**Hepatocyte Growth Factor Activator Inhibitor 2/Placental Bikunin (HAI-2/PB) Gene Is Frequently Hypermethylated in Human Hepatocellular Carcinoma**

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**ABSTRACT**

To identify methylation-mediated silencing of genes in hepatocellular carcinoma (HCC), we surveyed genes induced by treatment with 5-aza-2'-deoxycytidine (5-Aza-CdR) in six human hepatoma cell lines using cDNA microarray analysis and determined the methylation status of 5' CpG islands by bisulfite DNA sequencing or methylation-specific PCR. Fifty genes exhibited a >5-fold induction in response to treatment with 5-Aza-CdR in at least one of the hepatoma cell lines examined. Among these genes, the hepatocyte growth factor activator inhibitor 2/placental bikunin (HAI-2/PB) gene was maximally induced by 5-Aza-CdR in three of six cell lines studied (HLE, HuH7, and Hep3B). Bisulfite sequencing revealed that the 5' CpG island of this gene was densely methylated in HLE, HuH7, and Hep3B cells. After treatment with 5-Aza-CdR, re-expression and demethylation of HAI-2/PB gene were detected in these cells. These findings suggest that HAI-2/PB expression may be inappropriately repressed by promoter hypermethylation in HCC. Methylation-specific PCR analysis demonstrated that HAI-2/PB hypermethylation occurred in 21 of 26 HCC tumors (80.8%), whereas in the corresponding nontumorous liver tissues, it was found in 7 of 26 samples (26.9%). In addition, HAI-2/PB hypermethylation was not detected in any of the seven normal liver samples from individuals without HCC. Reverse transcription-PCR analysis demonstrated that promoter hypermethylation was associated with the reduced expression of the HAI-2/PB gene in HCC tumors. In conclusion, we have found that the HAI-2/PB gene is silenced by hypermethylation in human hepatoma cell lines by means of cDNA microarray analysis after 5-Aza-CdR treatment, and that HAI-2/PB hypermethylation occurs frequently in primary HCC tumors.

**INTRODUCTION**

Cytosine methylation is necessary for normal mammalian development (1) and is strictly regulated by DNA methyltransferases (2). Most housekeeping genes possess a hypomethylated CpG island in their promoter region, whereas de novo methylation of CpG islands is normally associated with gene silencing of X-linked (3) and imprinted (4) genes. In malignancies, however, this precise control of DNA methylation is frequently disrupted, resulting in inappropriate gene silencing by hypermethylation of promoter regions (5, 6). Aneuploidy of many types of genes including tumor suppressor genes, DNA repair genes, and genes related to metastasis and invasion has been shown to occur in neoplasms. Esteller et al. (7) recently analyzed the methylation status of a limited number of genes using a methylation-specific PCR (MSP) method in various cancers and reported that aberrant methylation of two or more genes occurred in each type of malignancy studied. Costello et al. (8) have also demonstrated that an average of 600 of the 45,000 CpG islands identified in the human genome were aberrantly methylated in tumors. These studies indicated that aberrant methylation of multiple genes might be involved in the development of malignancies. The 5' CpG islands of the E-cadherin, p16INK4a, SOCS-1, and 14-3-3 sigma genes have been reported to be hypermethylated in human hepatocellular carcinoma (HCC; Refs. 9–12). Similar to other gastrointestinal malignancies, multiple steps are thought to underlie the development of HCC. To discover aberrantly methylated genes in HCC, we surveyed genes induced by treatment with 5-aza-2'-deoxycytidine (5-Aza-CdR) in six human hepatoma cell lines using cDNA microarray analysis and determined the methylation status of 5' CpG islands by bisulfite DNA sequencing or MSP.

**MATERIALS AND METHODS**

**Cell Culture and 5-Aza-CdR Treatment.** The human hepatoma cell lines HLE, HuH7, HepG2, HuH6, and PLC/PRF/5 were obtained from the Health Science Research Resources Bank, and Hep3B was obtained from the Cell Resource Center for Biomedical Research. Cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. For demethylation experiments, cells were plated at a density of 1 × 10⁶ cells/100-mm dish and cultured for 24 h, followed by 96 h culture with 1 μM 5-Aza-CdR (Sigma Chemical Co., St. Louis, MO).

**Patients.** Liver tissues were obtained from 26 HCC patients (mean age, 64 ± 9 years) who underwent surgical resection (n = 25) or autopsy (n = 1). Among these patients, 9 were positive for hepatitis B virus surface antigen and 12 for hepatitis C virus core antibody, and the remaining five lacked evidence of either viral infection. On the basis of the histological findings, the 26 HCC tumors were classified as follows: 5 were well differentiated, 16 were moderately differentiated, and 5 were poorly differentiated. More than half the tumors (16 of 26) developed in cirrhotic livers. HCC samples and corresponding nontumorous liver tissues obtained by surgical resection or autopsy were immediately frozen at −80°C.

**cDNA Microarray Analysis.** cDNA microarray analysis was performed to identify genes up-regulated by 5-Aza-CdR treatment. Total RNA was extracted from 5-Aza-CdR-treated or untreated cell lines using TRIzol reagent (Invitrogen). cDNAs were then cohybridized to IntelliGene Human Cancer CHIP Version 2.1, which contained cDNAs from 557 genes known to be related to human malignancies (Takara). After being washed, the slides were scanned using an Affymetrix 428 Array Scanner (Takara), and signals were analyzed using BioDiscovery ImaGene Version 4.0 software (Takara). A subset of housekeeping genes, including glyceraldehyde-3-phosphate dehydrogenase and β-actin, were used for normalization.

**Reverse Transcription (RT)-PCR.** RT-PCR was performed to assess expression of hepatocyte growth factor activator inhibitor 2/placental bikunin (HAI-2/PB) transcripts in hepatoma cell lines. One μg of total RNA from 5-Aza-CdR-treated or untreated cell lines was subjected to a RT reaction using random oligonucleotide primers and SuperScript II reverse transcriptase (Invitrogen) in a 20-μl reaction volume for 60 min at 42°C. One μl of the RT reaction product was then amplified by PCR using the following primer set: sense, 5'- TTTCACGCTGGGAGAGGAGAC-3'; and antisense, 5'-AAACAGACCTGACGGCTTAAG-3'. PCR was performed in a 25-μl reaction volume for 27 cycles under the following conditions: 95°C for 30 s; 55°C for 30 s; and 72°C for 60 s. Five μl of the PCR product were run on a 3% agarose gel and stained with ethidium bromide.

Received 2/13/03; revised 9/12/03; accepted 10/9/03.

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visualized by SYBR Green (FMC, Rockland, ME) staining. The predicted size of the PCR product was 418 bp. RNA samples were also ampliﬁed using β-actin gene primers as a control. To analyze the expression of HAI-2/PB transcripts in the liver tissues, total RNA was extracted from primary HCC tumors or corresponding nontumorous liver tissues using TRIzol reagent, and RT-PCR analysis was then performed in the same way as mentioned above.

**Sodium Bisulﬁte DNA Sequencing.** To analyze CpG methylation of the 5’ region of the HAI-2/PB gene, genomic DNA extracted from each hepatoma cell line was modiﬁed by sodium bisulﬁte using the CpGenome DNA Modiﬁcation Kit (Intergen, Purchase, NY) according to the manufacturer’s instructions. The bisulﬁte-modiﬁed DNA was ampliﬁed by semi-nested PCR using the speciﬁc primers listed in Table 1. PCR products were then subcloned into the pCR2.1-TOPO vector using a TA cloning kit (Invitrogen) according to the manufacture’s instructions. To determine the CpG methylation status of the 5’ CpG island of the HAI-2/PB gene, 10 clones from each cell line were sequenced using an ABI PRISM Dye Deoxy Terminator Cycle Sequencing kit and analyzed on an ABI 377 DNA Sequencer (Applied Biosystems Japan, Tokyo, Japan).

**MSP.** To analyze the methylation status of the 5’ CpG islands of HAI-2/PB gene in HCC, MSP was performed. DNA was extracted from frozen liver tissues by using a QiAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. The extracted DNA was then modiﬁed with sodium bisulﬁte as mentioned above. For detection of aberrant methylation of the HAI-2/PB gene, the modiﬁed DNA was ampliﬁed using primers speciﬁc for the methylated sequence (Table 1). As quality control of the bisulﬁte modiﬁcation process, the bisulﬁte-modiﬁed DNA was also ampliﬁed using primers speciﬁc for the unmethylated sequence (Table 1). A hot-start PCR was performed in a 25-µl reaction volume using AmpliTaq Gold DNA Polymerase (Applied Biosystems Japan). Five µl of each of the products were run on a 3% agarose gel and visualized by SYBR Green staining.

**Transfection.** HAI-2/PB expression vector, pcDNA-HAI-2/PB, which contains the entire HAI-2/PB coding region (13) was transfected into hepatoma cells using Effectene Transfection Reagent (Qiagen), and the viable cell number was determined 72 h after transfection using the 3-(4,5-dimethyldiazol-2-yl)-5(3-carboxymethoxyphenyl)-2(4-sulfonyl)-2H-tetrazolium (MTS) assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay; Promega Corp., Madison, WI) according to the instructions of the manufacturer.

**Statistical Analyses.** The χ2 test was used for the analysis of categoric variables, and the unpaired Student’s t test was used for continuous variables. Results were considered statistically signiﬁcant at P < 0.05.

RESULTS

cDNA Microarray Analysis for 5-Aza-CdR-induced Gene. All six hepatoma cell lines showed a senescence-like phenotype after 5-Aza-CdR treatment. A ready-made cDNA microarray spotted with cDNAs from 557 genes known to be involved in human malignancies was used to analyze genes induced by the demethylation agent in the cell lines. Genes exhibiting a ≥5-fold increase in each cell line in response to 5-Aza-CdR treatment are listed in Table 2. The number of genes up-regulated after 5-Aza-CdR treatment varied from 7 for the PLC/PRF/5 cell line to 19 for Hep3B cells. In total, 50 genes were induced by the treatment in at least one of the hepatoma cells examined, and 14 genes were induced in multiple cell lines. The list (Table 2) consists of genes reported to be involved in apoptosis, cell growth, or cell adhesion. Regarding the 50 genes, putative transcription start sites for all but the Semaphorin gene were able to be determined by searching the National Center for Biotechnology Information human genome database. Analysis of the sequences revealed that 34 of 49 genes (69%) contained a putative 5’ CpG island (Table 2). Genes that were shown previously to be silenced because of aberrant methylation of their 5’ CpG islands in many types of malignancies were also demonstrated to be induced by 5-Aza-CdR treatment in the hepatoma cells, as tissue inhibitor of metalloprotease-3 (TIMP-3) and E-cadherin genes were up-regulated after treatment with the demethylating agent in Hep3B and HepG2 cells, respectively (Table 2). MSP analysis using primer set II reported previously (14, 15) veriﬁed that hypermethylation of the 5’ CpG islands of these genes correlated with transcriptional repression (data not shown).

**Methylation Status of CpG Islands in the 5’ Region of the HAI-2/PB Gene in Hepatoma Cells.** Of the genes induced by 5-Aza-CdR, the HAI-2/PB gene exhibited a maximum induction in three of six cell lines: 24-, 17-, and 142-fold in HLE, HuH7, and Hep3B cells, respectively (Table 2). Because there has been no report concerning the promoter hypermethylation of the HAI-2/PB gene, we focused on this gene for further analyses. RT-PCR using primers speciﬁcally amplifying the HAI-2/PB gene revealed that HAI-2/PB transcripts were poorly represented in HLE, HuH7, and Hep3B cells before treatment but were substantially up-regulated by the drug, in accordance with the data obtained from the cDNA microarray analysis (Fig. 1). The cell lines were classiﬁed into three groups according to the HAI-2/PB gene expression level as estimated by both microarray and RT-PCR analysis. Three cell lines, HLE, HuH7, and Hep3B, with low expression of HAI-2/PB exhibited a substantial increase in HAI-2/PB expression in response to 5-Aza-CdR, whereas HepG2 and HuH6, with intermediate levels of HAI-2/PB expression, exhibited a moderate increase (3- and 4-fold, respectively). PLC/PRF/5, with high initial expression of HAI-2/PB, exhibited no induction of HAI-2/PB expression after treatment with 5-Aza-CdR. To assess the methylation status of the CpG island in the 5’ region of HAI-2/PB, sodium bisulﬁte DNA sequencing was performed. The methylation status of the 49 CpG dinucleotides encompassing the promoter and exon 1 is shown in Fig. 2. The 5’ CpG island of the HAI-2/PB gene was densely methylated in HLE, HuH7, and Hep3B cells in which the expression of HAI-2/PB was maximally up-regulated by 5-Aza-CdR, whereas in PLC/PRF/5 cells, few CpG dinucleotides were methylated. In HepG2 and HuH6 cell-lines, methylation of the CpG island was limited either to a subpopulation or a speciﬁc region (Fig. 2). To easily assess the methylation status of the 5’ CpG island of HAI-2/PB, MSP analysis was performed. The primer set for MSP was designed on the basis of

<table>
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<th>Genomic positiona (bp)</th>
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<th>Product size (bp)</th>
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<td>154</td>
<td>50</td>
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a Nucleotide position relative to the transcription start site.
the CpG methylation status determined by bisulfite sequencing (Table 1). In HLE, HuH7, and Hep3B cells, the methylated allele was detected by MSP, but the unmethylated allele was not detected. However, in HepG2 and HuH6 cells, the unmethylated allele was observed as well as the methylated allele. In PLC/PRF/5 cells, in contrast, only the unmethylated allele was detectable (Fig. 3A). On the basis of these results, it is evident that MSP analysis can be used to determine the methylation status of the HAI-2/PB gene. Moreover, MSP analysis also demonstrated that the 5’CpG island of HAI-2/PB was partly demethylated in HLE, HuH7, and Hep3B cells after the treatment with 5-Aza-CdR (Fig. 3A), indicating that the HAI-2/PB gene may be transcriptionally repressed by promoter hypermethylation in these hepatoma cells.

**Hypermethylation of the HAI-2/PB Gene in HCC Tumors.** The occurrence of HAI-2/PB hypermethylation in primary HCC tumors was analyzed by MSP assay. The 5’CpG island of the HAI-2/PB gene was demonstrated to be hypermethylated in 21 of 26 (80.8%) tumors, whereas in corresponding nontumorous liver tissues, HAI-2/PB hypermethylation was detected in 7 of 26 (26.9%) samples (Fig. 3B). In 6 of these 7 cases, the corresponding tumor samples also possessed the methylated HAI-2/PB. The signal intensity of the MSP product for methylated sequence was the same or stronger than that for unmethylated sequence in most of the tumors where the 5’CpG island of HAI-2/PB was revealed to be hypermethylated. On the other hand, the signal was weak in the nontumorous liver samples, where the 5’CpG island of HAI-2/PB was assessed to be hypermethylated, suggesting that the occurrence of HAI-2/PB hypermethylation might be limited to a small population in these nontumorous liver tissues. In addition, all of the surrounding nontumorous liver samples with HAI-2/PB hypermethylation exhibited cirrhosis as determined by histological examination. Neither tumor size nor histological grade of HCC was correlated with the occurrence of HAI-2/PB hypermethylation. On the other hand, in terms of hepatitis virus association with HCC, the incidence of HAI-2/PB methylation was significantly lower in hepatitis B virus surface antigen-positive tumors (4 of 9) than that in hepatitis C virus-positive tumors (12 of 12; \( P = 0.003 \)). When MSP analysis was

![Fig. 1. RT-PCR analysis of HAI-2/PB expression in hepatoma cell lines. RNA samples were also amplified using β-actin gene primers as a control. +, 5-Aza-CdR-treated; −, untreated.](image-url)
performed on 7 additional nontumorous liver tissues obtained at the time of resection of metastatic liver tumors from colorectal cancer and showing no appearance of liver injury, HAI-2/PB hypermethylation was not detected in any of the samples (data not shown).

**HAI-2/PB Gene Expression in the Liver.** To investigate the correlation between the expression level of HAI-2/PB transcripts and the promoter methylation status in HCC tumors, we performed RT-PCR for detection of HAI-2/PB mRNA. HAI-2/PB transcripts were detectable to various degrees in all of the HCC tumors and surrounding nontumorous liver tissues examined. The amount of HAI-2/PB transcript was then compared between HCC and corresponding nontumorous liver tissues using semiquantitative RT-PCR under linear amplification conditions. With the exception of one case, all of the HCC tumors with HAI-2/PB methylation exhibited a marked reduction of HAI-2/PB gene expression as compared with the adjacent nontumorous liver tissues without HAI-2/PB methylation (Fig. 4). In a single case, HCC tissue exhibited a very low level of HAI-2/PB expression, although the methylated allele was undetectable by MSP, suggesting that other mechanisms such as homozygous deletion or somatic mutation may be responsible for the loss of transcription. These data suggest that the promoter hypermethylation might be closely related to the down-regulation of HAI-2/PB transcription in HCC tissues.

**Growth Inhibitory Effect of HAI-2/PB on Hepatoma Cells.** To investigate the effect of HAI-2/PB on the growth of hepatoma cells, HAI-2/PB expression vector was transiently transfected into hepatoma cells. Assessment of the viable cell number after 72-h culture using the MTS assay revealed that the growth of HuH7 and Hep3B cells was significantly inhibited by the transduction of HAI-2/PB, whereas the growth of HepG2, HuH6, and PLC/PRF/5 cells was not affected (Fig. 5).

**DISCUSSION**

To identify genes silenced in HCC because of hypermethylation, we surveyed genes induced by treatment with a demethylating agent in human hepatoma cell lines using cDNA microarray analysis. Fifty genes exhibited a >5-fold induction in response to the demethylating agent in at least one of the hepatoma cells examined, and 14 genes were induced in two or more lines. Microarray analysis revealed that a variety of genes were induced by 5-Aza-CdR treatment. Among the up-regulated genes, 34 of 49 genes (69%) were found to contain a putative 5′ CpG island. Moreover, genes that have been reported previously to be aberrantly methylated in human cancers, such as TIMP-3 and E-cadherin, were demonstrated to be induced by 5-Aza-CdR treatment in hepatoma cells, and correlation between promoter hypermethylation and transcriptional silencing was verified by means of MSP analysis.

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**Fig. 2.** Methylation status of 49 CpG dinucleotides in the promoter and exon 1 of the HAI-2/PB gene. Bisulfite sequencing was performed to determine the methylation status of the 5′ CpG island of the HAI-2/PB gene in each cell line indicated. Each circle indicates a CpG site in the primary DNA sequence, and each line of circles represents analysis of a single cloned allele. ● methylated CpG dinucleotides; ○ unmethylated CpG dinucleotides. Fold induction was calculated based on the results of the cDNA microarray analysis.

**Fig. 3.** MSP analysis of HAI-2/PB hypermethylation in HCC. A, MSP analysis was performed to assess the methylation status of the 5′ CpG island of the HAI-2/PB gene in hepatoma cells. +, 5-Aza-CdR-treated; −, untreated. B, representative results of MSP analysis of HAI-2/PB hypermethylation in primary HCC tumors and corresponding nontumorous tissues. T, tumor; NT, nontumorous liver tissue.

**Fig. 4.** Representative results of RT-PCR analysis of HAI-2/PB expression in primary HCC tumors and corresponding nontumorous liver tissues. T, tumor; N, nontumorous liver tissue.

**Fig. 5.** Effect of HAI-2/PB on cell growth. Hepatoma cells were transfected with pCIneo vector (empty vector, □) or pCIneo-HAI-2/PB (■). The number of viable cells was measured by MTS assay. Results are mean of independent experiments performed in triplicate; bars, SD. * P < 0.0001; † † P < 0.005.
Because genomic imprinting is known to be associated with DNA methylation, it is possible that some of the genes restored by the demethylating agent in these cell lines were imprinted genes in the liver. To assess this possibility, we examined the expression in normal liver for the following genes: HAI-2/PB, IGFBP2, IGFBP3, CTGF, Fibronectin 1, COL1A2, COL3A1, BIGH3, Cyclin A2, Cyclin G2, Lumican, Vimentin, API2, FGFR3, K-cadherin, Kiss-1, NCA, TOP2A, CDC2, and Versican. By using RT-PCR, we verified that 12 of the 20 genes were expressed in human normal liver (Table 2). Recently, Yano et al. (16) reported the gene expression profile of adult human liver as examined by cDNA array system. On the basis of their report, 31 of the 50 genes induced by the demethylating agent in our study appear to be expressed in normal liver (Table 2). Together with other reports describing positive expression in the liver for other genes such as SPARC (17), HB-EGF (18), PDGF β (19), and Vitronectin (20), 42 of the 50 genes (84%) were assumed to be expressed in adult human liver. Concerning genes with a putative 5′ CpG island, 31 of 34 genes (91%) were thought to be expressed in the liver, suggesting that most of the genes apparently induced by the demethylating agent might not be imprinted genes or tissue-specific genes inaccurately inactivated in the liver.

The HAI-2/PB gene was found to be suppressed before 5-Aza-CdR treatment and exhibited a maximum induction by the drug in three of six cell lines (HLE, HuH7, and Hep3B). According to the previous reports, HAI-2/PB is assumed to act as a negative regulator of hepatocyte growth factor (HGF) signaling and also as an inhibitor of plasmin, which is thought to play a crucial role in the invasion of HCC (21–24). HAI-2 has been identified as a kunitz-type, serine protease inhibitor that can specifically inhibit the function of HGF activator (HGFA; Ref. 21). HGF activator is identified as a serine protease that converts the inactive precursor of HGF to the active form (22), and hence HAI-2 is assumed to be a negative regulator of HGF signaling through its inhibition of HGF activator. HAI-2 was also independently reported as placental bikunin (PB) that showed broad inhibition spectra against various serine proteases such as plasmin, trypsin, tissue and plasma kallikreins, and factor X1a (23, 24). Although the physiological roles of HAI-2/PB in tumorigenesis are poorly understood, Hamasuna et al. (25) have recently reported that HAI-2/PB could possess a suppressive activity in the progression of human gliomas in vitro.

HGF functions as a mitogen, morphogen, and motogen for a variety of cells through interaction with c-met, a specific HGF receptor expressed on the cell surface (26–30). There are conflicting reports on the role of HGF in hepatocarcinogenesis (31–35). A number of studies have shown that c-met is overexpressed in HCC and correlated with poor prognosis (36–38). HGF levels in the liver and serum were also demonstrated to be higher in cirrhosis and correlated with the occurrence of HCC (39–41). Taken together, these studies suggest that the HGF/c-met signaling pathway might be involved in the development of HCC. Because plasminogen converted to plasmin by urokinase-type plasminogen activator induces the degradation of extracellular matrix and the migration of tumor cells, the urokinase-type plasminogen activator/plasmin system has been assumed to have important roles in the invasive process of many types of cancer, including HCC. Moreover, it has been demonstrated that the HGF-induced invasion of HCC is mediated by the up-regulation of urokinase-type plasminogen activator (42). Together with the inhibitory activity of HAI-2/PB against plasmin, HAI-2/PB might have a suppressive role in the progression of HCC through the regulation of HGF signaling. There have been no previous studies of HAI-2/PB expression in HCC. Here, we demonstrated that the 5′ CpG island of the HAI-2/PB gene was densely methylated in half of the hepatoma cell lines examined. Furthermore, 5-Aza-CdR treatment increased the expression of the HAI-2/PB transcripts exclusively in cells with HAI-2/PB hypermethylation, and the induction was accompanied by demethylation of the 5′ CpG island, suggesting that promoter hypermethylation may be associated with HAI-2/PB gene silencing. According to the transient transfection experiments, HAI-2/PB could function as a growth inhibitor for HuH7 and Hep3B cells in which HAI-2/PB is down-regulated by promoter hypermethylation. Further studies are required to elucidate how HAI-2/PB could inhibit the growth of these hepatoma cells.

The study of primary HCC tumors revealed that HAI-2/PB hypermethylation occurred in the majority of HCC tumors, indicating that it might not be an event specific for the cultured cell lines. In addition, HCC tumors with HAI-2/PB methylation showed a marked reduction in gene expression as compared with the adjacent nontumorous liver tissues without HAI-2/PB methylation, suggesting that promoter hypermethylation might be closely related to the down-regulation of HAI-2/PB transcription in HCC tumors as well as in the cultured cells. It is of note that the promoter hypermethylation of the HAI-2/PB gene was observed frequently in HCCs <3 cm in diameter and in well-differentiated HCCs as well as in advanced HCC.

In conclusion, we have found that the HAI-2/PB gene is silenced by promoter hypermethylation in human hepatoma cells by means of cDNA microarray analysis after 5-Aza-CdR treatment, and that HAI-2/PB hypermethylation occurs frequently in primary HCC tumors.

ACKNOWLEDGMENTS

We thank Dr. Makoto Arai for helpful discussion.

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