP1A1-regulatory factor(s) rather than CYP1A1 itself, although the mechanisms are still unclear.

We found *CYP1B1* promoter methylation in 2 of 40 (5%) primary colorectal cancers, but not in non-cancer tissues. The results reflected genuine DNA methylation status, because we examined pure cancer and normal epithelia using crypt isolation (19) and directly compared DNA methylation status between paired epithelia. We could not directly compare DNA methylation status with the CYP1B1 mRNA expression, because crypt isolation provided ethanol-fixed epithelia and it was difficult to obtain fresh mRNA samples. However, these cancer tissues exhibited a pattern of DNA methylation quite similar to that observed in SW48 and Caco-2 (Fig. 2). Therefore, the association between promoter methylation and transcriptional silencing of *CYP1B1* gene is most likely applicable to primary colorectal cancers. It was recently demonstrated that *CYP1B1* gene was hypermethylated in gastric cancers (28), while *CYP1B1* gene was hypomethylated in prostate cancers (17). Taken together with these studies, we suggest that aberrant expression of the *CYP1B1* gene is commonly regulated by promoter methylation in human cancers, independent of hormone response. Previous studies demonstrated frequent CYP1B1 overexpression in colorectal cancers (12,13). The CYP1B1 overexpression is, however, unlikely due to hypomethylation (demethylation) of the *CYP1B1* promoter, because normal colon tissues never showed promoter methylation in the present study. Therefore, certain other mechanisms, such as microRNA downregulation observed in a breast cancer cell line (29), may be associated with CYP1B1 overexpression in colorectal cancers. The present study showed that a minor, but a significant subgroup of colorectal cancers did undergo downregulation of CYP1B1 through its promoter methylation. Interestingly, it was found that AhR was a putative Wnt/ β -catenin target in prostate cancers (30). In addition, sulindac, one of the nonsteroidal anti-inflammatory drugs (NSAIDs), exerts its chemopreventive effects by inducing carcinogen metabolizing enzymes such as CYP1B1 through activating AhR (31). Therefore, aberrant expression of CYP1B1 by promoter methylation might play roles in colorectal carcinogenesis through Wnt/ β -catenin signaling pathway.

Cancer chemotherapy exhibits marked variability in drug response between individual patients. These differences mainly resulted from genetic polymorphisms of drug-metabolizing enzymes and drug transporters. For example, several genetic polymorphisms of UDP-glucuronosyltransferase 1A1 (UGT1A1), which acts as a detoxifier of irinotecan, provide clinically relevant determinants for predicting severe toxicity in cancer patients (32-35). On the other hand, drug response also depends on the biological characteristics of individual cancers, because cancer is highly heterogeneous by accumulating multiple genetic and epigenetic alterations. Indeed, downregulation of the UGT1A1 by promoter methylation occurred in cancer cells and can lead to increased toxicity of irinotecan (16). Therefore, we suggest that

CYP1B1 methylation, similar to *UGT1A1* methylation, can alter metabolic capacities of cancer cells and alter clinical response to chemotherapy. Although *CYP1B1* methylation is not a common event in colorectal cancers in the present study, the *CYP1B1* might play important roles in both cancer development and response to procarcinogen metabolism and chemotherapy in some colorectal cancers. Therefore, we suggest that molecular profiling of epigenetic DNA methylation status of drug-metabolizing enzymes, in combination with information of genetic polymorphisms, may better characterize cancers and provide more accurate prediction of drug response.

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