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Methylation of the Glutathione-S-Transferase P1 Gene Promoter Is Associated with Oxidative Stress in Patients with Chronic Hepatitis B

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Glutathione-S-transferase P1 (GSTP1) and glutathione-S-transferase M3 (GSTM3) catalyze the glutathione-related clearance of xenobiotics. The methylation of these gene promoters was associated with oxidative stress that induced liver damage. This study aims to explore the relationship among *GSTP1* and *GSTM3* methylation, DNA methyltransferases (DNMTs) expression, and oxidative stress in patients with chronic hepatitis B (CHB). We retrospectively enrolled 153 patients with CHB and 40 healthy controls (HCs). The *GSTP1* and *GSTM3* methylation status, DNMTs mRNA levels in peripheral mononuclear cells (PBMCs) and TNF-*α* and malondialdehyde (MDA) levels in plasma were detected. *GSTP1* methylation was significantly higher in patients with CHB than HCs ($P = 0.047$). Patients with HBeAg-positive CHB showed significantly higher *GSTP1* methylation than those with HBeAg-negative CHB ($P = 0.017$) and HCs ($P = 0.007$). No significant difference was observed between *GSTP1* methylation in HBeAg-negative CHB and HCs ($P = 0.191$). DNMT1 and DNMT3a mRNA levels were significantly higher in participants with *GSTP1* methylation than those without. In patients with CHB, the degree of *GSTP1* promoter methylation was significantly correlated with DNMT1 mRNA, DNMT3a mRNA, TNF-*α*, MDA, HBeAg, ALT, AST and TBIL. In contrast, no significant difference was found between *GSTM3* methylation in patients with CHB and HCs ($P = 0.079$). Meanwhile, no significant difference could be observed between *GSTM3* promoter methylation in patients with HBeAg-positive CHB and HBeAg-negative CHB ($P = 0.146$). Therefore, this study demonstrated that *GSTP1* hypermethylation was associated with DNMT1, DNMT3a overexpression and oxidative stress in patients with HBeAg-positive CHB.

Keywords: chronic hepatitis B; DNA methylation; DNA methyltransferases; glutathione-S-transferase M3; glutathione-S-transferase P1
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Introduction

Hepatitis B virus (HBV) infection is a life-threatening liver disease. Its clinical course can range from asymptomatic infection to liver failure (Lozano et al. 2012). Chronic hepatitis B (CHB) affects more than 350 million people globally (Lok and McMahon 2007). Nearly one-third of these people will eventually develop cirrhosis or hepatocellular carcinoma (HCC) (Chang and Chen 2015). It is estimated that 780,000 people die from HBV infection every year. Although the inner mechanism for HBV infection is still not fully understood, many studies have demonstrated that oxidative stress plays an important role in the development and progression of HBV infection (Bolukbas et al. 2005; Dikici et al. 2005; Lin et al. 2011).

Oxidative stress is mainly caused by enhanced production of reactive oxygen species and/or weakened antioxidant capacity. HBV infection can induce oxidative stress which causes apoptotic or necrotic death of hepatocytes (Waris et al. 2001). Tumor necrosis factor-alpha (TNF-α) and malondialdehyde (MDA), markers for oxidative stress, are elevated in patients with HBV infection (Lara-Pezzi et al. 1998a, b; Acar et al. 2009; Tsai et al. 2009). Our previous studies demonstrated that oxidative stress was associated with severity of HBV infection (Li et al. 2011a; Qi et al. 2012). Moreover, we found that *glutathione-S-transferase P1* (*GSTP1*) and *glutathione-S-transferase M3* (*GSTM3*) promoters were hypermethylated in acute-on-chronic hepatitis B liver failure, and the degree of methylation was associated with oxidative stress (Li et al. 2011a; Qi et al. 2012).

DNA methylation is catalyzed by methyltransferases (DNMTs), which can be divided into “de novo” DNMTs

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(DNMT3a and DNMT3b) and maintenance DNMT (DNMT1) (Bird and Wolffe 1999; Denis et al. 2011). DNMT3a and DNMT3b can recognize and methylate unmethylated DNA during embryogenesis and development (Okano et al. 1999). DNMT1 has a higher catalytic activity for semimethylating DNA to maintain DNA methylation patterns during replication (Robertson et al. 1999; Jones and Liang 2009). Based on the previous studies, we have assumed that HBV infection might promote the overexpression of DNMTs, resulting in hypermethylation of *GSTP1* and *GSTM3* promoter, which finally causes oxidative stress and liver damage.

Therefore, this study aimed to explore the relationship among *GSTP1* and *GSTM3* promoter methylation, DNMTs expression, and oxidative stress in patients with CHB.

Materials and Methods

Patients and controls

The present study retrospectively analyzed 153 patients with CHB, including 81 with hepatitis B e antigen (HBeAg)-positive CHB, 72 with HBeAg-negative CHB, from May 2012 to July 2014 at Department of Hepatology, Qilu Hospital of Shandong University. CHB was defined as the presence of hepatitis B surface antigen (HBsAg) for more than 6 months (Lok and McMahon 2007). The 40 healthy volunteers were also enrolled as healthy controls (HCs). Exclusion criteria included (1) co-infection with hepatitis C virus, hepatitis D virus, hepatitis E virus, hepatitis G virus and/or human immunodeficiency virus (HIV); (2) suffering from other liver diseases such as alcoholic hepatitis, autoimmune hepatitis and drug hepatitis; (3) receiving antioxidant agent or interferon therapy; and (4) complication with liver cirrhosis or HCC.

All co-authors had access to the study data and approved the final manuscript. All participants gave written informed consents under protocols approved by the local Research and Ethics Committee at Qilu Hospital of Shandong University, in accordance with the guidelines of the 1975 Declaration of Helsinki.

DNA extraction and RNA isolation from peripheral mononuclear cells (PBMCs)

EDTA-anticoagulated venous peripheral blood (5 ml) was collected from each participant. PBMCs were isolated by gradient centrifugation on Ficoll-Paque (Pharmacia Diagnostics, Uppsala, Sweden). Genomic DNA was extracted from PBMCs using QIAamp DNA Blood Mini Kit (QIAGEN, CA, USA) according to the standard protocol provided by the manufacturer. The extracted DNA was eluted in a total volume of 200 μL sterile water and stored at −20°C until use. RNA was extracted from PBMC via phenol chloroform isopropranol method according to the recommendations. Then, total RNA was resuspended in 20 μL of RNase free water and stored at −20°C until use.

Sodium bisulfite modification and methylation-specific polymerase chain reaction (MSP)

Bisulfite modification was conducted by EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA) according to the standard protocol recommended by the manufacturer. The modified DNA was amplified by MSP using primers specific for methylated and unmethylated *GSTP1* and *GSTM3*. The primers sequences were the same with those in previous studies (Esteller et al. 1998; Kollermann et al. 2006; Li et al. 2011a; Qi et al. 2012). The MSP-amplified region of *GSTP1* is from +78 to +168 within a CpG island in the 5′ region of *GSTP1* gene. The MSP-amplified region of *GSTM3* is from +75 to +218 within a CpG island in the 5′ region of *GSTM3* gene. The schematic representation of the *GSTP1* and *GSTM3* genes is shown in Fig. 1. MSP was performed in a total volume of 25 μL containing 1 μL bisulfite-treated DNA, 0.5 μL of each primer (10 μM), 10.5 μL nuclease-free water, and 12.5μL Premix Taq (Zymo Research, USA), which consisted of Taq DNA polymerase, reaction buffer, and deoxynucleotide triphosphate mixture. The PCR protocols of *GSTP1* and *GSTM3* were composed of an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 58°C (*GSTP1*) or 51.5°C (*GSTM3*) for 40 s, and primer extension at 72°C for 40 s. Water control without DNA was used in each round of PCR. PCR products were then electrophoresed on a 2%-agarose gel,

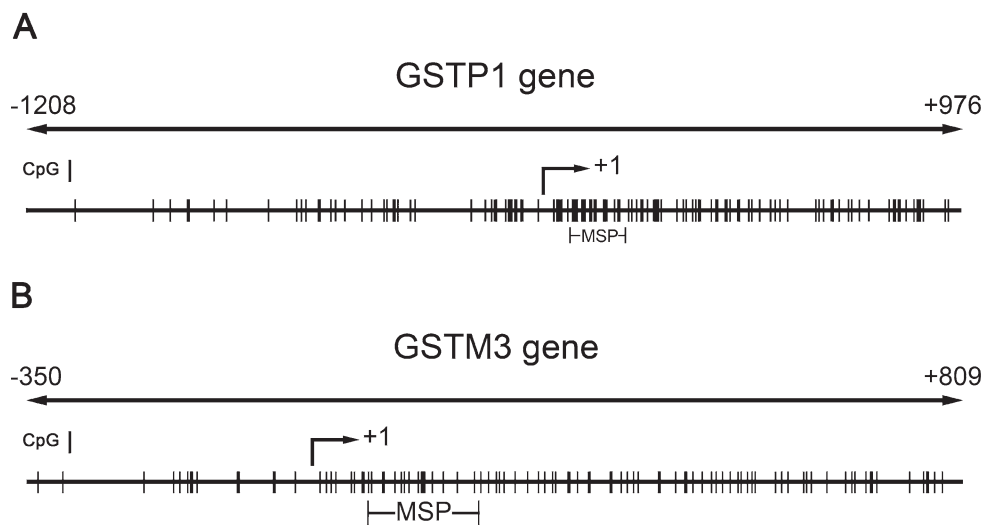

Fig. 1. Schematic representation of *GSTP1* and *GSTM3* genes. The transcriptional start site (curved arrow) and CpG site (short vertical lines) were shown.
A. MSP-amplified region of *GSTP1* (+78 to +168) is indicated.
B. MSP-amplified region of *GSTM3* (+75 to +218) is indicated.

Table 1. Primer sets for RT-qPCR.

Gene		Primer sequences (5'-...-3')
DNMT1	F	GAG CTA CCA CGC AGA CAT CA
	R	CGA GGA AGT AGA AGC GGT TG
DNMT3a	F	CACACAGAAGCATATCCAGGAGTG
	R	AGTGGACTGGGAAACCAAATACCC
DNMT3b	F	CCC ATT CGA GTC CTG TCA TT
	R	GGT TCC AAC AGC AAT GGA CT
β-actin	F	ATG GGT CAG AAG GAT TCC TAT GTG
	R	CTT CAT GAG GTA GTC AGT CAG GTC

M, methylation-specific primers; U, unmethylation-specific primers; F, forward; R, reverse; DNMT1, DNA methyltransferases 1; DNMT3a, DNA methyltransferases 3a; DNMT3b, DNA methyltransferases 3b.

stained with ethidium bromide, and visualized under UV illumination.

Quantitative real-time polymerase chain reaction (RT-qPCR)

RNAs were converted into cDNA using first-strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania). The mRNA levels of DNMT1, DNMT3a and DNMT3b were measured by RT-qPCR, which was performed on Lightcycler (Roche, Basel, Switzerland) using SYBR Green (Toyobo, Osaka, Japan). *β*-actin was used as the endogenous control. The real-time PCR reaction was performed as follows: the initial step was 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 60°C for 30 s and 72°C for 30 s. The primers sets were designed from a previous published study (Li et al. 2011b) and described in Table 1.

Enzyme-linked immunosorbent assay (ELISA)

All blood samples were collected and centrifuged at 2,000 rpm/min for 5 min, and the plasma obtained was stored at −80°C until subsequent experiments. Plasma level of MDA was determined using an ELISA kit (Cell Biolabs, Inc., USA) according to the manufacturers' instructions. Plasma level of TNF-*α* was determined using an ELISA kit (Abcam, USA) according to the manufacturers' instructions.

Clinicopathological data collection

The clinicopathological data were collected, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), HBeAg, HBsAg, HBV-DNA, and prothrombin time (PT). These markers were measured at Department of Laboratory Medicine, Qilu Hospital, Shandong University.

Statistical analysis

Statistical analyses were performed using SPSS version 16.0 (Chicago, IL, USA). The variables were expressed as median (centile 25; centile 75) or number (percentage). Comparison between groups was performed by Mann-Whitney U test or Chi-square test. Spearman rank correlation was used to evaluate correlations between two parameters. P value < 0.05 was considered statistically significant.

Results

General characteristics

From May 2012 to July 2014, 153 patients with CHB (81 with HBeAg-positive CHB and 72 with HBeAg-negative CHB) and 40 HCs were retrospectively enrolled at Department of Hepatology, Qilu Hospital of Shandong University. No significant differences of age and sex could be observed between CHB group and HCs. Patients with CHB showed significantly higher levels of Log_{10} [HBV-DNA], ALT, AST and TBIL than those in HCs (Table 2). Meanwhile, no significant differences of age, sex and TBIL could be observed between patients with HBeAg-positive CHB and HBeAg-negative CHB. Patients with HBeAg-positive CHB showed significantly higher levels of Log_{10} [HBV-DNA], ALT, and AST than those with HBeAg-negative CHB (Table 3).

Methylation status of GSTP1 and GSTM3 promoter in different groups

GSTP1 promoter methylation was detected in 16 patients with CHB (16/153, 10.5%), which was significantly higher than HCs (0/40, 0.0%; $P < 0.05$). The patients with HBeAg-positive CHB (13/81, 16.05%) showed significantly higher *GSTP1* methylation than those with HBeAg-negative CHB (3/72, 4.17%; $P = 0.017$) and HCs ($P = 0.007$). However, no significant difference could be observed in *GSTP1* methylation between HBeAg-negative CHB and HCs ($P = 0.191$) (Fig. 2).

GSTM3 promoter methylation was detected in 14 patients with CHB (14/153, 9.2%), but in none of the HCs (0/40, 0.0%). No significant difference was found between

Table 2. Clinical characteristics of patients with CHB and HCs.

Variables	CHB (n =153)	HCs (n = 40)	*P* value
Age (years)	40.0 (28.0-51.0)	41.5 (29.3-51.8)	0.694[a]
Sex (male/female)	105/48	11/29	0.636[b]
Log_{10} [HBV-DNA]	4.7 (2.8-6.4)	NA	
HBeAg (+/−)	81/72	0/40	< 0.001[b]
ALT (U/L)	89.0 (30.0-243.0)	18.5 (14.0-23.8)	< 0.001[a]
AST (U/L)	54.0 (32.0-114.5)	16.5 (12.3-21.8)	< 0.001[a]
TBIL (μmol/L)	18.3 (12.2-42.0)	8.9 (6.6-11.1)	< 0.001[a]

The ranges of each variable are also shown within parenthesis.
HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBIL, total bilirubin; NA, not available.
[a]Mann-Whitney U test.
[b]Chi-square test.

Table 3. Clinical characteristics of patients with HBeAg-positive CHB and HBeAg-negative CHB.

Variables	HBeAg-positive (n = 81)	HBeAg-negative (n = 72)	*P* value
Age (years)	40.0 (28.0-52.5)	39.0 (27.3-50.8)	0.745[a]
Sex (male/female)	58/23	47/25	0.400[b]
Log_{10} [HBV-DNA]	5.8 (4.0-7.2)	2.9 (2.5-5.0)	< 0.001[a]
ALT (U/L)	139 (53.5-312.5)	45.5 (23.0-143.5)	< 0.001[a]
AST (U/L)	67.0 (41.5-144.0)	38.0 (26.0-81.0)	< 0.001[a]
TBIL (μmol/L)	18.8 (11.6-68.3)	17.4 (12.5-31.0)	0.632[a]

The ranges of each variable are also shown within parenthesis.
HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBIL, total bilirubin.
[a]Mann-Whitney U test.
[b]Chi-square test.

the two groups ($P = 0.079$). Meanwhile, no significant difference could be observed between *GSTM3* promoter methylation in patients with HBeAg-positive CHB (10/81, 12.3%) and HBeAg-negative CHB (4/72, 5.6%, $P = 0.146$) (Fig. 2).

In this study, 6 patients with CHB (6/153, 3.9%) showed hypermethylation of both gene promoters, 5 of which were HBeAg-positive CHB and only 1 is HBeAg-negative CHB.

Association of GSTP1 promoter methylation with DNMTs mRNA expression

The DNMT1 mRNA level was significantly higher in patients with CHB [1.3×10^{-3} (6×10^{-4}-6.8×10^{-3})] than HCs [5×10^{-4} (1×10^{-4}-4.2×10^{-3}), $P < 0.01$). The DNMT3a mRNA level was significantly higher in patients with CHB [1.9×10^{-3} (8×10^{-4}-5.8×10^{-3})] than HCs [5×10^{-4} (2×10^{-4}-4.2×10^{-3}), $P < 0.01$]. The DNMT3b mRNA level was significantly higher in patients with CHB [8×10^{-5} (5×10^{-5}-2.8×10^{-4})] than HCs [4×10^{-5} (2×10^{-5}-1.3×10^{-4}), $P < 0.01$].

In patients with CHB, the methylation of *GSTP1* pro-

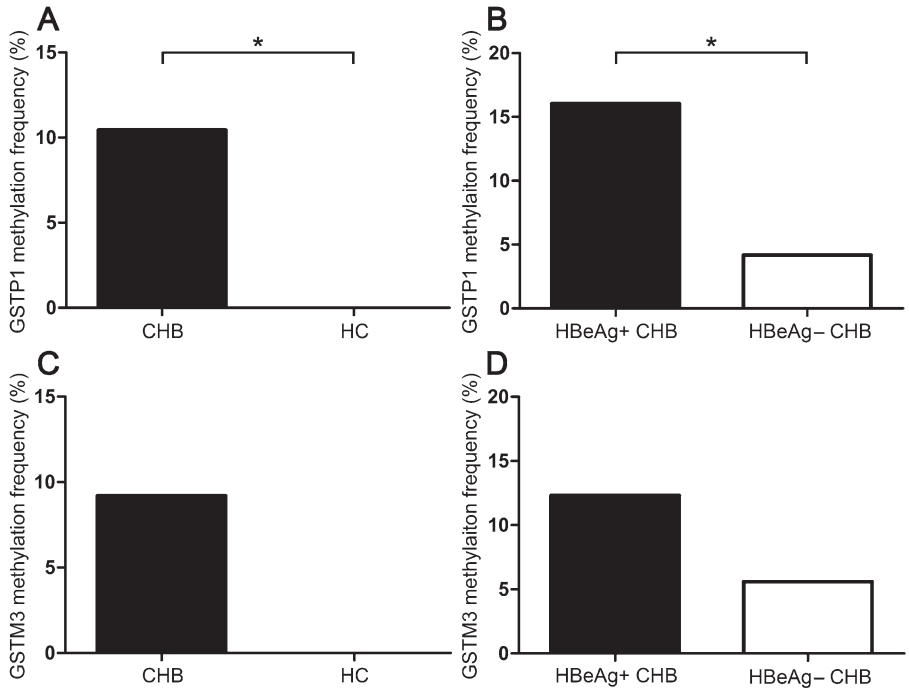

Fig. 2. The comparison of *GSTP1* and *GSTM3* methylation between different groups.
A. The comparison of *GSTP1* methylation between patients with CHB and HCs.
B. The comparison of *GSTP1* methylation between patients with HBeAg+ and HBeAg− CHB.
C. The comparison of *GSTM3* methylation between patients with CHB and HCs.
D. The comparison of *GSTM3* methylation between patients with HBeAg+ and HBeAg− CHB.

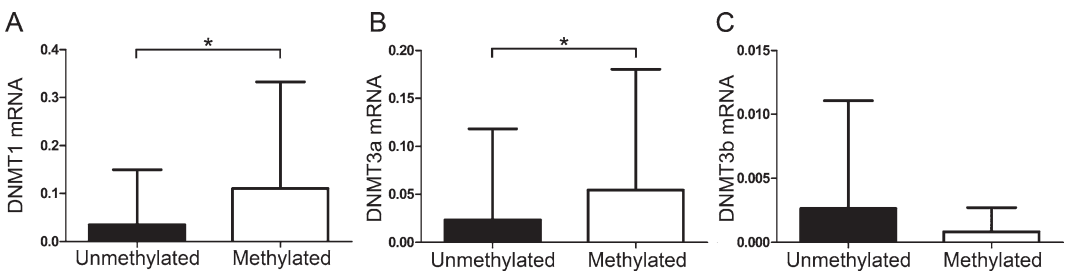

Fig. 3. The comparison of DNMTs expression between CHB patients with or without *GSTP1* methylation.
A. DNMT1 mRNA levels in CHB patients with or without *GSTP1* methylation.
B. DNMT3a mRNA levels in CHB patients with or without *GSTP1* methylation.
C. DNMT3b mRNA levels in CHB patients with or without *GSTP1* methylation.

moter was positively correlated with DNMT1 mRNA expression (rs = 0.217, $P = 0.007$) and DNMT3a (rs = 0.243, $P = 0.002$). However, no significant correlation could be found between *GSTP1* methylation and DNMT3b mRNA level (rs = 0.117, $P = 0.151$) (Fig. 3).

Plasma levels of MDA and TNF-α

The MDA level was significantly elevated in patients with CHB [205.0 (68.1-399.5) μmol/mg] compared with HCs [87.5 (24.0-198.8) μmol/mg, $P = 0.001$]. Meanwhile, patients with HBeAg-positive CHB [252.0 (86.7-413.0) μmol/mg] showed significantly higher MDA level than those with HBeAg-negative CHB [129.5 (48.4-355.8) μmol/mg; $P = 0.048$]. The TNF-α level was elevated in CHB [3.1 (1.5-5.4) pg/ml] compared with HCs [1.4 (1.0-1.9) pg/ml, $P < 0.001$]. Meanwhile, patients with HBeAg-positive CHB [4.0 (2.7-7.1) pg/ml] showed significantly higher TNF-α level than those with HBeAg-negative CHB [2.5 (1.1-3.7) pg/ml, $P < 0.001$].

The TNF-α level in patients with *GSTP1* methylation [4.8 (3.1-6.7) pg/ml] was significantly elevated compared with those with unmethylation groups [3.0 (1.5-5.3) pg/ml, $P = 0.025$]. However, no significant difference of MDA level could be observed between patients with *GSTP1* promoter methylation [355.0 (141.8-504.5) μmol/mg] and those without [197.0 (64.1-386.5) μmol/mg, $P = 0.085$].

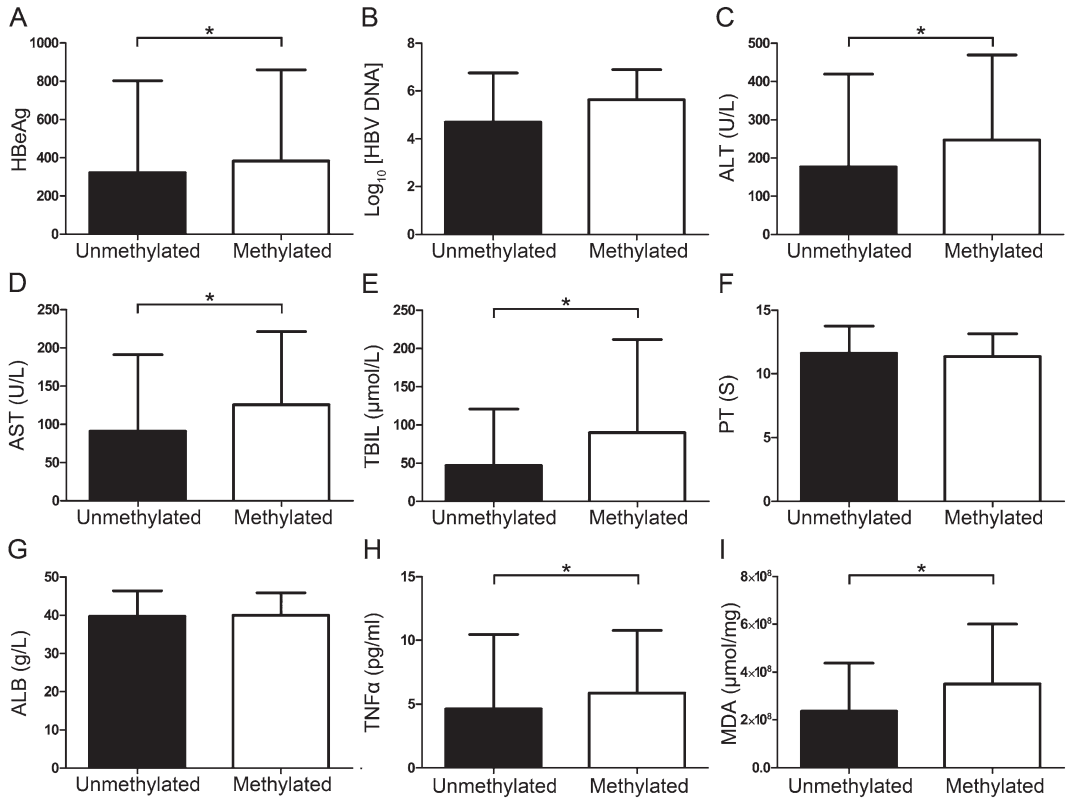

Fig. 4. The comparison of clinicopathological data between CHB patients with or without *GSTP1* methylation.
A. HBeAg in CHB patients with or without *GSTP1* methylation.
B. Log_{10} [HBV-DNA] in CHB patients with or without *GSTP1* methylation.
C. ALT level in CHB patients with or without *GSTP1* methylation.
D. AST level in CHB patients with or without *GSTP1* methylation.
E. TBIL level in CHB patients with or without *GSTP1* methylation.
F. Prothrombin time (PT) in CHB patients with or without *GSTP1* methylation.
G. ALB level in CHB patients with or without *GSTP1* methylation.
H. TNF-*α* level in CHB patients with or without *GSTP1* methylation.
I. MDA level in CHB patients with or without *GSTP1* methylation.

Correlation between GSTP1 methylation, DNMTs mRNA expression and clinicopathological features

In patients with CHB, *GSTP1* methylation was significantly positively correlated with TNF-*α* (rs = 0.182, P = 0.025), MDA (rs = 0.140, P = 0.085), HBeAg (rs = 0.194, P = 0.016), ALT (rs = 0.161, P = 0.047), AST (rs = 0.179, P = 0.026) and TBIL (rs = 0.173, P = 0.032). No correlation was found between *GSTP1* promoter methylation and PT (rs = −0.070, P = 0.441), albumin (ALB) (rs = −0.027, P = 0.749), and Log_{10} [HBV-DNA] (rs = 0.151, P = 0.062) (Fig. 4).

DNMT1 expression was positively associated with MDA (rs = 0.405, P < 0.001) and TNF-*α* (rs = 0.281, P < 0.001) level. DNMT3a expression was also positively correlated with MDA (rs = 0.391, P < 0.001) and TNF-*α* (rs = 0.370, P < 0.001) level. However, there was no significant association between DNMT3b expression and MDA (rs = 0.110, P = 0.177) or TNF-*α* (rs = 0.015, P = 0.857) level. Moreover, none of the three DNMTs had correlation with Log_{10} [HBV-DNA]: DNMT1, rs = 0.077, P = 0.343; DNMT3a, rs = 0.099, P = 0.225; and DNMT3b, rs = 0.113, P = 0.163.

Discussion

In this study, we found that hypermethylation of *GSTP1* promoter existed in patients with CHB. Patients with HBeAg-positive CHB showed significantly higher *GSTP1* methylation than those with HBeAg-negative CHB and HCs. However, no significant difference could be observed between HBeAg-negative CHB and HCs. In patients with CHB, *GSTP1* methylation was positively correlated with DNMT1 mRNA, DNMT3a mRNA, TNF-*α*, MDA, HBeAg, ALT, AST and TBIL. DNMT1 mRNA and DNMT3a mRNA levels were positively associated with MDA and TNF-*α* level.

Previous studies demonstrated that oxidative stress played an important role in the process of HBV infection (Bolukbas et al. 2005; Dikici et al. 2005; Lin et al. 2011). MDA and TNF-*α*, which were markers for oxidative stress, were significantly elevated in patients with CHB (Gutierrez-Ruiz et al. 2001; Acar et al. 2009; Tsai et al. 2009).

Glutathione-S-transferases (GSTs) can protect cells against damages from toxic substances (Hayes and McLellan 1999; Tew and Ronai 1999). *GSTP1*and *GSTM3*, which belong to the GSTs family, can be downregulated by aberrant methylation in their promoter regions (Zhong et al. 2002; Lee et al. 2003; Niu et al. 2009). In mammals, DNA methylation is mainly catalyzed by DNMT1, DNMT3a and DNMT3b. Several studies have reported that HBV could increase the expression of DNMT1 and DNMT3a, resulting in aberrant promoter methylation of E-cadherin, *p16INK4A*, *SFRP1*and *SFRP5* (Lee et al. 2005; Jung et al. 2007; Xie et al. 2014). Consistent with these studies, this study found that *GSTP1* promoter methylation was positively correlated with DNMT1 mRNA, DNMT3a mRNA, TNF-α, MDA, HBeAg, ALT, AST and TBIL in patients with CHB. Therefore, HBV infection might induce methylation of *GSTP1* and *GSTM3* promoters through activation of DNMT1 and DNMT3a.

Previous studies showed that oxidative stress might also be associated with HBV replication (Bolukbas et al. 2005). However, no correlation was observed between plasma MDA level and Log_{10} [HBV-DNA] (rs = 0.001, P = 0.994) in this study. Interestingly, patients with HBeAg-positive CHB displayed higher levels of TNF-α and MDA than those with HBeAg-negative CHB. HBeAg seroconversion is an important serologic end point for CHB. HBeAg-negative CHB patients had a sustained reduction of HBV-DNA (Fattovich et al. 2008), a lower incidence of cirrhosis and HCC (Lin et al. 2007), and a higher incidence of complication-free survival (Chu et al. 2004). In this study, *GSTP1* methylation was significantly higher in patients with HBeAg-positive CHB than those with HBeAg-negative CHB and HCs, which implied that *GSTP1* methylation might potentially serve as a biomarker for HBeAg seroconversion.

There were also several limitations in this study. We enrolled participants from one single center and the sample size was relatively small. Therefore, our findings still need further validation with large scale and multicenter studies prior to its clinical usage. The study was performed using PBMCs and plasma of the participants. However, we believed that studies with hepatocytes or liver tissue were also needed. Meanwhile, future studies with animal models might be useful for validating our findings.

Therefore, this study demonstrated that *GSTP1* hypermethylation was associated with DNMT1, DNMT3a overexpression and oxidative stress in patients with HBeAg-positive CHB. Therefore, *GSTP1* methylation might potentially serve as a biomarker for HBeAg seroconversion.

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Conflict of Interest

The authors declare no conflict of interest.

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