Silencing of GSTP1 Gene by CpG Island DNA Hypermethylation in HBV-associated Hepatocellular Carcinomas

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ABSTRACT

Purpose and Experimental Design: Glutathione S-transferases, enzymes that defend cells against damage mediated by oxidant and electrophilic carcinogens, may be critical determinants of cancer pathogenesis. In this report, we assess the role of epigenetic silencing of the GSTP1 gene, a gene encoding the \( \pi \)-class glutathione S-transferase, in the pathogenesis of hepatitis B virus (HBV)-associated hepatocellular carcinomas (HCC). The cell lines Hep3B, HepG2, and a cohort of 43 HBV-associated HCC tissue specimens and corresponding nontumor tissues were subjected to analysis for GSTP1 epigenetic alteration and expression. GSTP1 “CpG” island DNA hypermethylation in the liver cell lines, and the tissue specimens were determined by methylation-specific PCR and correlated with expression of the gene using reverse-transcription PCR, immunoblotting, and immunohistochemistry.

Results: GSTP1 CpG island DNA hypermethylation was detected in 28 of 43 (65.1%) HCC tissues and 4 of 40 (10%) corresponding nontumor tissues. GSTP1 protein was absent in those cases showing hypermethylation of the gene. Similarly, DNA from Hep3B and HepG2 cell lines displayed complete GSTP1 hypermethylation in the CpG island, and they failed to express GSTP1 mRNA and the corresponding protein product. Treatment of the cell lines with the DNA methyltransferase inhibitor 5-aza-deoxycytidine reversed the hypermethylation, and restored GSTP1 mRNA and polypeptide expression.

Conclusions: These data indicate that epigenetic silencing of GSTP1 gene expression by CpG island DNA hypermethylation is common in human HBV-associated HCC. In addition, somatic GSTP1 inactivation via CpG island hypermethylation may contribute to the pathogenesis of this malignancy.

INTRODUCTION

HCC is a common malignancy in many parts of the world, particularly the Far East and sub-Saharan Africa. Most HCC cases in our region arise in the setting of chronic HBV infection (1, 2). Dietary carcinogens, such as aflatoxin B1, also contribute to hepatic carcinogenesis (3, 4).

GSTs are a family of isoenzymes that play an important role in protecting cells from cytotoxic and carcinogenic agents. GSTs catalyze the conjugation of glutathione with electrophilic compounds, including carcinogens and exogenous drugs (5), resulting in less toxic and more readily excreted metabolites. GSTs are encoded by different genes at different loci and are organized into several distinct classes: \( \alpha \), \( \mu \), \( \pi \), and \( \theta \) (6). These enzymes may help defend hepatocytes against a variety of potentially promutagenic stresses, including reactive oxygen species associated with chronic hepatic inflammation and reactive electrophilic compounds associated with the hepatic metabolism of dietary carcinogens (7–10).

GST\( \pi \) is of particular interest in the study of cancer biology. GST\( \pi \) is expressed in normal tissues at various levels in different cell types, and abnormal GST\( \pi \) activity and expression have been reported in many tumors, including those of the breast and kidney (11–14). A recent study of the expression of several different GST isoenzymes in HCC specimens from China revealed an overall reduction of GST levels in HCC tissue (15). The decrease in GST levels in HCC specimens was particularly marked in specimens that were found to contain HBV DNA (15). Somatic GSTP1 DNA hypermethylation changes have been detected in many human cancers (16, 17), including HCC (17). However, in the previous report (17) only a limited number of tumor tissues were included, hypermethylation status in tumor tissues was not compared with their corresponding nontumor tissues, and HBV DNA status in those tissues were not determined. As a result, the correlation of decreased GST level (15) with the epigenetic alteration of GSTP1 gene and the development of HBV-associated HCC remains unclear.

The aims of the present study were to assess the status of the GSTP1 expression in HCC cell lines and tissues, and the possible role of a GSTP1 expression defect in the development of HBV-associated HCC.

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3 The abbreviations used are: HCC, hepatocellular carcinoma; GST, glutathione S-transferase; RT-PCR, reverse transcription-PCR; HBV, hepatitis B virus; PBMC, peripheral blood mononuclear cell; GST\( \pi \), \( \pi \)-class GST; MSP, methylation-specific PCR; DNMT, DNA methyltransferase; 5-aza-dC, 5-aza-deoxycytidine.
MATERIALS AND METHODS

Patients and HCC Tissue Specimens. Surgically resected HCC and nontumor liver tissues from 54 Chinese patients with HCC were collected from Hong Kong (Prince of Wales Hospital) and Shanghai (Zhongshan Hospital). Tissue samples were used for DNA extraction and for subsequent analysis of HBV DNA by Southern blot hybridization and PCR analysis. PBMCs from 10 healthy blood donors were also included as negative controls. DNA from the tissues and the cell lines, and PBMCs were prepared as described previously (18, 19). Serum was collected from each of the 54 patients for analysis of HBV markers using commercial kits (Abbott Laboratories, North Chicago, IL). All of the DNA samples were tested for HBV DNA by Southern hybridization or PCR using multiple primers in different parts of the HBV genome; details of the results were included in our previous report (19). Forty-three tumor and 40 corresponding nontumor specimens, in which an integrated form of HBV DNA was detected, were chosen for this study.

Cell Lines and Reagents. Two human HCC cell lines (HepG2 and Hep3B) were propagated in vitro in MEM growth medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. For the demethylating experiment, HepG2 and Hep3B cells at an exponentially growing stage were seeded at a density of 3 × 10⁶/100-mm culture dish. The cells were allowed to attach overnight before the addition of appropriate concentration of freshly prepared 5-aza-dC (Sigma Chemical Co.). For HepG2 cells, medium was supplemented with 5 μM of 5-aza-dC, whereas Hep3B cells were supplemented with 2 μM of the same agent.

Detection of GSTP1 CpG Island DNA Hypermethylation by MSP. Bisulfite modification of DNA (1 μg) was carried out by the CpGenome DNA Modification kit (Intergen, Purchase, NY) according to manufacturer’s instructions. Modified DNA samples were precipitated with ethanol and resuspended in TE buffer [10 mM Tris and 1 mM EDTA (pH 7.5)]. The bisulfite-modified DNA samples were amplified by primers specific for GSTP1, and methylated and unmethylated sequences (16, 20). The methylated and unmethylated primer sequences had been reported previously (16). PCR was performed in 25-μl reaction volumes containing 1 × PCR buffer, 2.4 mM each of the deoxynucleoside triphosphates, 1 μM of each primer, and 1 unit of Taq polymerase (AmpliTaq Gold; PE Applied Biosystems, Foster City, CA). The temperature profiles for the amplification were: 5 min at 95°C, 40 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 45 s, extension at 72°C for 1 min, and a final extension step of 5 min at 72°C. Negative control (water without DNA) was included in each amplification protocol. PCR products (8 μl) from each of the samples was loaded onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination.

RT-PCR. First-strand synthesis of cDNA was performed by using an oligodeoxynucleotidylate primer and Superscript II reverse-transcriptase according to the instruction (Life Technologies, Inc.). A 5-μg aliquot of total cellular RNA was used for each reverse transcription reaction, and one-tenth of this reaction was used for PCR. Primers targeting exons 6 and 7 of GSTP1 and exons 1 and 3 of β-actin were used according to a previous report (21). The PCR for GSTP1 was set for 38 cycles consisting of 94°C for 30 s, 1 min at gradually decreasing temperatures using a touch-down protocol (4 cycles each at 62°C and 60°C; and 30 cycles at 58°C), and 74°C for 5 min. RT-PCR products were electrophoresed in a 2% agarose gel and visualized by ethidium bromide staining.

Western Blot Analysis. For analysis of GSTP1 protein, cells grown for 3 days in the present or absence of 5-aza-dC were harvested by trypsinization and washed with PBS. Cells were lysed on ice for 30 min in radioimmunoprecipitation assay buffer in PBS containing 1% Igepal CA-630 (Sigma Chemical Co.), 0.5% sodium deoxycholate, 0.1% SDS, 100 μg phenylmethylsulfonyl fluoride, 30 μl aprotinin, and 1 μg sodium orthovanadate/1 ml of radioimmunoprecipitation assay buffer (Santa Cruz Biotechnology, Inc.). Lysate were cleared by centrifugation at 10,000 g for 10 min at 4°C. The supernatant fluid was collected, and the protein content of the cleared lysate was determined by the method of Lowry assay (Bio-Rad, Hercules, CA). Approximately 70 μg of protein from each sample was separated on 8% SDS-PAGE and transferred to hybond enhanced chemiluminescence nitrocellulose membrane (Amersham). Immunoblot analysis was performed with rabbit polyclonal IgG directed against the human GST π class (Immunotech) using standard protocols. Western blot analyses were performed using an enhanced chemiluminescence-based photoblot system (Amersham).

Immunohistochemical Staining for GSTπ. To detect GSTπ polypeptides in HCC tissues, formalin-fixed, paraffin-embedded HCC tissue specimens were cut into tissues sections, deparaffinized, hydrated, and stained for the presence of GSTπ polypeptides with specific rabbit antiserum (Immunotech, Mar- seilles, France) using an immunoperoxidase technique (22). Expression was determined by a single investigator (C. L.), who did not have any knowledge of the molecular analysis of these samples.

RESULTS

GSTP1 CpG Island DNA Hypermethylation and GSTπ Expression in HCC Tissues. To study whether GSTP1 CpG island hypermethylation changes led to absence of GSTπ expression in human HCC cells, a series of 43 HBV-associated HCC tissue specimens were analyzed for GSTP1 CpG island DNA hypermethylation and correlated with polypeptide expression by immunohistochemical staining using anti-GSTπ antisemum. We surveyed the CpG island hypermethylation status in CpG island located upstream of the transcription start site of GSTP1 gene by MSP. Representative results of the application of the assay to the analysis of somatic GSTP1 are displayed in Fig. 1. GSTP1 CpG island DNA hypermethylation was detected in 28 of 43 (65.1%) HCC tissues and 4 of 40 (10%) corresponding nontumor tissues, whereas, GSTP1 CpG island hypermethylation was not detected in any of PBMCs from normal subjects.

To determine whether the CpG island DNA hypermethylation in HCC tissues might be correlated with the loss of GSTπ polypeptide expression, immunohistochemical staining was carried out on 27 HCC tissues available. Typical immunohistochemical staining results are shown in Fig. 2. In each of the
HCC tissue specimens, liver tissue adjacent to HCC lesions displayed characteristic morphological changes of hepatitis and cirrhosis. In this context, low level GST protein polypeptide was detected in hepatocytes and other cells (e.g., fibroblasts and inflammatory cells) in all of the cases, whereas bile duct cells tend to express abundant GST protein. In 21 of 27 (77.8%) HCC cases examined, HCC cells appeared devoid of GST protein expression (Table 1). In the remaining 6 HCC cases, each appeared to contain occasional stained cells (≤5%) for GST protein. Of the 21 tumors that lacked GST protein expression, 18 (85.2%) displayed GSTP1 promoter hypermethylation, whereas none of the 6 tumors that expressed GST protein was methylated at the GSTP1 locus (P < 0.001 by Fisher’s exact test).

GSTP1 CpG Island DNA Hypermethylation Correlates with Its Gene Expression in Human HCC Cell Lines. To determine whether diminished or absent GST protein expression in human HCC cells might be the result of somatic alterations affecting the GSTP1 gene, human Hep3B and HepG2 were assessed for GSTP1 gene expression. Using MSP analysis, presence of abnormal GSTP1 CpG island hypermethylation was detected in all of the alleles of both Hep3B and HepG2 cell DNA (Fig. 3). Using RT-PCR and immunoblotting, Hep3B and HepG2 cells were found not to express either GSTP1 mRNA or polypeptide (Fig. 4A). To ascertain whether the GSTP1 DNA CpG island hypermethylation was associated with transcriptional silencing of the GSTP1 gene, Hep3B and HepG2 cells were exposed to 5-aza-dC, an inhibitor of DNMTs, for 72 h. As expected, after 72-h treatment, unmethylated CpG island hypermethylation DNA was detected in both of the cell lines (Fig. 4B). GSTP1 mRNA expression in Hep3B and HepG2 cells began to appear at 24 h and increased in abundance by 72 h. GST protein polypeptide expression was restored along with re-expression of mRNA after 5-aza-dC treatment at 72 h. These data indicate that GSTP1 CpG island hypermethylation in Hep3B and HepG2 cell lines is the likely cause for the absence of GST protein expression.

DISCUSSION

Most cases of HCC in China are HBV-related. Our data demonstrate silencing (i.e., failure of gene expression) of the GSTP1 gene by GSTP1 promoter hypermethylation in nearly two-thirds of such cases, and a lack of GST protein expression in an even higher percentage (77.8%). In contrast to tumor tissues, GST protein was detectable in the corresponding nontumorous tissues in all of the cases analyzed, suggesting that silencing of the GSTP1 gene by CpG island DNA methylation may play an important role in the development of HBV-associated HCC. Consistent with our observations, Tchou et al. (17) have also reported that aberrant methylation is a common event in both HBV-associated and HCV-associated primary liver cancer. The mechanism by which genomic DNA methylation patterns become altered in human cancer cells has not been established. However, overexpression of DNA methyltransferase (DNMT1) in mammalian cells in vitro has been reported to promote both de novo DNA methylation and transformation (23, 24). When compared with normal liver tissues, increases in DNMT1 mRNA expression have been detected in HCC tissues and in liver tissues that showed hepatitis (25). Additional types of mammalian DNMTs, capable of de novo DNA methylation, have been identified recently and shown to be expressed in liver tissues (26). It is possible that the abnormality in DNMT activity during chronic hepatitis B infection may contribute to the abnormal de novo CpG island hypermethylation changes affecting GSTP1, E-cadherin, p16, and other genes, which accumulate during the pathogenesis of human HCC (17, 27–29).

The role for GSTP1 in the development of HCC remains to be ascertained. The GST protein was down-regulated in the two liver cancer cell lines analyzed in the present study. We also confirmed that the GST protein nonexpressing cell lines Hep3B and HepG2 were fully methylated at the GSTP1 promoter. Using the demethylating agent 5-aza-dC, the expression of GSTP1 RNA and protein could be restored in Hep3B and HepG2 cells when the GSTP1 promoter region was partially demethylated. Similarly, in HCC tissue samples, the presence of the epigenetic alteration in GSTP1 was associated with a loss of expression of GST protein as determined by immunohistochemistry, whereas all of the tumors that expressed GST protein were unmethylated at the GSTP1 promoter. A few tumors showed loss of expression without hypermethylation. This might be because of post-transcriptional differences, which have been described in human breast cell lines (30). However, the overall strong association between hypermethylation and loss of expression suggests a causative role for aberrant methylation of the GSTP1 promoter and the silencing of the gene in the majority of liver cancers lacking the GST protein expression. We speculate that hepatocytes that contain inactivated GSTP1 genes may be prone to genomic damage when exposed to carcinogens such as dietary aflatoxins (3–10). In line with such a notion, HBV infection and aflatoxin are thought to be largely responsible for the high incidence of HCC in southeast China and Southern Africa (3). The metabolites of the mycotoxin, the ultimate carcinogen of aflatoxin B1, have been associated with mutation at codon 249 of the p53 tumor suppressor gene (31).

Interestingly, silencing of the GSTP1 gene appears to be restricted to liver, prostate, breast, and renal carcinomas. Is there any relationship among prostate, breast, kidney, and liver? One plausible explanation is that all of the tumors derived from these tissues are related, although at different levels, to steroid hormone exposure. A putative pathway involving DNA damage induced by GSTP1 promoter hypermethylation is as follows: the metabolism of estrogens to catechol estrogen quinones gener-
ates electrophilic intermediates (32) that are neutralized by their binding to glutathione in the presence of GSTπ. Epigenetic inactivation of GSTP1 might lead to the accumulation of these compounds that covalently bind to DNA, forming apurinic stable adducts and perhaps mutations. However, additional confirmation of such a hypothesis is necessary. It would be interesting to determine the role of hypermethylation-associated inactivation of GSTP1 in antineoplastic drug sensitivity. Several studies suggest that GSTπ contributes to the resistance to doxorubicin (33), whereas the reduction of GSTπ levels using an antisense approach has been shown to result in increased sensitivity (34). Furthermore, the use of GST class-selective inhibitors and the depletion of reduced glutathione by buthionine sulfoximine sensitizes cancer cells to a variety of antineoplastic drugs (35). Because alkylating agents and anthracyclines are commonly used in HCC chemotherapeutic regimens, the response of tumors may be affected by GSTP1 promoter hypermethylation and the subsequent loss of expression.

The finding of GSTP1 hypermethylation associated with human HCC has significant implications for the detection of HCC. Because the prevalence of the hypermethylation in tumor and nontumor tissues, as revealed in this study, is different, PCR-based strategies targeting GSTP1 CpG island DNA hypermethylation changes may be able to detect HCC DNA in liver

**Table 1** GSTP1 CpG island methylation and gene expression in the 27 HCC cases

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<th>GSTP1 gene expression</th>
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<td>Nontumor</td>
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**Fig. 2** Immunohistochemical staining for GSTπ. Paraffin sections of HCC were deparaffinized, hydrated, and stained with specific rabbit polyclonal antibodies to detect the expression of GSTπ. A representative HCC case shows that GSTπ was negative in HCC cells (right side), weak positive in adjacent nontumor (cirrhosis) tissue (left side); whereas bile duct cells in the nontumor tissues were strongly positive for GSTπ (arrow).

**Fig. 3** Methylation analysis of GSTP1 in cell lines HepG2 and Hep3B. MSP results are expressed as unmethylated GSTP1-specific bands (U) or methylated GSTP1-specific bands (M). DNA was extracted from a cell that was grown for 3 days in the presence (+) or absence (−) of 5 μM 5-aza-dC for HepG2 cell and 2 μM 5-aza-dC for Hep3B cell. Water was used as negative control.
biopsy tissues and in serum or plasma of HCC patients (36). To achieve such a goal, the precise GSTP1 CpG island methylation patterns in normal liver tissue, liver tissue with hepatitis and/or cirrhosis, and from HCC tissue will need to be determined and analyzed in a quantitative manner.

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