Methylation of the Constitutive Androstane Receptor Is Involved in the Suppression of CYP2C19 in Hepatitis B Virus–Associated Hepatocellular Carcinoma

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ABSTRACT

Hepatocellular carcinoma (HCC), one of the most dangerous malignancies with an increasing incidence and a high mortality rate, represents a major international health problem. HCC progression is known to involve genome-wide alteration of epigenetic modifications, leading to aberrant gene expression patterns. The activity of CYP2C19, an important member of the cytochrome P450 superfamily, was reported to be compromised in HCC, but the underlying mechanism remains unclear. To understand whether epigenetic modification in HCC is associated with a change in CYP2C19 activity, we evaluated the expression levels of CYP2C19 and its transcription factors by quantitative real-time polymerase chain reaction using mRNA extracted from both primary hepatocytes and paired tumor versus nontumor liver tissues of patients infected with hepatitis B virus (HBV). DNA methylation was examined by bisulfite sequencing and methylation-specific polymerase chain reaction. Our results indicated that CYP2C19 could be regulated by e-box methylation of the constitutive androstane receptor (CAR). Decreased CYP2C19 expression in tumorous tissues of HBV-infected patients with HCC was highly correlated with suppressed expression and promoter hypermethylation of CAR. Our study demonstrates that aberrant CAR methylation is involved in CYP2C19 regulation in HBV-related HCC and may play a role in liver tumorigenesis.

Introduction

Although tremendous efforts have been made in the fight against hepatocellular carcinoma (HCC), HCC is the fifth most prevalent cancer worldwide and is currently the third leading cause of cancer mortality (Jemal et al., 2011). Hepatitis B virus (HBV) infection, hepatitis C virus infection, and alcohol abuse are the main causes of HCC, and HBV infection accounts for 60% of HCC in the Chinese population (Yang et al., 2014). A recent World Health Organization report estimates that 90 million people in China (almost 7% of the population) are chronically infected with HBV (Liu et al., 2016).

HCC progression was traditionally considered a gradual multistep process of normal cells evolving into tumor cells caused by mutations that led to altered hepatocellular phenotypes (Thorgerisson and Grisham, 2002; Lesset et al., 2014). However, evidence accumulated in the past few decades suggests that altered epigenetic modifications also play a pivotal role in HCC development (Nishida and Goel, 2011). DNA methylation, which refers to the addition of a methyl group to the cytosine within a CpG dinucleotide, is the most intensively studied epigenetic mechanism in mammals. The cancerous tissues in HCC are reported to possess a unique methylation landscape that differs from adjacent tissues. In HCC, the methylome feature is roughly characterized by global hypomethylation and gene-specific DNA hypermethylation, which contribute to genomic instability and inactivation of tumor-suppressor genes, respectively (Hereeg and Paliwal, 2011). Since advanced HCC is highly lethal and incurable, there is an urgent need to understand the tumorigenesis process and to search for new diagnostic biomarkers and novel therapeutic targets.

CYP2C19 is one of the most important members of the cytochrome P450 (P450) superfamily and is responsible for the metabolism of a variety of clinically important drugs such as mephenytoin, diazepam, and some barbiturals (Griskevicius et al., 2003). The genetic polymorphisms of CYP2C19 have been well studied. Individuals can be classified as extensive metabolizers, intermediate metabolizers, poor metabolizers, and ultra-rapid metabolizers based on the catalytic capacity of the enzyme, which largely depends on the different alleles individuals carry (Upptunguderi et al., 2012). The regulatory mechanism of CYP2C19 is currently characterized by several nuclear receptors and transcription factors, including the constitutive androstane receptor (CAR), pregnane X receptor, glucocorticoid receptor α, and hepatocyte nuclear factor-3y. Of these, CAR seems to play a central role and has a strong association with the basal expression level of CYP2C19 (Wortham et al., 2007). It has been predicted that epigenetics might also play a role in CYP2C19 regulation, and some CpG sites exist in the

ABBREVIATIONS: 5-aza-dC, 5-aza-2'-deoxycytidine; BSP, bisulfite sequencing polymerase chain reaction; CAR, constitutive androstane receptor; HCC, hepatocellular carcinoma; HEK293, human embryonic kidney 293; HBV, hepatitis B virus; HK2, human kidney-2; P450, cytochrome P450.
5'-flanking region of the CYP2C19 gene but their functional importance remains to be further elucidated (Ingelman-Sundberg et al., 2007).

CYP2C19 expression is reported to be decreased in advanced cancer, including breast cancer, lung cancer, and HCC, which is also accompanied by compromised enzymatic activity (Tsunedomi et al., 2005; Helsby et al., 2008). Despite researchers’ extensive efforts to investigate the underlying mechanism over the past years, altered expression of CYP2C19 in HCC does not appear to be fully accounted for by known polymorphisms, inflammatory factors, or growth hormones (Helsby et al., 2008).

This study aimed to investigate the possible epigenetic mechanism of decreased CYP2C19 expression in HBV-infected patients with HCC. Since CYP2C19 is also important in the disposition of a number of chemotherapeutic agents, including cyclophosphamide, thalidomide, and bortezomib, elucidating the epigenetic modulation of CYP2C19 might also help improve understanding and prediction of chemotherapy outcomes for patients with HCC.

Materials and Methods

Cell Culture and Tissue Samples. HepG2 and human embryonic kidney 293 (HEK293) cells were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Carlsbad, CA), supplemented with 10% fetal bovine serum (Life Technologies) with InVitroGRO CP medium. The medium was changed every other day. Cells were harvested after 5 days treatment for DNA and RNA isolation.

Treatment of Primary Hepatocytes with 5-Aza-2'-Deoxycytidine. Primary hepatocytes were seeded at a density of 5 × 10^5 cells/ml in collagen-coated, six-well plates (Life Technologies) with InVitroGRO CP medium. The medium was changed the next day to remove the unattached cells. After 24 hours of plating, drug incubation started with 1 μM and 2 μM 5-aza-2'-deoxycytidine (5-aza-dC; Sigma-Aldrich, St. Louis, MO) (Christman, 2002) in InVitroGRO HI medium supplemented with 25 ng/ml epidermal growth factor (Sigma-Aldrich) to stimulate proliferation. The control group was treated with 0.1% dimethylsulfoxide. The medium was changed every other day. Cells were harvested after 5 days treatment for DNA and RNA isolation.

Semi-quantitative Real-Time Polymerase Chain Reaction. mRNA was extracted from tissue samples and cultured cells using the RNeasy Mini Kit (Qiagen, Valencia, CA). Five-hundred nanograms of mRNA was used in a reverse transcribed polymerase chain reaction (PCR) performed with the PrimeScript RT Reagent Kit (Takara, Tokyo, Japan). Quantitative real-time PCR was then performed using SYBR Premix Ex Taq II (Takara) and the StepOnePlus system (Applied Biosystems, Foster City, CA). Primers for quantitative real-time PCR are listed in Table 1. Relative quantitation of gene expression was calculated with the 2^(-ΔΔCt) method as described previously (Schmittgen and Livak, 2008).

DNA Methylation Analysis. Genomic DNA from tissue samples or cells was isolated using the DNeasy Blood and Tissue Kit (Qiagen). DNA was considered qualified with a ratio of absorbance at 260 nm and 280 nm (A260/A280) between 1.7 and 1.9. Samples were stored in elution buffer at −80°C before use.

Sodium bisulfite modification of DNA samples was conducted using an EZ DNA Methylation-Gold Kit (Zymo, Orange, CA). The location of all CpG sequences examined in this study is shown in Fig. 1. PCR amplification was done with Taq polymerase (Takara) in a 50-μl reaction mixture with 2 μl modified DNA as the template. PCR amplification started with 10 cycles of touchdown PCR of 30 seconds at 94°C, 30 seconds at 60°C to 55°C (−0.5°C per cycle), and then 45 seconds at 72°C and 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 45 seconds at 72°C, followed by an extension of 10 minutes at 72°C. Amplicons were then purified and subcloned into pMD18-T vector (Takara) according to the manufacturer’s instructions. Ligation products were transformed into Escherichia coli DH5α cells and 10–15 random colonies with recombiant plasmids were subjected to Sanger sequencing (Sangon, Shanghai, China).

Methylation-specific PCR was also conducted using the modified DNA as a template. The following touchdown PCR program was applied: 10 cycles of touchdown PCR of 30 seconds at 94°C, 30 seconds at 58°C to 53°C (−0.5°C per cycle), 20 seconds at 72°C and 25 cycles of 30 seconds at 94°C, 30 seconds at 53°C, and 30 seconds at 72°C, followed by a final extension of 10 minutes at 72°C. Ten-microliter PCR products were shown on 2% agarose gel containing ethidium bromide. Primers for methylation analysis are listed in Table 2.

Plasmid Construction. pCpGL-CYP2C19 (+1977 to +64), pCpGL-CAR-CGI (+146 to +1971), pCpGL-CAR-2E (+175 to +1971), and pCpGL-CAR-1E (+384 to +447) were constructed with a CpG-free luciferase reporter vector named pCpGL-basic (a kind gift from Prof. Michael Rehli, University Hospital, Regensburg, Germany) (Fig. 2A) (Klug and Rehli, 2006). pCpGL-CYP2C19 contains an approximately 2.0-kb sequence of the 5'-flanking promoter region of CYP2C19, pCpGL-CAR-CGI contains an approximately 3.5-kb sequence with a

<table>
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<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (5’-3’)</th>
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<tr>
<td>CYP2C19</td>
<td>NM_000769.2</td>
<td>GAAAATTTGAAATGAAAAACATTAGGATT</td>
<td>CGAGGGTTGTGATGTCCATC</td>
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<td>CAR</td>
<td>NM_005122.4</td>
<td>GCAGCTGTGGAATCTGTCGA</td>
<td>CAGGTCGCGTCAGAGAAAG</td>
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<td>GRe</td>
<td>NM_000176.2</td>
<td>GGGGAAAGGAGGATGGGA</td>
<td>GGTGTCGAAGTGGGAGG</td>
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<tr>
<td>PXR</td>
<td>NM_022002.2</td>
<td>CAAAGCGGAAGAAAGTTGACG</td>
<td>CTGGTCTGTCAGTGCGAGCA</td>
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<tr>
<td>GATA-4</td>
<td>NM_00130809.4</td>
<td>CTGGCCCTGTCATCTCAGTAC</td>
<td>GGTCGCGCAAGGAATTTAGG</td>
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<tr>
<td>HNF-3y</td>
<td>NM_004497.2</td>
<td>ATGCTGGGCTGCTGAGAAT</td>
<td>CAGGTTACACGTCTTCA</td>
</tr>
<tr>
<td>HNF-4a</td>
<td>NM_178849.2</td>
<td>GGTGCTGGGCGGAGATGATA</td>
<td>TCTAGTTAATAGGAGGAAGG</td>
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<tr>
<td>GAPDH</td>
<td>NM_002046.5</td>
<td>GAAAGTTGGAAGTGGAGTC</td>
<td>CAAAGTTGTACATTGAGTACC</td>
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</tbody>
</table>

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GR, glucocorticoid receptor; HNF, hepatocyte nuclear factor; PXR, pregnane X receptor.
Epigenetic Study of CYP2C19 in Hepatocellular Carcinoma

CpG island on the 5′-flanking region and both e-box binding sites close to the transcription start site and translation start site of CAR, respectively. In pCpGL-CAR-2E, the CpG island region was deleted from the sequence of pCpGL-CAR-CGI; in pCpGL-CAR-1E, another approximately 200-bp sequence that contains the first e-box binding site was deleted. In addition, a full-length CAR coding DNA sequence amplified from mRNA (primers CDS-F/R) was ligated with the 5′-flanking region as well as the untranslated exon 1 and intron 1 (amplified by primers Flanking-F/R) by overlap PCR. The approximately 3-kb ligated fragment was then inserted into a CpG-free expression vector, pCpGfree-mcs (Fig. 2B) (InvivoGen, San Diego, CA), to construct pCpGfree-CAR. Primers used for plasmid construction are listed in Table 3. All constructs were confirmed by Sanger sequencing (Sangon).

In Vitro Methylation of Plasmid DNA. Plasmids were methylated using M.SsI (New England Biolabs, Beverly, MA) according to the manufacturer’s instructions. Two micrograms of plasmid DNA was incubated in a 40-μl volume with 2 μl (4 U/μl) SsSI and 160 μM S-adenosyl methionine at 37°C for 6 hours. The control group of plasmids also underwent the same incubation but in the absence of SsSI. The reaction was stopped by heating at 65°C for 20 minutes. Both the methylated group and the control group of plasmid DNA were purified with the Axygen PCR Clean-Up Kit (Axygen, Hangzhou, China).

Transient Transfection Assay. HepG2, HEK293, or HK2 cells were seeded in 24-well plates at a density of 2.5 × 10⁵/ml. The transient transfection was carried out using Lipofectamine2000 (Life Technologies) when cells reached 70%–80% confluence. Reporter plasmids and expression plasmids were transfected at 500 ng and 250 ng per well, respectively, with 50 ng pRL-TK per well as the internal control. Medium was refreshed 6 hours after transfection. Luciferase activity was assessed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) 48 hours after transfection.

Statistical Analysis. Quantitative PCR results are shown as 2^(-ΔΔCt) using glyceraldehyde 3-phosphate dehydrogenase as a housekeeping gene. The methylation percentage of bisulfite sequencing polymerase chain reaction (BSP) analysis was calculated as the number of methylated cytosines divided by the total number of cytosines on the CpG site in all amplicons. The methylation percentage of a sequence was determined as the average percentage of all CpG sites within this region. Comparisons of the methylation percentage and mRNA expression between groups were analyzed by a paired t test using GraphPad Prism software (version 5.0; GraphPad Software Inc., San Diego, CA). The Pearson correlation was used to determine the correlation between CYP2C19 and CAR expression. Differences or correlations were considered statistically significant at P < 0.05, P < 0.01, and P < 0.001.

Results

Increased CYP2C19 and CAR mRNA in Primary Hepatocytes after 5-Aza-dC Treatment. To determine whether DNA methylation is a potential mechanism of CYP2C19 regulation in the liver, a DNA methyltransferase inhibitor (5-aza-dC) was applied to the primary cultures of human hepatocytes. Quantitative real-time PCR analysis revealed an increase in CYP2C19 and CAR expression in a dose-dependent manner, whereas other transcription factors were not significantly affected (Fig. 3). To investigate whether demethylation led to the elevated expression level of CYP2C19 and CAR in primary hepatocytes, BSP was performed on the promoter regions as shown in Fig. 1. BSP results revealed a moderate methylation frequency on the CpG-poor promoter of CYP2C19 (52.5%) as well as the two e-box binding sites on CAR (50% and 47.5%). After

### TABLE 2

<table>
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<th>Amplicon</th>
<th>Gene ID</th>
<th>Forward Primer (5′-3′)</th>
<th>Reverse Primer (5′-3′)</th>
</tr>
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<td>Primers for BSP</td>
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<td>CYP2C19 segment 1</td>
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<td>1557</td>
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<td>ACCCACCAAATATTTAAAAACCTTAAAC</td>
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<td>1557</td>
<td>GATTTTTTGTAGAAATATTTATAGT</td>
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<td>CAR segment 4</td>
<td>9970</td>
<td>TGGTTAGAGTTAGGAGTGTGTTAAAG</td>
<td>AATACCTACACCTATTTACCA</td>
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<td>CAR segment 5</td>
<td>9970</td>
<td>TAGTTTAGGAGTTAGGAGTTTTATAG</td>
<td>ATACCTACACCTATTTACCA</td>
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<td>CAR segment 6</td>
<td>9970</td>
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<td>Primers for MSP</td>
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<tr>
<td>CAR-MSP-M</td>
<td>9970</td>
<td>GTGGGTTAGGTTACAGTGGTTTATAG</td>
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<td>CAR-MSP-U</td>
<td>9970</td>
<td>TGTTTTGGTTAGGTTACAGTGGTTTATAG</td>
<td>TACTAAATATACAAATACATTTCCCGGT</td>
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MSP, methylation-specific polymerase chain reaction.
5-aza-dC treatment, the methylation frequency of these three regions decreased to 38.3%, 13.3%, and 42.5%, respectively (Fig. 4, A–C). The CpG island on the CAR promoter, which was highly methylated (92.5%), also showed decreased methylation frequency (72.5%) after exposure to 5-aza-dC (Fig. 4D). BSP results showed an overall decreased DNA methylation level on the promoter of CYP2C19 and CAR after 5-aza-dC treatment. These results suggest that DNA methylation is a regulatory mechanism of CYP2C19 in the liver and CAR might also be involved.

**TABLE 3**

Primer sequences for plasmid construction

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<tr>
<th>Amplicon</th>
<th>Gene ID</th>
<th>Forward Primer (5'-3')</th>
<th>Reverse Primer (5'-3')</th>
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<tr>
<td>pCpGL-CYP2C19</td>
<td>1557</td>
<td>GGactagtATTATTCTAAAGAGAGCAAC</td>
<td>CGggatccTTAAGACAACCGTGAG</td>
</tr>
<tr>
<td>pCpGL-CAR-2E</td>
<td>9970</td>
<td>GGactagtGAAATCTGTTGGGGACCAAAGACTAA</td>
<td>CGggatccCTGTGTCCATCAGACAAACATTCAG</td>
</tr>
<tr>
<td>pCpGL-CAR-1E</td>
<td>9970</td>
<td>GGactagtCCTGTAATCCCAGCACTTTGGGAAGCC</td>
<td>CGggatccCTGTGTCCATCAGACAAACATTCAG</td>
</tr>
<tr>
<td>pCpGFree-CAR</td>
<td>9970</td>
<td>Flanking-F CTCTCCTCTTCTCTAGCGAGGAGCGG</td>
<td>Flanking-R TACTGGGCATGACGCTCGTGGG</td>
</tr>
<tr>
<td>NM_005122.4</td>
<td></td>
<td>CDS-F CGTGACGCATGCCAGTAGGGAG</td>
<td>CDS-R GAgaatctCCTCACAGTCAGATCTCCTG</td>
</tr>
</tbody>
</table>

CDS, coding DNA sequence.
Decreased mRNA Expression of CYP2C19 and CAR in Tumorous Tissues. Expression of CYP2C19 and CAR in tumorous and adjacent tissues is shown as grouped column scatter plots with means ± S.E. in Fig. 5A. The average expression of CYP2C19 in tumorous tissues (0.39 ± 0.20) was significantly decreased compared with the normal tissues (2.4 ± 0.40, P < 0.001). In addition, the average expression of CAR also decreased significantly (0.40 ± 0.085 versus 1.40 ± 0.15, P < 0.001).

Alterations of the expression levels of CYP2C19 and CAR were correlated, with a Pearson correlation coefficient of 0.31 (R² = 0.09, P < 0.05) (Fig. 5C).

Elevated Methylation Level of CpG Sites on the CAR Promoter in Tumorous Tissues. BSP analysis of segment 5 on CAR was performed using DNA from paired tumor and adjacent tissue samples. Representative BSP results are shown in Fig. 6A and the corresponding
relative mRNA expression is shown in Fig. 5B. Clearly, in BSP analysis, CpG 3, CpG 5, and CpG 6 in segment 5 around the transcription start site of CAR had a higher methylated status in tumorous tissue compared with adjacent tissue. Among these three sites, CpG 3 is a potential e-box binding site, whereas CpG 5 and CpG 6 are two adjacent CpG sites 121 bp downstream of CpG 3. In addition, methylation-specific PCR within segment 5 also revealed a higher methylation level in tumorous tissues (Fig. 6B). Interestingly, in the case of sample 246564, the expression levels of CAR and CYP2C19 were increased while methylation status was decreased in tumorous tissues (Fig. 6A). No significant methylation difference was detected between tumorous and adjacent tissues in the other segments analyzed using BSP (data not shown).

In Vitro Methylation on the CAR Promoter Leads to Decreased CAR-Mediated CYP2C19 Expression in HepG2 Cells. Results of the dual-luciferase reporter assay showed that the CpG-poor 2-kb promoter of CYP2C19 was not affected by methylation (Fig. 7, A, C, D) in all tested cell lines, suggesting that these sporadic CpG sites are irrelevant to the methylation mechanism. The unmethylated pCpGL-CAR-CGI was 2.5-fold higher in relative luciferase activity compared with the methylated group in HepG2 cells. This fold change remained after the deletion of the CpG island, as shown by the unmethylated versus methylated pCpGL-CAR-2E (2.2-fold), but was greatly reduced after deletion of the 200-bp fragment containing the e-box binding site near the transcription start site (in the first intron). Our results indicate that this e-box binding site was essential in the methylation-regulating mechanism of CAR, whereas the e-box close to the translation start site was not involved. However, no difference between the methylated and unmethylated groups was found in HK2 or HEK293 cells (Fig. 7, C and D).

To explore whether the methylation-mediated decrease of CAR could exert an effect on CYP2C19 expression, either methylated or unmethylated pCpGfree-CAR was cotransfected with pCpGL-CYP2C19 in HepG2 cells. A 2.3-fold higher luciferase activity was observed in the unmethylated pCpGfree-CAR group (Fig. 7B), which further supported the finding that CYP2C19 could be suppressed through hypermethylation caused by a decrease in CAR expression.

Discussion

HBV infection remains a key public health issue in China according to the World Health Organization’s 2014 World Cancer Report (Bernard et al., 2014). HBV-induced hepatocarcinogenesis includes proteomic disruption, viral integrations, and aberrant epigenetic modifications (Ji et al., 2014). Our study demonstrates that DNA demethylation leads to elevated CAR and CYP2C19 expression in primary hepatocytes. This finding suggests that DNA methylation is a potential mechanism of CAR and CYP2C19 regulation in the liver. This notion is further supported by suppressed expression of CAR and CYP2C19 in Chinese patients with HBV-related HCC.

Since CAR is a member of the nuclear receptor superfamily and is a central modulator of oxidative and conjugative enzymes and transporters that are involved in drug disposition and metabolism, it is conceivable that the suppression of CAR plays an important role in the altered expression of CYP2C19 and possibly other drug metabolism targeting genes. P450s participate in the activation and metabolism of a large number of chemotherapeutic agents such as docetaxel (Shou et al., 1998) and cyclophosphamide (Griskevicius et al., 2003). Thus, CAR suppression mediated by hypermethylation may lead to disturbance of the drug metabolizing system. Therefore, the methylation status of CAR might serve as a biomarker of altered pharmacokinetics. Indeed, other studies have confirmed that several CAR-regulating P450 genes were also suppressed along with decreased CAR expression in end-stage HCC.
(Chen et al., 2014), but no significant decrease in CAR and related P450 genes was found in HBV cirrhosis samples. This finding suggests that the sharp decline in expression of these genes might occur when liver cirrhosis evolves into carcinoma. However, our results only offered a Pearson correlation coefficient of 0.31 between CYP2C19 and CAR expression, in contrast with a stronger correlation (0.693) observed in healthy liver samples (Wortham et al., 2007). Since downregulation of metabolism-related genes is demonstrated to be preferentially linked with HBV-related HCC rather than hepatitis C virus–related HCC (Okabe et al., 2001; Iizuka et al., 2002), it is possible that the virus itself
Fig. 6. Methylation analysis of segment 5 near the CAR transcription start site. (A) Comparison of methylation status of CpG sites within segment 5 between HCC tumorous tissues (circles) and adjacent normal tissues (triangles). (B) Methylation-specific PCR analysis of the e-box binding site within segment 5. DNA samples from tumorous and normal tissue are shown (denoted as C and N, respectively). Segment 5 amplified by PCR was used as either methylated control DNA (Me, M.SssI treated) or unmethylated control DNA (Un). Methylation-specific and unmethylation-specific primers are shown as M and U, respectively.
might have a direct effect on the expression of certain genes. Furthermore, many novel mechanisms have been discovered in recent years. The participation of hasa-miR-29a-3p in CYP2C19 regulation in liver cells is one example of an epigenetic mechanism (Yu et al., 2015). MicroRNA may also participate in disrupting the correlation between CAR mRNA and protein levels and may thus lead to the poor correlation between CAR and CYP2C19 mRNA levels. Further study is warranted to build a comprehensive map of how CYP2C19 is regulated in HCC.

Since there was a 2-fold decrease in luciferase activity after deletion of the 200-bp sequence containing the e-box at the first intron as indicated in the reporter gene assay, this sequence may act as an enhancer element of CAR. This hypothesis warrants further investigation. We performed the same in vitro methylation and reporter gene assay in three cell lines (HepG2, HEK293, and HK2). Interestingly, only HepG2 showed decreased luciferase activity in methylated pCpGL-CAR-2E. No difference between methylated and unmethylated pCpGL-CAR-2E was detected in either HEK293 or HK2 cells. Our results indicate that certain factors specific to HepG2 cells may be involved in this methylation-related regulation. Biobase (http://www.biobase-international.com/) predicted possible binding of C-myc and upstream translation factor 1 to this sequence, however, quantitative real-time PCR results show that C-myc and upstream translation factor 1 are abundant in all three cell lines (data not shown). Since there are very limited data available regarding the transcriptional regulation of CAR, the exact function of this sequence and the candidate binding proteins remain unclear.

An e-box is a special DNA sequence (CANNTG) found in some promoter regions in eukaryotes that acts as a binding site for regulating proteins (Massari and Murre, 2000). It has been reported that methylation of the CpG dinucleotide within the e-box sequence can block the binding of N-Myc to its targeting gene promoters in vivo, thus contributing to the cell type–specific expression pattern of certain genes such as caspase-8 and the epidermal growth factor receptor (Perini et al., 2005). Since two e-box sequences (CACGTG) are located near the transcription start site and translation start site of CAR, respectively, methylation on the CpG dinucleotide is likely to play a role in modulating CAR expression. In our study, 29 liver tumor samples showed decreased CYP2C19 and CAR expression along with elevated DNA methylation around the e-box binding site compared with adjacent normal liver samples. Only in sample 246564 did the situation reverse. Further scrutiny into this patient’s diagnostic information did not reveal anything unusual. Clearly, this distinct phenomenon awaits more data in order to be better understood. However, the reversed correlation between CYP2C19 and CAR expression levels and DNA methylation was also observed in sample 246564, which confirms that DNA methylation status might serve as a potential marker for predicting changes in gene expression.

Nevertheless, other factors that contribute to the epigenetic regulation of CYP2C19 cannot be excluded, since the distal promoter region of CYP2C19 is unidentified and more transcription factors might exist. However, reduced CAR expression by e-box methylation at least partially accounts for CYP2C19 suppression in the HBV-infected HCC tissue samples.

It has been well documented that DNA methylation in different genomic contexts exerts different effects on gene transcription (Jones, 2012). Much attention has been paid to the CpG-rich promoters during the past years, whereas CpG-poor transcription start sites have been

Fig. 7. Effect of DNA methylation on the transcription activity of CYP2C19 and CAR promoter constructs. (A, C, and D) The pCpGL plasmids were cotransfected with pRL-TK in HepG2, HEK293, or HK2 cells. The pCpGL plasmids were either methylated in the presence (methylated) or absence (unmethylated) of M.SssI. (B) The CpG-free constructs were cotransfected with pCpGL-2C19 and pRL-TK in HepG2 cells. The CpG-free constructs were either methylated in the presence (methylated) or absence (unmethylated) of M.SssI. The relative luciferase activity was calculated as the ratio of firefly luciferase to Renilla luciferase. Data are shown as means ± S.D. with three replicate wells for each group. Data are representative of three independent experiments. **P < 0.01.
overlooked. In fact, non-CpG island methylation seems to be more dynamic and therefore more changeable during development or pathogenesis. For example, the CpG-poor LAMB3 promoter was shown to be hypomethylated in primary bladder tumors. In vitro methylation of the promoter can directly lead to transcription silencing (Han et al., 2011). Furthermore, enhancers (which can reside at variable distances from promoters and are mostly CpG poor) also tend to show variable methylation status.

Lempiäinen et al. (2011) demonstrated that long-term activation of CAR in mice can lead to HCC, which is accompanied by a switch in the methylomes. However, whether CAR plays a direct role in human HCC remains controversial. Cancer pathogenesis is a complex process in which multiple genetic and epigenetic factors are regulated normally. This complexity poses a formidable challenge to holistic integration of information when the investigation is limited to only a certain gene. Traditionally, epigenetic research on cancer has mostly focused on tumor suppressors or oncogenes. Only in recent years has the necessity of building a comprehensive epigenome map been realized. Compared with the well established Human Genome Project, the International Human Epigenome Consortium was launched recently in 2010 (http://ihec-epigenomes.org/) and aims to increase understanding of how the molecular aspects.

A major goal of epigenetics is to help elucidate the dynamic and therefore more changeable during development or pathogenesis. For example, the CpG-poor LAMB3 promoter was shown to be hypomethylated in primary bladder tumors. In vitro methylation of the promoter can directly lead to transcription silencing (Han et al., 2011). Furthermore, enhancers (which can reside at variable distances from promoters and are mostly CpG poor) also tend to show variable methylation status.

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References


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