Hypermethylation of the N-Myc Downstream-Regulated Gene 2 Promoter in Peripheral Blood Mononuclear Cells is Associated with Liver Fibrosis in Chronic Hepatitis B

Xin-Yuan Liu,1 Yu-Chen Fan,1,2 Shuai Gao,1 Jing Zhao,1 Feng Li,1 Jun Zhang1 and Kai Wang1,2

1Department of Hepatology, Qilu Hospital of Shandong University, Jinan, Shandong, China
2Institute of Hepatology, Shandong University, Jinan, Shandong, China

DNA methylation is a fundamental epigenetic modification to regulate gene expression. N-Myc downstream-regulated gene (NDRG) 2 is a cytoplasmic protein and participates in the pathogenesis of liver fibrosis. In this study, the mRNA expression and methylation status of NDRG2 was evaluated in patients with chronic hepatitis B (CHB). The study included 143 CHB patients and 65 normal controls (NC). The mRNA expression of NDRG2 in peripheral blood mononuclear cells (PBMCs) was detected by quantitative real-time polymerase chain reaction. The methylation status of the NDRG2 promoter in PBMCs was detected by methylation-specific polymerase chain reaction. The NDRG2 mRNA level was lower in the CHB group than in the NC group (p < 0.001). Methylation frequency of the NDRG2 promoter was significantly higher in CHB patients than in the NC group (52.44% vs. 26.15%, p < 0.001). Importantly, the relative expression levels of NDRG2 mRNA were significantly lower in the methylated group than in the unmethylated group in both CHB patients and NC (p < 0.001). Furthermore, a lower mRNA level and hypermethylation of NDRG2 were associated with liver fibrosis and inflammation grade in CHB. The aspartate aminotransferase-to-platelet ratio index (APRI) score is widely used to predict liver fibrosis. The mRNA expression levels and methylation status of NDRG2 showed a better score compared to APRI for discriminating the severity of liver fibrosis. In conclusion, hypermethylation of NDRG2 in PBMCs was correlated with decreased mRNA expression and with liver fibrosis. The methylation status of the NDRG2 promoter in PBMCs is a potential noninvasive biomarker to predict the severity of liver fibrosis.

Keywords: chronic hepatitis B; inflammation; liver fibrosis; methylation; NDRG2

Introduction

Hepatitis B virus (HBV) infection is the leading cause of chronic liver disease worldwide, with nearly 350 million people infected. Particularly, HBV infection causes more than 500,000 deaths annually and is the 15th most common cause of death worldwide (Liaw and Chu 2009; Trepo et al. 2014). Persistent viral replication and repetitive hepatic injury will ultimately develop into cirrhosis, liver failure, portal hypertension, and hepatocellular carcinoma (McMahon 2009).

N-Myc downstream-regulated gene 2 (NDRG2) is a prominent member of the NDRG family, which includes four members, NDRG1-4. NDRG2 is a cytoplasmic protein that is down-regulated by Myc and is involved in cell growth and differentiation, stress, and hormonal responses (Boulkroun et al. 2002; Shen et al. 2008). NDRG2 is widely expressed in many tissues such as the liver, heart, and central nervous system (Okuda and Kondoh 1999; Okuda et al. 2008). NDRG2 is decreased in many types of tumor tissues and inhibits the growth, proliferation, and invasion of tumors. Therefore, NDRG2 is a candidate tumor suppressor gene (Lee et al. 2008; Furuta et al. 2010). NDRG2 is a potential regulator of liver fibrosis and can regulate liver regeneration via the cell cycle and apoptosis (Hu et al. 2004; Liu et al. 2007; Shon et al. 2009). Increased NDRG2 expression inhibits hepatic stellate cell (HSC) activation, promotes degradation of the extracellular matrix, and regulates regeneration of the liver (Wang et al. 2006; Yang et al. 2010; Yang et al. 2011). HSCs play an important role during the development of liver fibrosis and are regulated by multiple pathways and factors, primarily through transforming growth factor (TGF)-β1/Smad signaling (Dooley et al. 2001; Friedman 2004; Bataller and Brenner 2005; Inagaki and Okazaki 2007). A previous study found that NDRG2 inhibited basal and TGF-β1-
mediated HSCs activation by reducing Smad3 phosphorylation (Yang et al. 2011). Considering the independent role of NDRG2 in the TGF-β1/Smad signaling pathway in regulating fibrogenesis, we have hypothesized that NDRG2 is an important molecule involved in the progression of fibrosis caused by chronic HBV infection.

DNA methylation is a fundamental epigenetic modification of DNA that regulates the transcription and affects the development of common diseases (Egger et al. 2004; Feinberg 2008). Epigenetic mechanisms are regulated throughout life and served as an interphase between the genetic background and environmental influences (Murphy et al. 2013; Zeybel et al. 2015). NDRG2 methylation has been detected in colorectal cancer and gastric cancer (Ling et al. 2015; Hong et al. 2016). Whether NDRG2 methylation influences the transcription of NDRG2 and is related to the progression of liver fibrosis and inflammation in chronic hepatitis B (CHB) patients remains unclear.

In this study, we first evaluated the expression of NDRG2 mRNA in peripheral blood mononuclear cells (PBMCs) of CHB patients by quantitative real-time polymerase chain reaction (RT-qPCR). Next, we used methylation-specific polymerase chain reaction (MSP) to investigate the methylation status of NDRG2 in PBMCs and identified whether the methylation pattern affects the regulation of NDRG2 mRNA expression.

Methods

Patients and controls
One hundred forty-three patients with CHB and 65 normal controls (NC) participated in this study. All the CHB patients had undergone percutaneous liver biopsies in the Department of Hepatology, Qilu Hospital of Shandong University from January 2012 to May 2015. CHB patients were diagnosed as being positive for hepatitis B surface antigen for at least 6 months. A history of concomitant chronic hepatitis C, human immunodeficiency virus or autoimmune liver disease, alcoholic liver disease, nonalcoholic fatty liver disease, and other causes of chronic liver disease were exclusion criteria. All participants signed the written informed consent, and this study protocol was approved by the Ethics Committee of Qilu Hospital of Shandong University.

Plasma collection and peripheral blood mononuclear cells (PBMCs) isolation
Peripheral venous blood (10 mL) was obtained from each participant. EDTA was used as an anticoagulant. After centrifugation, plasma was collected and then stored at −80°C. Gradient centrifugation was used to isolate PBMCs via Ficoll-Paque (Pharmacia Diagnostics, Uppsala, Sweden) according to the manufacturer’s protocol. PBMCs were then stored at −20°C until use.

DNA and RNA extraction from PBMCs
DNA was extracted using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer’s protocol and stored at −20°C. The phenol-chloroform-isopropanol method was used to extract total RNA, which was resuspended in 20 μL of nuclease-free water.

Quantitative real-time polymerase chain reaction
PBMCs were lysed in 1 mL of Trizol reagent (Invitrogen, Carlsbad, CA, USA). PrimerScript™ RT Reagent Kit (Perfect Real Time; Takara, Japan) was used to convert total RNA into complementary DNA (cDNA). The conditions for this reaction were 70°C for 5 min for denaturation and 42°C for 60 min for reverse transcription. cDNA products were subjected to RT-qPCR to detect the level of NDRG2 mRNA. β-Actin was used as a control in each run. The primer sequences were obtained from previous studies (Lee et al. 2008). A volume of 10 μL in the RT-qPCR system included 0.2 μL each primer, 4.1 μL nuclease-free water, 5 μL 10 × SYBR Green (Toyobo, Osaka, Japan), and 0.5 μL cDNA. RT-qPCR was performed as follows: initial step at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, 58°C for 30 s, and 72°C for 30 s. Data were analyzed using LightCycler Software 2.0 (Roche Diagnostics, Basel, Switzerland). The results were determined using the comparative (2−ΔΔCt, ΔΔCt = Ct(NDRG2) − Ct(β-Actin)) method.

Sodium bisulfite modification and methylation-specific polymerase chain reaction
The EZ DNA Methylation-Gold Kit™ (Zymo Research, USA) was used to treat DNA according to the manufacturer’s instructions. A total of 20 μL modified DNA was obtained as a template for MSP or stored at −20°C. Methylated and unmethylated primers of NDRG2 for MSP were used to amplify the bisulfite-modified DNA. The MSP primer sequences were obtained from previous studies (Lee et al. 2008). The expected size of the PCR product was 132 bp (Table 1). The MSP-amplified region of NDRG2 ranged from sites −329 to −213 within a CpG island in the 5’ region of NDRG2. The methylation pattern of the NDRG2 promoter region is shown in Fig. 1. A mixture of 12.5 μL Premix Taq (Zymo Research, Irvine, CA, USA), 10.5 μL nuclease-free water, 0.5 μL of each primer, and 1 μL bisulfite-treated DNA was used in a 25-μL MSP reaction system. The PCR protocol involved an initial denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 40 s, and primer extension at 72°C for 40 s, finally, extension was conducted at 72°C for 10 min. The negative control contained water without DNA. Total PCR products (7 μL) were then electrophoresed on a 2% agarose gel, which was stained with GelRed and visualized under UV illumination.

Liver biopsy
All patients received ultrasound-guided liver biopsy by professional physicians in the Department of Hepatology, Qilu Hospital, Shandong University. The samples were stained with hematoxylin and eosin and Masson’s trichrome for light microscopic examination by pathologists. Scheuer’s classification system was used for histological grading of liver necro-inflammation activity (G0-G4) and staging of liver fibrosis (S0-S4) (Scheuer 1991).

Clinical features
Blood samples were obtained before liver biopsies. Clinico-pathological data were collected from the Department of Laboratory Medicine, Qilu Hospital, Shandong University, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), total bilirubin (TBIL), platelet count (PLT), prothrombin time activity (PTA), hepatitis B surface antigen, hepatitis B e antigen (HBeAg), hepatitis B e antibody, and HBV-DNA. Aspartate aminotransferase-to-platelet ratio index (APRI)
scores were calculated according to the formula: APRI = AST/ULN × 100/platelet (10^9/L) (Wai et al. 2003). Detailed information is shown in Table 2.

Statistical analysis

G*Power software 3.1 was used to calculate the sample size. The alpha value was 0.05 and statistical power was 80% (β = 0.2). All data were analyzed using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). Baseline characteristics of the participants were measured by Mann-Whitney U test and Chi-square test. Changes in mRNA concentration were measured by Mann-Whitney U test and one-way ANOVA. Chi-square test was used to analyze the methylation status among different inflammation and fibrosis levels. The ability of the NDRG2 methylation status, NDRG2 mRNA level or APRI score to predict fibrosis severity in CHB patients was assessed by receiver operating characteristic (ROC) curve analysis. P value < 0.05 was considered statistically significant.

Results

General characteristics

Our study included 143 CHB patients with liver puncture and 65 normal controls. The clinical and virological characteristics are shown in Table 2.

NDRG2 mRNA levels in PBMCs of CHB patients and normal controls

The mRNA level was measured in PBMCs by RT-qPCR assays. The results showed that the NDRG2 mRNA level was dramatically lower in the CHB group than in NC (p < 0.001). Next, linear correlation analysis and Mann-Whitney U test were performed. The NDRG2 mRNA levels showed a significant negative correlation with age (r = −0.244, p = 0.003). Significant correlations were observed between mRNA level and HBV-DNA (r = 0.232, p = 0.022). However, the NDRG2 mRNA level showed no significant relationship with sex, ATL, AST, TBIL, GGT, ALB, or HBeAg (Fig. 2a-j).

Methylation frequency of NDRG2 promoter in CHB patients and normal controls

The methylation frequency of the NDRG2 promoter in PBMCs was significantly increased in CHB patients compared with the NC group (52.44% vs 26.15%, p < 0.001,
Fig. 3a). Products of MSP are shown in Fig. 3c. However, no correlation was observed between the methylation of NDRG2 and age ($p = 0.160$), sex ($p = 0.951$), ALT ($p = 0.763$), AST ($p = 0.990$), GGT ($p = 0.920$), TBIL ($p = 0.760$), ALB ($p = 0.935$), HBeAg ($p = 0.90$), or HBV-DNA ($p = 0.150$).

Hypermethylation of NDRG2 promoter down-regulates NDRG2 mRNA expression

Considering that the alteration of promoter methylation status is one of the mechanisms influencing gene transcription, we compared the NDRG2 mRNA level in subjects with and without methylation. The results showed that the relative expression of NDRG2 mRNA was significantly decreased in methylated subjects compared to unmethylated subjects in both CHB group and NC group ($p < 0.001$, Fig. 3b). The data supported our hypothesis.

Correlation between NDRG2 promoter methylation and liver fibrosis progression

According to fibrosis levels, CHB patients were divided into 5 groups including S0, S1, S2, S3, and S4. We

<table>
<thead>
<tr>
<th>Variable</th>
<th>CHB group (n = 143)</th>
<th>NC group (n = 65)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>33.0 ± 8.6</td>
<td>31.3 ± 8.5</td>
<td>0.092*</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>95/48</td>
<td>40/25</td>
<td>0.974*</td>
</tr>
<tr>
<td>HBV-DNA(+/-)</td>
<td>98/45</td>
<td>0/65</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>HBsAg (+/-)</td>
<td>143/0</td>
<td>0/65</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>HBeAg (+/-)</td>
<td>82/61</td>
<td>0/65</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>HBeAb (+/-)</td>
<td>31/112</td>
<td>0/65</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>182.8 ± 286.5</td>
<td>17.3 ± 10.5</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>103.8 ± 155.1</td>
<td>15.2 ± 7.1</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>TBIL (μmol/l)</td>
<td>23.6 ± 28.9</td>
<td>10.7 ± 6.5</td>
<td>0.037*</td>
</tr>
<tr>
<td>ALB (g/l)</td>
<td>44.5 ± 4.6</td>
<td>43.5 ± 5.2</td>
<td>0.971*</td>
</tr>
<tr>
<td>GGT (U/l)</td>
<td>75.6 ± 94.0</td>
<td>30.1 ± 8.3</td>
<td>0.082*</td>
</tr>
<tr>
<td>PLT(10⁹/L)</td>
<td>160.2 ± 64.0</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

Liver fibrosis stage

<table>
<thead>
<tr>
<th>Stage</th>
<th>CHB</th>
<th>NC</th>
</tr>
</thead>
<tbody>
<tr>
<td>S0</td>
<td>43</td>
<td>NA</td>
</tr>
<tr>
<td>S1</td>
<td>51</td>
<td>NA</td>
</tr>
<tr>
<td>S2</td>
<td>22</td>
<td>NA</td>
</tr>
<tr>
<td>S3</td>
<td>15</td>
<td>NA</td>
</tr>
<tr>
<td>S4</td>
<td>12</td>
<td>NA</td>
</tr>
</tbody>
</table>

Inflammation grade

<table>
<thead>
<tr>
<th>Grade</th>
<th>CHB</th>
<th>NC</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0</td>
<td>19</td>
<td>NA</td>
</tr>
<tr>
<td>G1</td>
<td>76</td>
<td>NA</td>
</tr>
<tr>
<td>G2</td>
<td>36</td>
<td>NA</td>
</tr>
<tr>
<td>G3</td>
<td>9</td>
<td>NA</td>
</tr>
<tr>
<td>G4</td>
<td>3</td>
<td>NA</td>
</tr>
</tbody>
</table>

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HbsAg, hepatitis B surface antigen; HbeAg, hepatitis B e antigen; HbeAb, hepatitis B e antibody; TBIL, total bilirubin; CHB, chronic hepatitis B; HBV-DNA, Hepatitis B virus-DNA; M, male; F, female; ALB, albumin; CHB, chronic hepatitis B; NC, normal control; PLT, platelet count; GGT, gamma-glutamyl transpeptidase; NA, not applicable.

#Mann–Whitney U test; *Chi-square test.
NDRG2 Methylation in Chronic Hepatitis B

compared the NDRG2 mRNA levels among the S0, S1, S2, S3, and S4 groups. The data revealed significant differences between S0 and the other groups ($p < 0.001$), S1 and the other groups ($p < 0.001$), S2 and S3 ($p < 0.003$), and S2 and S4 ($p = 0.019$). The data suggest that the decreased NDRG2 mRNA level is related to liver fibrosis progression.

Next, we compared the NDRG2 promoter methylation status among the S0, S1, S2, S3, and S4 groups. There were significant differences between S0 and S1 ($p = 0.032$), S0 and S2 ($p < 0.001$), S0 and S3 ($p < 0.001$), S0 and S4 ($p < 0.001$), S1 and S2 ($p = 0.017$), S1 and S3 ($p = 0.007$), and S1 and S4 ($p = 0.023$) (Fig. 4a, b).

**Correlation between NDRG2 promoter methylation and liver inflammation in CHB patients**

We divided CHB patients into 3 groups including G0, G1, and G2+G3+G4 according to different inflammation grades. Next, we compared the NDRG2 mRNA level among different groups. Significant differences were observed between the NC group and G2+G3+G4 group ($p < 0.001$), G0 and G2+G3+G4 group ($p < 0.001$), and G1 group and G2+G3+G4 group ($p < 0.001$). Next, we compared the methylation frequency of NDRG2 in different groups, and significant differences were observed between the NC group and G2+G3+G4 group ($p < 0.001$), G0 and G2+G3+G4 group ($p = 0.018$), and G1 group and G2+G3+G4 group ($p < 0.001$) (Fig. 4c, d).

**Methylation of NDRG2 promoter is an independent predictor for assessing the severity of liver fibrosis in CHB patients**

To determine whether the methylation status of NDRG2 and NDRG2 mRNA level can be used to predict the severity of liver fibrosis, we performed multivariate logistic regression analysis. Mild fibrosis was considered as G < 2 and S < 2, while the severe group was G ≥ 2 or S ≥ 2. The results showed that methylation of the NDRG2 promoter and mRNA level were associated with fibrosis severity ($p = 0.03$, $p < 0.001$, respectively). We evaluated the diagnostic performance for advanced fibrosis (G ≥ 2 or S ≥ 2) to assess the clinical applicability of this test. The ROC curve for NDRG2 mRNA level, NDRG2 methylation,
APRI score were evaluated. The APRI score is a widely used non-invasive model to predict liver fibrosis. The areas under the ROC curve (AUC) were 0.846 (SE 0.040; 95%SI 0.768-0.924) for the NDRG2 mRNA level and 0.722 (SE 0.044; 95%SI 0.635-0.809) for NDRG2 methylation, which were higher than the APRI score of 0.706 (SE 0.045; 95%SI 0.617-0.795) (Fig. 5).

Discussion
In this study, we first determined NDRG2 mRNA expression and NDRG2 methylation status in CHB patients who underwent liver biopsy. We found lower expression level of NDRG2 mRNA and aberrant hypermethylation of NDRG2 in CHB patients compared to normal controls. With the progression of liver fibrosis stages and inflammation level, the methylation frequency increased gradually. Hypermethylation of NDRG2 promoter decreased gene transcription in CHB patients. Moreover, NDRG2 promoter methylation status and the NDRG2 mRNA level are predictive of the severity of liver fibrosis independently in CHB patients.

We first detected the NDRG2 mRNA level and methylation status of NDRG2 in patients at different liver fibrosis stages. We found that with the progression of liver fibrosis, mRNA expression was decreased. However, methylation frequency was increased. Therefore, methylation-induced promoter inactivation may be the primary cause of the frequent down-regulated NDRG2 mRNA expression in CHB patients.

NDRG2 plays a major role in liver fibrosis in the following four aspects: 1. by suppressing the activation of HSCs (Yang et al. 2011); 2. by regulating liver extracellular matrix degradation (Wang et al. 2006; Yang et al. 2011); 3. by regulating the growth of liver cells (Yang et al. 2010); and 4. through resistance to hypoxia stress damage (Corpechot et al. 2002; Wang et al. 2008). We found that both NDRG2 hypermethylation and down-regulated expression were correlated with liver fibrosis stages. Moreover, there were significant differences between early stages and severe stages. There was no significant difference between S3 and S4. In the S3 and S4 group, the mRNA level was maintained at a low level, which may result from the inactivation of a large number of HSCs. The variation in methylation was negatively related to mRNA expression in different stages, suggesting that hypermethylation may decrease NDRG2 transcription.

PBMCs are associated with inherent immunity and play an important role in CHB progression (Zhang et al. 2011). These cells are recruited into the liver and participate in liver fibrosis. Thus, PBMCs provide a non-invasive approach for studying liver fibrosis. In our study, PBMCs were also found to be related to inflammation, indicating that NDRG2 is not only related to HSC activation, but also inflammation. The prevalence of HBV infection is of great concern worldwide. It has been reported that 8-20% untreated patients will develop cirrhosis within 5 years.
Additionally, untreated patients with decompensated cirrhosis have a poor prognosis, and the 5-year survival rate is only 14-35% (Liaw et al. 2008). Thus, accurate determination of fibrosis degree is required for doctors to manage this disease. APRI score was initially derived from patients with chronic hepatitis C and subsequently widely adopted to help rationalize liver biopsy for CHB patients (Wai et al. 2003). For diagnosis, we found that the status of NDRG2 mRNA level and methylation status performed significantly better than APRI score in determining the severity of liver fibrosis.

The present study has some limitations. First, we did not detect the correlation between NDRG2 and different sites in CHB patients. We will further evaluate the function of NDRG2. In addition, intrahepatic methylation is unclear, and we will detect the methylation status and gene expression in liver tissues in the future. Moreover, NDRG2 promoter methylation was also found in some normal controls, challenging the specificity of our results. We will perform sequencing to determine the exact methylated and unmethylated loci within the amplified region.

In conclusion, our study demonstrated that aberrant
NDRG2 methylation occurred in the PBMCs of CHB patients, which was associated with transcriptional repression of this gene. Moreover, the expression of NDRG2 mRNA and promoter methylation performed significantly better than APRI score in discriminating the severity of liver fibrosis and is a potential noninvasive biomarker for predicting liver fibrosis.

Acknowledgments
This work was supported by grants from the Key Project of Chinese Ministry of Science and Technology (2012ZX10002007, 2013ZX10002001), National Natural Science Foundation of China (81371832), Science and Technology Development Plan of Shandong Province (2015GSF118145), and the Fundamental Research Funds of Shandong University-Clinical Research Project of Qilu Hospital (2014QLKY11).

Conflict of Interest
All authors declare no conflict of interest.

References


