Methylation status of the stimulator of interferon genes promoter in patients with chronic hepatitis B

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Abstract

The stimulator of interferon genes (STING) plays a crucial role in the recognition of a viral infection and subsequent stimulation of an immune response. However, it is unclear whether methylation of the STING promoter affects STING transcription and response to antiviral therapy. The present study determined the methylation status of the STING promoter in patients with chronic hepatitis B (CHB).

This study included 198 participants, of which 159 participants had CHB and 39 were healthy controls (HCs). Methylation-specific polymerase chain reaction was performed to detect the methylation status of the STING promoter. Reverse transcription-quantitative polymerase chain reaction was performed to determine STING mRNA level in peripheral blood mononuclear cells. The methylation frequency of the STING promoter was significantly higher and STING mRNA level was lower in the patients with CHB than in the HCs. Presence of hepatitis B virus (HBV) DNA was independently correlated with an increased risk of STING promoter methylation. Virological response frequency was higher in the patients with CHB having methylated STING promoters than in those having unmethylated STING promoters. However, there was no significant difference in the virological response frequency between ADV-treated patients having methylated and unmethylated STING promoters. These results indicate that the hypermethylation of the STING promoter and thus the transcriptional repression of STING weaken the effect of STING in inhibiting HBV replication and decreases the effectiveness of antiviral therapy.

Abbreviations: ADV = adefovir, AKP = alkaline phosphatase, ALB = albumin, ALT = alanine aminotransferase, AST = aspartate aminotransferase, BUN = blood urea nitrogen, CHB = chronic hepatitis B, Cr = creatine, DNMTs = DNA methyltransferases, ETV = entecavir, GGT = y-glutamyl transferase, HBeAg = hepatitis B e antigen, HBsAg = hepatitis B surface antigen, HBV = hepatitis B virus, HCs = healthy controls, MSP = methylation-specific polymerase chain reaction, PBMCs = peripheral blood mononuclear cells, PT = prothrombin time, PTA = prothrombin activity, RT-qPCR = reverse transcription-quantitative polymerase chain reaction, STING = the stimulator of interferon genes, TBL = total bilirubin.

Keywords: antiviral therapy, chronic hepatitis B, DNA methylation, hepatitis B virus DNA, the stimulator of interferon genes

1. Introduction

Hepatitis B virus (HBV) infection is an important global public health problem affecting approximately 2 billion people.[1]

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Approximately 240 million people are chronically infected with HBV worldwide[2,3], hepatitis B and its associated complications, such as cirrhosis and hepatocellular carcinoma, account for 686,000 deaths annually.[4] An immune response induced during the course of HBV infection promotes hepatic injury.[4] HBV-infected hepatocytes function as target cells and are essential players in inducing host immune response.[5] However, it is unclear why patients with chronic hepatitis B (CHB) show an immunocompromised status.

The stimulator of interferon genes (STING) plays a crucial role in the recognition of a viral infection and subsequent stimulation of an immune response. Patients with CHB show the persistent presence of HBV in their hepatocytes or sera.[6,7] STING contributes to the induction of an early immune response against HBV infection, which may help in more effective clearance of the virus by host.[8] Some studies have suggested that cyclic GMP-AMP synthase (cGAS)-STING pathway plays a role in inhibiting HBV replication in hepatocytes.[9,10] Furthermore, STING activation in liver-resident immune regulatory cells and hepatocytes suppresses HBV replication.[11] One study has reported that STING deficiency in hepatocytes promotes HBV infection.[12] Epigenetics defines all heritable changes in gene expression occurring during meiosis and mitosis without altering the DNA sequence. DNA methylation is an epigenetic modification that regulates the transcription of genes and affects the development of common diseases.[13,14] DNA methylation involves the
addition of a methyl group to the cytosine residue in CpG dinucleotides and leads to gene silencing if it occurs in the promoter region of a gene.15 Hypermethylation of CpG dinucleotides in gene promoters interferes with gene transcription and down-regulates gene expression.16,17 Patients with CHB do not express appropriate levels of STING.18 Therefore, it is reasonable to suspect that the STING promoter is hypermethylated in patients with CHB. However, it is unclear whether the methylation of the STING promoter affects STING transcription and is associated with response to antiviral therapy in patients with CHB.

At present, bisulfite modification and methylation-specific polymerase chain reaction (MSP) are commonly used methods for detecting DNA methylation.19 In the present study, we performed MSP to investigate the methylation status of the STING promoter in peripheral blood mononuclear cells (PBMCs) of patients with CHB and healthy controls (HCs). Moreover, we performed reverse transcription-quantitative polymerase chain reaction (RT-qPCR) to assess STING mRNA expression in the PBMCs of the patients with CHB and HCs. Furthermore, we examined whether the methylation status of the STING promoter affected the regulation of STING mRNA expression and response to antiviral therapy.

2. Methods

2.1. Study population

The study enrolled 218 participants, of which 179 participants had CHB, which was confirmed by performing clinical and laboratory evaluations, and 39 participants were HCs. Patients with CHB were enrolled according to the practice guidelines for managing CHB established in the 2018 update of the American Association for the Study of Liver Diseases. Patients were diagnosed with CHB based on the presence of HBsAg for at least 6 months and at elevated alanine aminotransferase (ALT) levels.20 All the patients with CHB had increased serum ALT levels (>40 U/L). These patients visited the Department of Hepatology, Qilu Hospital of Shandong University, from January 2016 to January 2018. However, 20 patients with CHB were excluded from the study because they had co-existing hepatocellular carcinoma (14 patients), fatty liver disease (4 patients), and hepatitis C infection (2 patients). Thus, the present study included 159 patients with CHB. Of these, 83 patients received antiviral therapy and 76 patients did not receive antiviral treatment. Of the 83 patients who received the antiviral therapy, were previously treated for 1 to 6, 12 to 24, and >24 months, respectively. Written informed consent was obtained from all the study participants in accordance with the Declaration of Helsinki,21 and study protocols were approved by the local research and ethics committee of the Qilu Hospital of Shandong University.

2.2. Genomic DNA extraction and bisulfite conversion

Genomic DNA was extracted from the PBMCs of the study participants by using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer’s protocol, and was stored at −20°C until further use for performing bisulfite conversion. The concentration and purity of the extracted DNA were measured using Eppendorf Biophotometer (Brinkmann Instruments, Westbury, NY). Bisulfite conversion was performed by treating the genomic DNA with EZ DNA Methylation-Gold Kit (Zymo Research Corp, Orange, CA), according to the manufacturer’s instructions. Finally, 20 µL modified DNA was immediately used as a template for MSP or was stored at −20°C.

2.3. Methylation-specific polymerase chain reaction

The stimulator of interferon genes promoter-specific methylated and unmethylated primers were designed using MethPrimer according to recommended criteria.22 The sequences of these primers are as follows: methylated forward primer: 5′-TTAGGTTGGAGTGAATGTACC-3′, methylated reverse primer: 5′-AAATTAATAAAGTAAATACAGCA-3′, unmethylated forward primer: 5′-TTAGGTGGAGTGAATGTATGTA-3′, and unmethylated reverse primer: 5′-AAAAATTAACATACATAAAACATAC-3′. Amplification was performed in a 25-µL reaction mixture containing 1 µL bisulfite-modified DNA, 0.5 µL of each primer (10 µM), 10.5 µL nuclease-free water, and 12.5 µL Premix Taq (Zymo Research Corp). PCR was performed using the following conditions: initial denaturation at 95°C for 10 minutes; 40 cycles of denaturation at 94°C for 30 seconds, annealing at 60.5°C for methylated STING primers) or 58°C (for unmethylated STING primers) for 30 seconds, primer extension at 72°C for 30 seconds; and final extension at 72°C for 10 minutes. Water without the modified DNA was used as a negative control. PCR products were electrophoresed on 2% agarose gels stained with Gelred (Biotium, CA) and visualized under UV illumination. Each reaction was replicated 3 times.

2.4. RNA extraction from PBMCs and RT-qPCR

Total RNA was extracted from the PBMCs of the study participants by using phenol-chloroform-isopropanol method, according to a recommended protocol, and was suspended in 20 µL RNase-free water. Next, cDNA was synthesized using a first-strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania), according to the manufacturer’s instructions. RT-qPCR to measure STING mRNA level was performed using SYBR Green (Toyobo, Osaka, Japan) and Agilent Technologies Stratagene Mx3005P instrument (Stratagene, La Jolla, CA), with the β-actin gene as an internal control and the following condition: initial denaturation at 95°C for 30 seconds, followed by 45 cycles of denaturation at 95°C for 5 seconds, annealing at 60°C for 30 seconds, and a final extension at 72°C for 30 seconds. The RT-qPCR was performed using the following primers: STING forward: 5′-GAGATCTCTCTGACTGTTGAA-3′, STING reverse: 5′-GCGCAGATATCCGATGTA-3′, β-actin forward: 5′-CACAGATGCAATGCCGTC-3′, and β-actin reverse 5′-AGTCTTGTCCGATGTCACGT-3′. Each reaction was conducted in triplicate, and mRNA expression was evaluated using a comparative (2−ΔΔCT) method.

2.5. Clinical features of the study participants

The levels of serum biochemical markers, including aspartate aminotransferase (AST), ALT, γ-glutamyl transferase (GGT), alkaline phosphatase (AKP), total bilirubin (TBIL), albumin (ALB), blood urea nitrogen (BUN), and creatinine (Cr), were determined using COBAS Integra 800 (Roche Diagnostics, Penzberg, Germany). Serum HBV DNA load was determined using ABI 7300 PCR System (Applied Biosystems, Foster City, CA), and HBsAg and HBeAg levels were determined using COBAS 6000 analyzer series (Roche Diagnostics, Basel, Switzerland). Prothrombin time (PT) and prothrombin activity (PTA) were quantified using ACL TOP 700 (Instrument Laboratory, Lexington, MA). All
clinical features were determined at the Department of Laboratory Medicine, Qilu Hospital of Shandong University.

2.6. Statistical analysis
Quantitative variables are expressed as median (centile 25; centile 75), and categorical variables are expressed as number (%). All the data were analyzed using SPSS 21 software (SPSS, Inc., Chicago, IL). The baseline characteristics of the participants were assessed using Mann–Whitney U test and chi-square test. The relationship between the methylation status of the STING promoter and the clinical features of the patients with CHB was investigated using multivariate logistic regression analysis. Univariate and multivariate logistic regression analyses were used to investigate the relationship of the virological response of the patients with CHB with clinical features, STING promoter methylation status, and drug treatment. Spearman correlation was used to analyze the association between the quantitative and categorical variables. Biochemical response was defined as the normalization of serum ALT level. Serological response was defined as the loss of HBeAg and seroconversion to anti-HBe in patients with HBeAg-positive CHB infection or loss of HBsAg and seroconversion to anti-HBs. Virological response was defined as the absence of serum HBV DNA during therapy or reduction in serum HBV DNA level to >1 log IU/mL after 24 weeks of oral antiviral therapy in adherent patients. All the responses to antiviral therapy were recorded after 12 weeks of therapy.[2]

3. Results
3.1. General characteristics of the study participants
This study included 159 patients with CHB and 39 HCs. The basic demographic and clinical characteristics of the study participants are shown in Table 1. Significant differences were observed between the patients with CHB and the HCs with respect to ALT (P < .001), AST (P < .001), GGT (P < .001), AKP (P = .001), TBIL (P = .003), ALB (P < .001), BUN (P = .003), and Cr (P = .001) levels; PT (P < .001); and PTA (P < .001). However, no difference was observed between the patients with CHB and the HCs with respect to gender (P = .297) and age (P = .230).

3.2. Methylation status of the STING promoter in the patients with CHB and HCs
The methylation status of the STING promoter was determined by performing MSP. Hypermethylated STING promoter was detected in the PBMCs of 111 out of 159 (69.81%) patients with CHB and 9 out of 39 (23.08%) HCs. The methylation frequency of the STING promoter was significantly higher in the patients with CHB than in the HCs (P < .001; Fig. 1A). STING promoter methylation was detected in 64 of the 83 patients with CHB who received the antiviral therapy and in 47 of the 76 patients with CHB who did not receive the antiviral therapy. Moreover, the methylation frequency of the STING promoter was higher in the patients who received the antiviral therapy than in those who did not receive the antiviral therapy (P = .036; Fig. 1B). However, no significant difference in the methylation frequency of the STING promoter was observed between entecavir (ETV) and adefovir (ADV)-treated patients with CHB and among patients with a history of antiviral therapy (Fig. 1C and D). Fig. 1E shows a typical representative MSP assay of STING promoter methylation.

3.3. Correlation between the methylation of the STING promoter and the clinical features of patients with CHB
Table 2 shows the association between the methylation of the STING promoter and the clinical features of the patients with CHB.

| Table 1 |
| --- |
| General characteristics of the enrolled participants. |
| **Variables** | **CHB (n=159)** | **HCs (n=39)** | **P** |
| Gender (M/F) | 126/33 | 28/11 | .297<sup>a</sup> |
| Age (years) | 45.00 (35.00–53.00) | 42.00 (55.50–46.00) | .230<sup>a</sup> |
| HBsAg (+/–) | 153/6 | NA | — |
| HBeAg (+/–) | 93/66 | NA | — |
| HBV DNA (+/–) | 134/25 | NA | — |
| ALT (IU/L) | 66.00 (28.00–214.00) | 23.00 (16.00–28.00) | <.001<sup>b</sup> |
| AST (IU/L) | 51.00 (27.00–121.00) | 26.00 (19.00–30.00) | <.001<sup>b</sup> |
| GGT (IU/L) | 56.00 (26.00–121.00) | 32.00 (20.00–39.00) | <.001<sup>b</sup> |
| AKP (IU/L) | 88.00 (71.00–119.00) | 78.00 (68.00–85.50) | .001<sup>b</sup> |
| TBIL (µmol/L) | 18.70 (11.60–50.90) | 15.20 (9.70–16.75) | .003<sup>b</sup> |
| ALB (g/L) | 42.80 (37.70–46.60) | 47.70 (45.40–50.25) | <.001<sup>a</sup> |
| SUN (µmol/L) | 4.06 (3.55–5.41) | 5.80 (3.80–6.60) | .003<sup>a</sup> |
| Cr (mg/dL) | 65.00 (55.00–73.00) | 71.00 (62.50–82.50) | .001<sup>b</sup> |
| PT (s) | 12.40 (11.16–13.80) | 10.70 (10.10–11.40) | <.001<sup>b</sup> |
| PTA (%) | 78.00 (67.00–86.00) | 94.00 (88.00–101.50) | <.001<sup>b</sup> |
| Methylation, n (%) | 111 (69.81) | 9 (23.08) | <.001<sup>b</sup> |
| Antiviral therapy, Y/N | 83/76 | NA | — |
| Antiviral drugs, ETV (N)/ADV (N) | 51/32 | NA | — |
| Antiviral history | | | |
| 1–6 mos (N)/8–12 mos (N)/12–24 mos (N)/24 m (N) | 22/32/16/13 | NA | — |

Categorical variables are expressed as number (%). Data are shown as median (centile 25; centile 75). ADV = adefovir, AKP = alkaline phosphatase, ALB = albumin, ALT = alanine aminotransferase, AST = aspartate aminotransferase, BUN = blood urea nitrogen, Cr = creatine, CHB = chronic hepatitis B, ETV = entecavir, F = female, GGT = γ-glutamyl transferase, HBsAg = hepatitis B e antigen, HBeAg = hepatitis B surface antigen, HCs = healthy controls, M = male, N = without antiviral therapy, NA = not available, PT = prothrombin time, PTA = prothrombin activity, TBIL = total bilirubin, Y = with antiviral therapy.

<sup>a</sup> Chi-square test.
<sup>b</sup> Mann–Whitney U test.
Figure 1. (A) The methylation frequency of the STING promoter in the PBMCs of the patients with CHB and the HCs; ***P < .001. (B) The methylation frequency of the STING promoter in the patients treated with or without the antiviral therapy; *P < .05. (C and D) The methylation frequency of the STING promoter in the patients receiving the different antiviral drugs and with a different antiviral treatment history. (E) A representative image showing the methylation of the STING promoter by performing MSP. A 50-bp marker was used. CHB = chronic hepatitis B, HCs = healthy controls, M = methylated sequence, MSP = methylation-specific polymerase chain reaction, NC = negative control, PBMCs = peripheral blood mononuclear cells, STING = the stimulator of interferon genes, U = unmethylated sequence.

Table 2

| Parameters               | Methylated (n = 111) | Unmethylated (n = 48) | P     |
|--------------------------|----------------------|-----------------------|-------|
| Sex (MF)                 | 87/24                | 39/9                  | .682a |
| Age (y)                  | 45.00 (35.50–53.00)  | 43.50 (35.00–52.00)   | .910b |
| HbsAg (+/−)              | 105/6                | 47/1                  | .349a |
| HBeAg (+/−)              | 68/43                | 26/22                 | .403a |
| Hbv DNA (+/−)            | 96/13                | 36/12                 | .035b |
| ALT (U/L)                | 90.00 (28.00–266.00) | 45.50 (29.50–77.00)   | .040b |
| AST (U/L)                | 60.00 (51.00–139.00) | 35.50 (26.75–76.75)   | .043b |
| GGT (U/L)                | 62.00 (30.00–144.00) | 41.00 (24.00–105.50)  | .035b |
| TBIL (µmol/L)            | 95.00 (72.00–123.00) | 79.00 (67.50–102.75)  | .030b |
| ALB (g/L)                | 42.15 (36.18–45.73)  | 43.80 (39.95–46.95)   | .085b |
| BUN (µmol/L)             | 3.98 (3.41–5.36)     | 4.12 (3.60–5.80)      | 642b  |
| Cr (mg/dL)               | 65.00 (64.00–71.00)  | 65.00 (66.00–76.00)   | .320b |
| PT (s)                   | 12.45 (11.63–14.28)  | 12.10 (11.50–13.30)   | .424b |
| PTA (%)                  | 78.00 (75.00–85.00)  | 78.00 (71.50–85.00)   | .882b |

Data are shown as median (centile 25; centile 75).
Categorical variables are expressed as number (%).

| STING methylation status in PBMCs |
|-----------------------------------|

- ALP = alkaline phosphatase, ALB = albumin, ALT = alanine aminotransferase, AST = aspartate aminotransferase, BUN = blood urea nitrogen, CHB = chronic hepatitis B, Cr = creatine, F = female, GGT = γ-glutamyl transferase, HBeAg = hepatitis B e antigen, HBsAg = hepatitis B surface antigen, M = male, NA = not available, PBMCs = peripheral blood mononuclear cells, PT = prothrombin time, PTA = prothrombin activity, STING = the stimulator of interferon genes, U = unmethylated sequence.

a Chi-square test.
b Mann–Whitney U test.
CHB. STING promoter methylation was significantly correlated with HBV DNA (P = .035), ALT (P = .040), AST (P = .043), GGT (P = .035), and AKP (P = .030) levels. HBV DNA positivity was higher in the patients with CHB having methylated STING promoters than in those having unmethylated STING promoters (P = .035). However, no correlation was observed between STING promoter methylation and sex; age; HBsAg, TBIL, ALB, BUN, and Cr levels; PT; and PTA (P > .05 for all).

Results of multivariate logistic regression analysis indicated that only HBV DNA was independently correlated with an increased risk of STING promoter methylation (P = .027), whereas sex; age; HBsAg, ALT, AST, GGT, AKP, TBIL, ALB, BUN, and Cr levels; PT; or PTA were not correlated with an increased risk of STING promoter methylation (Table 3).

### 3.4. STING mRNA level in PBMCs

The STING mRNA level was evidently lower in the patients with CHB than in the HCs (P < .001; Fig. 2A). Meanwhile, STING mRNA level was significantly lower in both the patients with CHB (P = .023; Fig. 2B) and HCs (P < .001; Fig. 2C) having methylated STING promoters than in those having unmethylated STING promoters. Moreover, STING mRNA level was lower in the patients showing HBV DNA positivity than in those showing HBV DNA negativity (P = .046; Fig. 2D). However, no obvious difference in STING mRNA level was observed between the patients showing HBsAg positivity and negativity (Fig. 2E and F, respectively). The methylation status of the STING promoter was negatively correlated with the STING mRNA level in patients with CHB (r = –0.204, P = .010). We also analyzed the correlation between the STING mRNA level and the clinical features of the patients with CHB to determine the possible correlation between STING promoter methylation and CHB severity. No evident correlation was observed between the STING mRNA level and HBsAg level (r = 0.101, P = .203); HBsAg level (r = 0.075, P = .350); AST, GGT, AKP, TBIL, and ALB levels; PT; and PTA. However, the STING mRNA level was negatively correlated with ALT level (r = –0.218, P = .006) and HBV DNA (r = –0.159, P = .046), and was positively correlated with BUN (r = 0.214, P = .017) and Cr level (r = 0.191, P = .033) (Fig. 2G–P).

### 3.5. Response of the patients with CHB to antiviral therapy

Because some patients missed their follow-up visits, the outcomes of only 63 patients with CHB (41 ETV-treated patients and 22 ADV-treated patients) were used to evaluate responses to the antiviral therapy. A marked difference in the virological response was observed between the ETV and ADV-treated patients with CHB (P = .023; Table 4); however, no difference was observed in the biochemical and serological responses of these patients. The virological response frequency was higher in the ETV-treated patients than in the ADV-treated patients (P = .023). However, the virological response frequency was lower in the ETV-treated patients with methylated STING promoters than in those with unmethylated STING promoters (P = .046). However, no significant difference in the virological response frequency was observed between the ADV-treated patients with methylated and unmethylated STING promoters (P = .127). Moreover, no significant difference in the virological response frequency was observed in the 63 patients with CHB with methylated and unmethylated STING promoters (P = .464; Fig. 3).

Results of the univariate analysis showed no association between the virological response and clinical characteristics of the patients with CHB, except for ALT (P = .011) and AST (P = .012) levels (Table 5). Multivariate logistic regression analysis was performed to assess the association of virological response with some clinical characteristics of the patients with CHB (P < .2), STING mRNA level, STING promoter methylation status, and antiviral therapy. Results of the multivariate logistic regression analysis showed that only ALT level (P = .038) and antiviral therapy (P = .011) were associated with the virological response (Table 6).

Univariate analysis of the ETV-treated patients with CHB showed no association between the clinical characteristics of these patients and virological response (Table 7). Therefore, ALT and AST levels (P < .2), STING mRNA level, and STING promoter methylation status were used to perform multivariate logistic regression analysis. Results of this analysis showed that only STING promoter methylation status (P = .027) was associated with and that ALT level (P = .991), AST level (P = .912), and STING mRNA level (P = .329) were not associated with the virological response. The number of ADV-treated patients with CHB was very less to perform univariate and multivariable logistic regression analyses for assessing the association of the clinical characteristics, STING mRNA level, STING promoter methylation status, and antiviral therapy with virological response in these patients.

### 4. Discussion

To our knowledge, this is the first study to investigate the methylation status of the STING promoter in patients with CHB. In the present study, we found that the methylation frequency of the STING promoter was higher in the patients with CHB than in the HCs. Moreover, we found that the methylation frequency of the STING promoter was higher in the patients with CHB receiving antiviral therapy than in those not receiving the antiviral therapy. The STING mRNA level was lower in the
patients with CHB than in the HCs. Moreover, the STING mRNA level was lower in the patients with CHB having methylated STING promoters than in those having unmethylated STING promoters. Furthermore, we found that the methylation status of the STING promoter was negatively correlated with the STING mRNA level. HBV DNA positivity was evidently higher in the patients with CHB having methylated STING promoters than in those having unmethylated STING promoters; moreover, presence of HBV DNA was correlated with an increased risk of

![Figure 2.](image)

**Table 4**

| Variables                  | ETV (n=41) | ADV (n=22) | P   |
|----------------------------|------------|------------|-----|
| Biochemical response (R/N) | 22/19      | 18/4       | .640* |
| Serological response (R/N) | 4/37       | 3/19       | .190* |
| Virological response (R/N) | 32/9       | 11/11      | .023* |

ADV = adefovir, CHB = chronic hepatitis B, ETV = entecavir, N = not responsive, R = responsive.

*Chi-square test.
STING promoter methylation. In addition, HBV DNA positivity was negatively correlated with the STING mRNA level. The virological response was higher in the ETV-treated patients with CHB than in ADV-treated patients with CHB. Among the ETV-treated patients, the virological response frequency was lower in the patients having methylated STING promoters than in those having unmethylated STING promoters. Therefore, we concluded that STING methylation suppressed the effect of STING in inhibiting HBV replication and thus could be a potential therapeutic target for treating patients with CHB.

Hepatitis B virus is an enveloped double-stranded DNA virus, and a complex interaction between the host immune system and the replicating virus leads to HBV infection.\(^{[23,24]}\) Cytoplasmic DNA activates cGAS to produce cGAMP, which binds to STING to induce type I interferons.\(^{[26,27]}\) A recent study reported that STING agonists inhibited HBV replication and thus could be a potential therapeutic target for treating patients with CHB.

A previous study suggested that some therapies influence DNA methylation status.\(^{[28]}\) Similarly, the present study showed that the methylation frequency of the STING promoter was higher in the patients receiving the antiviral therapy than in those not receiving the antiviral therapy. However, no difference in the methylation frequency of the STING promoter was observed between the ETV and ADV-treated patients with CHB. Therefore, we hypothesized that these antiviral drugs increased the DNA methylation status by themselves or by affecting HBV DNA replication. Interestingly, we found that the methylation frequency of the STING promoter increased with time; however, the hypermethylation of the STING promoter reduced STING mRNA expression by decreasing gene transcription.\(^{[29,30]}\)

### Table 5

Univariate analysis of clinicopathological parameters with virological responsive in patients with CHB.

| Variables      | Coefficient | OR  | 95% CI    | P      |
|----------------|-------------|-----|-----------|--------|
| Sex            | -0.098      | 0.907 | 0.246–3.357 | .883   |
| Age            | 0.031       | 1.032 | 0.984–1.081 | .195   |
| HBeAg          | 0.871       | 2.389 | 0.142–40.315 | .546   |
| ALT            | 0.017       | 0.983 | 0.971–0.996 | .011   |
| AST            | 0.020       | 0.980 | 0.965–0.996 | .012   |
| GGT            | -0.002      | 0.998 | 0.993–1.003 | .420   |
| AKP            | -0.011      | 0.989 | 0.975–1.004 | .145   |
| ALB            | -0.013      | 0.987 | 0.972–1.001 | .077   |
| BUN            | 0.012       | 1.010 | 0.984–1.011 | .751   |
| Cr             | 0.001       | 1.001 | 0.967–1.035 | .972   |
| PT             | -0.016      | 0.984 | 0.818–1.183 | .862   |
| PTA            | 0.008       | 1.008 | 0.972–1.045 | .682   |

ARF = alkaline phosphatase, ALB = albumin, ALT = alanine aminotransferase, AST = aspartate aminotransferase, BUN = blood urea nitrogen, CHB = chronic hepatitis B, CI = confidence interval, Cr = creatine, GGT = γ-glutamyl transferase, HBsAg = hepatitis B surface antigen, HBeAg = hepatitis B e antigen, OR = odds rate, PT = prothrombin time, PTA = prothrombin activity, TBL = total bilirubin.

### Table 6

Multivariate logistic regression analysis in patients with CHB.

| Variables        | Coefficient | OR  | 95% CI    | P      |
|------------------|-------------|-----|-----------|--------|
| Age              | -0.007      | 0.993 | 0.918–1.075 | .888   |
| HBeAg            | -1.567      | 0.205 | 0.019–27.56 | .203   |
| ALT              | -0.038      | 0.963 | 0.920–0.998 | .038   |
| AST              | 0.005       | 1.005 | 0.972–1.040 | .760   |
| AKP              | 0.019       | 1.019 | 0.994–1.045 | .135   |
| Methylated status| -1.438      | 0.237 | 0.028–2.040 | .237   |
| HBV DNA level    | 50.091      | 5.680E+21 | 0.000–523858 | .105   |
| Antiviral therapy| 3.300       | 27.106 | 1.081–8750 | .195   |

ARF = alkaline phosphatase, ALT = alanine aminotransferase, AST = aspartate aminotransferase, CHB = chronic hepatitis B, CI = confidence interval, HBsAg = hepatitis B surface antigen, OR = odds rate, PT = prothrombin activity, TBL = total bilirubin.
We observed that the patients with CHB having promoter (evidently associated with the methylation status of the STING studies, the results of the present study suggest that ETV = GGT present study, STING promoter methylation was significantly associated with the virological response frequency in the patients with CHB having methylated STING promoters. Thus, the hypermethylation of the STING promoter can be used to develop potential new prevention and treatment strategies for patients with CHB, especially for patients who are sensitive to ETV therapy.

### Author contributions

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