Methylation of the Glutathione-S-Transferase M3 Gene Promoter is Associated with Oxidative Stress in Acute-on-Chronic Hepatitis B Liver Failure

Li Qi,1,2 Zhi-Qiang Zou,3 Li-Yuan Wang,1 Shuai Gao,1 Yu-Chen Fan,1,2 Bo Long,3 Yan-Mei Guo,3 Ai-Ling Xu,3 Jie Han,1 Tao Li1 and Kai Wang1,2

1Department of Hepatology, Qilu Hospital of Shandong University, Jinan, P.R. China
2Institute of Hepatology, Shandong University, Jinan, P.R. China
3Department of Hepatology, Yantai Infectious Disease Hospital, Yantai, P.R. China

Chronic hepatitis B (CHB) is a major cause for liver disease worldwide, ranking as the first cause for liver cirrhosis and hepatocellular carcinoma. Acute-on-chronic hepatitis B liver failure (ACHBLF) is most commonly caused by acute severe exacerbation during CHB virus infection. The pathophysiology of ACHBLF is still poorly understood. Glutathione-S-transferase (GST) M3 belongs to GSTs superfamily and it has been demonstrated to contribute to oxidative stress-mediated liver damage. The present study was aimed to determine the potential association between GSTM3 promoter methylation and oxidative stress in ACHBLF patients. Thirty ACHBLF patients, 30 CHB patients and 10 healthy controls were included in this study. Methylation of GSTM3 promoter was determined using methylation-specific PCR (MSP) method. Plasma biomarkers for oxidative stress including malondialdehyde (MDA) and GST were detected by enzyme-linked immunosorbent assay (ELISA). Model for end-stage liver disease (MELD) scoring system was used for predicting the severity and prognosis of liver failure. ACHBLF patients had significant higher GSTM3 promoter methylation rate than CHB patients (30% versus 6.7%, $\chi^2 = 5.455, P = 0.020$). Plasma MDA and GST levels were significantly increased in ACHBLF patients compared with CHB patients. Meanwhile, MDA, MELD scores and mortality rate were significantly higher in methylated group than those in unmethylated group of ACHBLF patients. Furthermore, plasma MDA levels were positively correlated with MELD scores of ACHBLF ($r = 0.588, P = 0.001$). In conclusion, the methylation of GSTM3 promoter may contribute to oxidative stress-associated liver damage and correlate with the disease severity in ACHBLF.

Keywords: acute-on-chronic hepatitis B liver failure; glutathione-S-transferase; malondialdehyde; oxidative stress; promoter methylation


Chronic hepatitis B virus (HBV) infection is a major cause for liver diseases worldwide, ranking as the first cause for liver cirrhosis and hepatocellular carcinoma, especially in Asia (McClune and Tong 2010). Acute-on-chronic hepatitis B liver failure (ACHBLF) is an acute deterioration of chronic liver disease caused by HBV infection with high mortality (Sarin et al. 2009). As a result of the high incidence of chronic HBV infection, ACHBLF accounts for more than 80% of all the acute-on-chronic liver failure (ACLF) patients and is the most common liver failure in China (Liver Failure and Artificial Liver Group, Chinese Society of Infectious Diseases, Chinese Medical Association and Severe Liver Diseases and Artificial Liver Group, Chinese Society of Hepatology, Chinese Medical Association 2006; Zou et al. 2008). At present, the underlying molecular mechanism for the pathogenesis of ACHBLF is still uncertain but current hypotheses have suggested that systemic inflammatory response and oxidative stress may underlie the transition from a stable cirrhotic state to progressive liver injury and end-organ failure which is accompanied by encephalopathy, hemorrhage or hepatorenal syndrome and associated with significant morbidity and short-term mortality (Sen et al. 2004).

Oxidative stress is a disturbance in the oxidant-antioxidant balance leading to potential cellular damage (Ha et al. 2010). The imbalance may result from a lack of antioxidant capacity caused by disturbances in production and distribution, or by an overabundance of reactive oxygen species (ROS) from other factors (Dröge 2003). ROS alterations in different signaling pathways may modulate gene expres-
sion, cell adhesion, cell metabolism, cell cycle and cell death. These events may induce oxidative DNA damage, which in turn increases chromosomal aberrations associated with cell transformation (Choi and Ou 2006). Oxidative stress contributes to the progression and deterioration of viral hepatitis and can be seen in various forms of HBV infection (Bolukbas et al. 2005; Nair et al. 2010). Malondialdehyde (MDA) is an end-product of lipid peroxidation and always employed as a serum marker of oxidative stress (Acar et al. 2009).

Glutathione-S-transferases (GSTs) are broadly expressed family of phase II isoenzymes that protect against endogenous oxidative stress, as well as exogenous potential toxins (White et al. 2008). They detoxify a variety of electrophilic compounds, including oxidized injury of lipid and DNA products generated by ROS damage to intracellular molecules (Hayes et al. 2005). Several GST variants are principally expressed in the liver whose primary functions include detoxification and metabolism. In the liver, inflammation is related to a variety of insults, including HBV, alcohol, and hepatitis C virus (HCV), which are sources of ROS (White et al. 2008). GST has been proposed to protect against HBV-related liver injury, which is partly manifested as extensive oxidative DNA damage (Hagen et al. 1994). Cytosolic and membrane-bound forms of GSTs are encoded by two distinct supergene families. At present, eight distinct classes of the soluble cytoplasmic mammalian GSTs have been identified: alpha, kappa, mu, omega, pi, sigma, theta, and zeta (Strange et al. 2001). The GSTP1 gene encodes the pi class of enzymes. Dysfunction of the GSTP1 gene through aberrant DNA methylation has been demonstrated in several human tumors (Esteller et al. 2001; Hopkins et al. 2007). GSTM3 belongs to mu-class subfamily. It is different from GSTP1 in biological significance. GSTM3 plays a pivotal role in conjugation and detoxification of environmental carcinogens (Hayes and Pulford 1995) and has strong antioxidant function (Hayes and McLellan 1999). GSTM3 intron 6 rs1799735, a 3-base-pair deletion polymorphism, produces a binding site for the transcription factor YY1 (Yin Yang 1), which influences the expression of GSTM3 (Shi et al. 1997). The 3-base-pair deletion in GSTM3 may reduce transcript expression (Inskip et al. 1995) and the ability of enzyme neutralizing the ROS.

So if the gene expression of GSTM3 is inhibited, its antioxidant function may be impaired, and abundant ROS and subsequent oxidative stress may be generated, thus tissues damage including the liver damage can be caused. DNA promoter methylation is a hot topic at present. It plays an important role in the regulation of gene expression and may be a common and crucial mechanism for the dysfunction of antioxidative members (Peng et al. 2009). It has been reported that DNA methylation of GSTs is correlated with reduced gene expression (Peng et al. 2009). So the suppression of GSTM3 gene may be caused by DNA methylation of CpG island in its promoter region. GSTM3 gene has been researched to be associated with some carcinomas, such as hepatocellular carcinoma, prostatic carcinoma and bladder carcinoma (Schnakenberg et al. 2000; Medeiros et al. 2004; Ladero et al. 2007), but there is no study of GSTM3 gene promoter methylation on chronic liver disease, especially on ACHBLF. Wang et al. (2006) found aberrant promoter methylation of GSTP1 in serum of liver cirrhotic patients. Our previous study has found aberrant promoter methylation of GSTP1 in ACHBLF patients (Li et al. 2011). GSTM3 is closely related with GSTP1 (Wu et al. 2008), but they have different biological significance, so it is significant to conduct a specific research on GSTM3. Based on the above understandings, it is reasonable to assume that GSTM3 gene may display aberrant promoter methylation and contribute to the oxidative stress-associated liver injury in ACHBLF.

Therefore, this study aimed to determine the potential association between GSTM3 promoter methylation and oxidative stress in ACHBLF patients.

Materials and Methods

Patients and controls

A total of 30 ACHBLF patients, 30 chronic hepatitis B (CHB) patients and 10 healthy volunteers as controls were recruited from December 2009 to February 2011 in Qilu Hospital of Shandong University and Yantai Infectious Disease Hospital. Patients with ACHBLF were defined according to the following inclusion criteria (Liver Failure and Artificial Liver Group, Chinese Society of Infectious Diseases, Chinese Medical Association: 2001). Patients with CHB infection were considered as exclusion criteria.

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Plasma collection and genomic DNA extraction

Plasma was obtained from peripheral blood by centrifugation and stored at −80°C for detection of MDA and GST levels by enzyme-linked immunosorbent assay (ELISA). Genomic DNA was extracted using TRIzol reagent (Invitrogen, USA). DNA concentrations were measured at 260 nm. The quality of DNA was assessed by agarose gel electrophoresis. 

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extracted from peripheral blood by QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA) following the standard protocol provided by the manufacturer and stored at −20°C.

Detection of GSTM3 promoter methylation by methylation-specific PCR (MSP)

MSP was used to detect GSTM3 promoter methylation status. In detail, DNA (500-1,000 ng) bisulfite modification was performed by CpGenome™ DNA Modification Kit (Intergen Company, Purchase, NY, USA), standard protocol provided by the manufacturer was exactly followed. Methylated and unmethylated primers specific for GSTM3 promoter (Wu et al. 2008) were used to amplify the bisulfate-modified DNA samples (Table 1). MSP mixture contained bisulfite modified DNA 100 ng and 2.5 μl 10 × PCR buffer, 200 μmol/L of each dNTP, 1 μmol/L of each primer and 1 U of Taq polymerase (Zymo Taq™ DNA Polymerase, Zymo research CORP. USA). The mixture was incubated for 10 minutes at 95°C, followed by 40 cycles of denaturing at 95°C for 30 seconds, annealing at 51.5°C for 30 seconds, extension at 72°C for 1 minute and a final extension at 72°C for 7 minutes. Water without DNA was used as negative control. PCR products were analyzed by 2% agarose gels, stained with ethidium bromide and visualized under UV illumination.

ELISA for detection of plasma MDA and GST levels

Plasma MDA and GST levels of normal controls, CHB and ACHBLF patients were detected in the present study. OXISelect™ MDA Adduct ELISA kit (Cell Biolabs, INC, USA) and GST ELISA kit (ShangHai Lengton Bioscience Co., LTD, China) were employed and standard protocol provided by the manufacturer was exactly followed.

Statistical analysis

The data were analyzed using SPSS 13.0 statistical software (SPSS Inc., Chicago, IL). The difference of GSTM3 promoter methylation status between ACHBLF and CHB patients was analyzed by Chi-squared test. Laboratory parameters differences between ACHBLF and CHB patients, GSTM3 promoter methylated group and unmethylated group of ACHBLF patients were analyzed by Independent-Samples T test. Independent-Samples T test was also used to compare the differences of plasma MDA, GST levels and MELD scores between the two groups of ACHBLF patients. The mortality rate between methylated group and unmethylated group of ACHBLF patients was analyzed by Fisher’s exact test. The correlations between MDA, GST levels and MELD scores were analyzed by correlation analysis. All statistical analyses were two-sided, and P value < 0.05 was considered to be statistically significant.

Results

Basic characteristics

There were 30 ACHBLF patients with a mean age of 47.0 ± 11.6 years old, 30 CHB patients of 43.1 ± 11.9 years old, and 10 normal controls participating in the present study. There were no significant differences in gender, drinking habits and age between ACHBLF group and CHB group, while significantly differences in plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), TBIL and PTA levels were found between the two groups (P < 0.05 or P < 0.001), but there was also no significant difference in HBV DNA copies (P = 0.123) (Table 2).
Aberrant methylation of GSTM3 gene promoter in the patients

The methylation of GSTM3 promoter was detected in 9 patients of 30 ACHBLF patients (30%) and 2 of 30 CHB patients (6.7%). Significant difference was found between the two groups ($\chi^2 = 5.455$, $P = 0.020$). Fig. 1 represented the typical result of GSTM3 promoter methylation analysis by MSP. Importantly, no GSTM3 promoter methylation was detected in the 10 normal controls.

Table 3. Laboratory parameters between methylated group and unmethylated group in ACHBLF.

<table>
<thead>
<tr>
<th>Group</th>
<th>Methylated group ($n = 9$)</th>
<th>Unmethylated group ($n = 21$)</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (IU/L)</td>
<td>376.311 ± 240.150</td>
<td>576.024 ± 606.040</td>
<td>0.351</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>333.289 ± 314.251</td>
<td>423.281 ± 350.262</td>
<td>0.512</td>
</tr>
<tr>
<td>PTA (%)</td>
<td>24.811 ± 10.697</td>
<td>33.919 ± 4.604</td>
<td>0.035</td>
</tr>
<tr>
<td>TBIL (µmol/L)</td>
<td>527.922 ± 113.564</td>
<td>321.581 ± 93.048</td>
<td>0.000</td>
</tr>
<tr>
<td>Log10 (HBV DNA)</td>
<td>5.579 ± 1.934</td>
<td>5.256 ± 1.141</td>
<td>0.650</td>
</tr>
<tr>
<td>MDA (pmol/mg)</td>
<td>16.956 ± 2.155</td>
<td>11.369 ± 5.553</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>GST (mIU/L)</td>
<td>1,381.678 ± 236.841</td>
<td>1,262.253 ± 202.782</td>
<td>0.170</td>
</tr>
<tr>
<td>MELD scores</td>
<td>22.706 ± 2.669</td>
<td>18.765 ± 4.808</td>
<td>0.029</td>
</tr>
<tr>
<td>Mortality (%)</td>
<td>55.6</td>
<td>14.3</td>
<td>0.032</td>
</tr>
</tbody>
</table>

Values are given as mean ± s.d.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; PTA, prothrombin time activity; TBIL, total bilirubin; HBV, hepatitis B virus; MDA, malondialdehyde; GST, glutathione-S-transferase; MELD, model for end-stage liver disease.

Plasma levels of MDA

MDA levels were significantly higher in ACHBLF patients than those in CHB patients ($13.045 \pm 5.416$ vs. $9.387 \pm 5.332$ pmol/mg, $P = 0.011$), and they were also higher in methylated group than those in unmethylated group of ACHBLF patients ($16.956 \pm 2.155$ vs. $11.369 \pm 5.553$ pmol/mg, $P < 0.001$) (Tables 2 and 3).

Plasma levels of GST

GST levels were significantly higher in ACHBLF patients than those in CHB patients ($1,318.081 \pm 273.887$ vs. $666.832 \pm 181.267$ mIU/L, $P < 0.001$), but no significant
difference was found between methylated group and unmethylated group of ACHBLF patients (1,381.678 ± 236.841 vs. 1,262.253 ± 202.782 mIU/L, P = 0.170) (Tables 2 and 3).

Correlation of methylation status and oxidative stress with the severity of ACHBLF and its clinical value to predict the prognosis of ACHBLF

MELD scores were significantly higher in methylated group than those in unmethylated group of ACHBLF patients (22.706 ± 2.669 vs. 18.765 ± 4.808, P = 0.029) (Table 3). Eight patients died of ACHBLF (26.7%), among them 5 patients belonging to methylated group (55.6%) and 3 patients belonging to unmethylated group (14.3%). The mortality rate was significantly higher in methylated group than that in unmethylated group of ACHBLF patients (P = 0.032) (Table 3). MDA levels were positively correlated with MELD scores of ACHBLF (r = 0.588, P = 0.001) (Fig. 3), while we did not find any correlation of GST levels with MELD scores of ACHBLF (r = 0.115, P = 0.546) (Fig. 4).

Discussion

In this study, we firstly demonstrated the presence of GSTM3 gene promoter methylation in ACHBLF patients. Nine cases of 30 (30%) ACHBLF and 2 cases of 30 (6.7%) CHB patients displayed GSTM3 promoter methylation. The frequency of GSTM3 promoter methylation was significantly higher in ACHBLF than that in CHB patients (P = 0.020). The aberrant DNA hypermethylation of gene promoter regions is an important epigenetic mechanism that
regulates gene expression leading to down-regulation and silencing of several genes (Eads et al. 2001; Schulmann et al. 2005; Clement et al. 2006). In our study, the methylation of GSTM3 gene promoter may be associated with gene silencing. Because GSTM3 is an antioxidative enzyme, so the dysfunction of GSTM3 gene caused by promoter methylation may result in the dysfunction of cellular anti-oxidative system and contribute to oxidative stress-associated liver damage (Armstrong 1997). Impaired liver cells fail to correct the exogenous and endogenous oxidative stresses, as thus ROS can occur. In this case, the accumulation of ROS will produce oxidative DNA damage that plays an important role in DNA mutagenesis and cell death (Loft and Poulsen 1996; Jackson and Loeb 2001; Storz 2005). Long-term HBV infection can cause regional methylations of some gene promoters (Lee et al. 2005; Jung et al. 2007). DNA promoter methylation may exacerbate the pathogenic progress of HBV infection. Our result presented the higher frequency of GSTM3 promoter methylation in ACHBLF patients, just as GSTP1 in our previous study (Li et al. 2011). So the methylation of GSTM3 genes promoter may play a potential role in the pathogenesis of ACHBLF. Although we can not conclude that GSTM3 promoter methylation has more advantages than GSTP1, at least it can be used as a prognostic indicator in ACHBLF. As for which one is better in estimating the prognosis, we should make further clinical research.

The plasma levels of MDA as an important marker of oxidative stress were significantly higher in ACHBLF patients than those in CHB patients ($P = 0.011$). They were also significantly higher in methylated patients than those in unmethylated patients of ACHBLF ($P < 0.001$). These demonstrated the enhanced oxidative stress in ACHBLF patients in our present study. As an antioxidase, GST levels were significantly higher in ACHBLF patients than those in CHB patients ($P < 0.001$). Since GST can be rapidly released into circulation after hepatocellular injury, it also can be used as a more rapid, more specific and more sensitive indicator of liver injury than the determination of conventional liver enzymes (Schmidt et al. 1999; Suehiro et al. 2004). Advantages of GST in the detection of hepatocellular injury include its low molecular weight (51 kDa), high cytosolic concentration (4%-5% of all hepatocellular protein) and brief half-life in circulation (< 90 min) (Arslan et al. 2009). It was consist with our result that GST levels were obviously increased in ACHBLF patients ($P < 0.001$). But GST levels showed no significant difference between methylated group and unmethylated group of ACHBLF patients ($P = 0.170$). It may be due to the relative small sample size and need further investigation.

MSP is a relatively simple, sensitive, convenient and specific method to detect promoter methylation of certain genes. It has low demand for the quality and quantity of DNA and can be used for the detection of microscale DNA (Palmisano et al. 2000). As a molecular marker system, the use of aberrant gene methylation seems to offer a potentially powerful approach to population-based screening for the detection of human diseases. Therefore, detection of GSTM3 promoter methylation status by MSP in CHB patients is possible to help physicians discriminate individuals who possess the possibility of developing to ACHBLF.

Meanwhile, we found significant difference in MELD
scores between methylated group and unmethylated group of ACHBLF patients ($P = 0.029$), methylated group has higher mortality rate than unmethylated group ($P = 0.032$). MELD scoring system was a widely accepted and used method in predicting the prognosis of liver failure. It has now been validated as a predictor of survival in patients with a wide variety of liver diseases (Said et al. 2004). Our results strongly suggested that GSTM3 promoter methylation status might influence the severity and prognosis of ACHBLF. In our study we also found GSTM3 promoter methylated patients had significant higher TBIL levels and significant lower PTA levels than unmethylated ACHBLF patients ($P < 0.001$ and $P = 0.035$, respectively). TBIL and PTA are well-known prognostic indicators of chronic liver diseases. So increased GSTM3 promoter methylation in predicting the prognosis of ACHBLF might be consistent with the traditional methods of TBIL and PTA.

In our study, we also found that MDA levels were positively correlated with MELD scores of ACHBLF ($r = 0.588$, $P = 0.001$). MDA is a product of lipid peroxidation, which is widely validated as an oxidative stress parameter. Increased MDA levels in ACHBLF indicate that oxidative stress is increased in these patients. It suggests that oxidative stress is associated with the severity of ACHBLF. Oxidative DNA damage is enhanced in ACHBLF patients. MDA levels may be a prognostic indicator of the severity. It further implies that oxidative stress plays an important role in the pathogenesis of ACHBLF. HBV-induced oxidative stress may be enhanced in ACHBLF patients through increasing ROS production, lowering cellular antioxidant levels, thus may contribute to the inflammatory development of ACHBLF. According to our results, HBV can not only increase the generation of ROS, but also down-regulate the expression of certain antioxidant genes by promoter methylation such as GSTM3. Based on the above findings, we presume that the epigenetic aberrant methylation of GSTM3 gene promoter observed in ACHBLF cases may play a role in lipid peroxidation and facilitate oxidative injury to liver cells. As basic information regarding the role of oxidative stress in disease development and the mechanisms underlying ROS-related cellular toxicity continue to emerge, these findings will lead to more rational antioxidant therapeutic approaches and be of major therapeutic value in protecting the liver, especially for those with high MDA levels in plasma (Acar et al. 2009).

In our study, there are some limitations to improve. To the best of our knowledge, this is the first study to analyze the involvement of GSTM3 gene promoter methylation in ACHBLF. Although the case number is limited, it shows a certain scientific issues. In order to validate these problems, we should collect more samples to participate in this study. Thus, the exact mechanism of GSTM3 gene promoter methylation and oxidative stress in the prognosis of ACHBLF should be explored in a multi-center, large, perspective cohort. But taking into account the retrospective feature of this study, we just compared the difference of mortality between methylated group and unmethylated group. The present study suggests that GSTM3 gene promoter methylation could be used as prognostic indicators, although more clinical evidence and the specific study design are essential to be carried out in future research.

In conclusion, our results first demonstrated that GSTM3 gene promoter methylation existed in ACHBLF patients. Meanwhile, MDA, MELD scores and mortality rate were significantly higher in methylated group than those in unmethylated group of ACHBLF patients. MDA levels were positively correlated with MELD scores of ACHBLF. It is therefore conceivable that GSTM3 gene promoter methylation may contribute to oxidative stress-associated liver damage in ACHBLF and be correlated with the severity of ACHBLF. However, the molecular mechanism of GSTM3 gene promoter methylation and whether it is specific to ACHBLF remains for further investigation.

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Conflict of Interest

The authors have no conflict of interest.

References


