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***Retrospective Study***

**Demethylation of tumor necrosis factor-α converting enzyme promoter associated with high hepatitis B e antigen level in chronic hepatitis B**

Wang ZL *et al.* HBeAg level in CHB

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**Informed consent:** All study participants provided informed written consent prior to study enrollment.

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**Data sharing:** Technical appendix, statistical code, and data set available from the corresponding author atwangdoc876@126.com. Participants gave informed consent for data sharing.

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**Abstract**

**AIM:** To evaluate tumor necrosis factor-α converting enzyme (TACE) methylation status in patients with chronic hepatitis B (CHB).

**METHODS:** Eighty patients with hepatitis B e antigen (HBeAg)-positive CHB, 80 with HBeAg-negative CHB, and 40 healthy controls (HCs) were randomly enrolled in this study. Genomic DNA was extracted from peripheral blood mononuclear cells and methylation status of TACE promoter was determined by methylation-specific polymerase chain reaction. The clinical and laboratory parameters were collected.

**RESULTS:** One hundred and thirty of 160 patients with CHB (81.25%) and 38 of 40 HCs (95%) displayed TACE promoter methylation. The difference was significant (*χ*2 = 4.501, *P* < 0.05). TACE promoter methylation frequency in HBeAg-positive CHB (58/80, 72.5%) was significantly lower than in HBeAg-negative CHB (72/80, 90%; *χ*2 = 8.041, *P* < 0.01) and HCs (*χ*2 = 8.438, *P* < 0.01). However, no significant difference was observed in the methylation frequency between HBeAg-negative CHB and HCs (*χ*2 = 0.873, *P* > 0.05). In the HBeAg-positive group, TACE methylation frequency was significantly negatively correlated with HBeAg (*r* = -0.602, *P* < 0.01), alanine aminotransferase (*r* = -0.461, *P* < 0.01) and aspartate aminotransferase (*r* = –0.329, *P* < 0.01).

**CONCLUSION:** Patients with HBeAg-positive CHB have aberrant demethylation of TACE promoter, which might potentially serve as a biomarker for HBeAg seroconversion.

**Key words:** Tumor necrosis factor-α converting enzyme; Methylation; Chronic hepatitis B; Methylation-specific polymerase chain reaction; Biomarker

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**Core tip:** We retrospectively recruited 80 patients with hepatitis B e antigen (HBeAg)-positive chronic hepatitis B (CHB), 80 with HBeAg-negative CHB, and 40 healthy controls. We evaluated tumor necrosis factor-α converting enzyme (TACE) promoter methylation status in peripheral blood mononuclear cells and analyzed the association between TACE methylation status and clinical features. Aberrant demethylation of TACE promoter in HBeAg-positive CHB was associated with high HBeAg, alanine aminotransferase and aspartate aminotransferase levels. These findings imply that demethylation of TACE promoter might potentially serve as a biomarker for HBeAg seroconversion.

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**INTRODUCTION**

With geographical variations, hepatitis B virus (HBV) has chronically infected > 350 million people throughout the world and results in 500000 to 700000 deaths annually[1,2]. China has an estimated 120 million people chronically infected with HBV[3]. Nearly 15%–40% of HBV carriers will progress to life-threatening complications including liver cirrhosis[4], decompensated liver disease[5], or hepatocellular carcinoma (HCC)[6,7]. Hepatitis B e antigen (HBeAg) seroconversion is a crucial step during the progression of HBV infection. However, there has been no sensitive and effective model for predicting the occurrence of HBeAg seroconversion until now.

DNA methylation is one of the most important epigenetic mechanisms, which donates the addition of a methyl group to DNA. It is widespread in the human genome and mainly occurs at cytosine adjacent to guanine (CpG dinucleotides)[8]. DNA methylation in gene promoter regions often results in long-term silencing of gene expression[9]. Meanwhile, aberrant promoter demethylation is usually associated with overexpression of genes that might participate in pathogenesis of many diseases[10]. Previous studies demonstrated that aberrant demethylation in promoter region of genes occurs in many diseases and might be used as biomarkers[11, 12].

Tumor necrosis factor (TNF)-α-converting enzyme (TACE) is a modular transmembrane protein with a zinc-dependent catalytic domain[13].The main function of TACE is to mediate cleavage of substrates such as TNF-α, TNF receptor type I and type II[13-15]. It is well documented that the overexpression of TACE is associated with several human diseases, including liver cancer. The human TACE promoter has not been definitely characterized to date. In the present study, we recognized the region within 2000 bp upstream of the transcriptional initiation site as a presumptive promoter region for TACE gene, as reported previously[16-18]. GenBank indicates that there are four CpG islands located on the TACE promoter sequence (Figure 1). Therefore, aberrant methylation of TACE gene promoter may occur in patients with chronic hepatitis B (CHB) and may be associated with its disease progression.

In this study, we determined the methylation status of TACE promoter in patients with HBeAg-positive CHB or HBeAg-negative CHB and healthy controls (HCs), and evaluated the relationship between TACE promoter methylation status and clinical features.

**MATERIALS AND METHODS**

***Patients and controls***

Eighty patients with HBeAg-positive CHB, 80 with HBeAg-negative CHB and 40 HCs were randomly enrolled from July 2012 to June 2014 at the Department of Hepatology, Qilu Hospital of Shandong University, China. Chronic HBV infection was defined as the presence of hepatitis B surface antigen (HBsAg) for > 6 mo prior to the beginning of this study[2]. The HCs were recruited from among eligible blood donors, who had no history of liver diseases. Patients were excluded if they met any of the following criteria: (1) co-infection with hepatitis C virus or HIV; (2) other liver diseases such as autoimmune hepatitis and alcoholic hepatitis; (3) receiving antioxidant agent or interferon therapy; (4) pregnancy; and (5) or complication with decompensated liver disease or HCC. Prior to sample collection, informed consent was obtained from each participant and the study was approved by the local Ethics Committee of Qilu Hospital of Shandong University.

***Peripheral blood mononuclear cells isolation and DNA extraction***

Peripheral blood mononuclear cells (PBMCs) were isolated using gradient centrifugation via Ficoll-Paque (Pharmacia Diagnostics, Uppsala, Sweden) and stored at –20 °C until use. Genomic DNA was extracted from PBMCs using QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, United States) and stored at –20 °C.

***Sodium bisulfite modification and methylation-specific polymerase chain reaction***

Extracted DNA was treated with sodium bisulfite using an EZ DNA methylation kit (Zymo Research, Orange, CA, United States) and then used to perform methylation-specific polymerase chain reaction (MSP). Methylated and unmethylated primers specific for *TACE* promoters were designed using MethPrimer (Table 1)[19]. There were four CpG islands in the promoter region of the TACE gene. The reasons for choosing this area included: (1) the primers contained at least one CpG site at the most 3’-end; (2) the primers in M pair and *U* pair contained the same CpG sites within their sequence; and (3) two sets of primers had similar product Tm values, which were 68.1 °C for M pair and 67.3 °C for *U* pair[18]. The selected primer sets were used to amplify the bisulfite-modified DNA in our study. The M pair primers amplified the –1831 to –1686 site of the 5’-UTR of the *TACE* gene (+1 for the transcriptional start site). Meanwhile, the U pair primers amplified the –1832 to –1686 site of the 5’-UTR of the TACE gene (Figure 1). MSP was performed in a total volume of 25 μL containing 1 μL bisulfite-treated DNA, 0.5 μL of each primer (10 μmol/L), 10.5 μL nuclease-free water, and 12.5 μL Premix Taq (Zymo Research, Irvine, CA, United States), which consisted of Taq DNA polymerase, reaction buffer, and dNTP mixture. The PCR protocol was composed of an initial denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 40 s, and primer extension at 72 °C for 40 s; followed by final extension at 72 °C for 10 min. Water blank without DNA was used as in each round of PCR. PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and visualized under UV illumination.

***Clinicopathological data collection***

Fasting venous blood was collected from each subject. HBeAg was detected by an automatic analyzer (Cobas 6000; Roche Diagnostics, Basel, Switzerland). HBV DNA level was measured by a real-time PCR system (ABI 7300; Applied Biosystems, Foster City, CA, United States). The serum biochemical markers (Cobas Integra 800; Roche Diagnostics, Mannheim, Germany) included alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), albumin and creatinine. Hemostasis markers (ACL TOP 700, Instrument Laboratory, Lexington, MA, United States) included prothrombin time-international normalized ratio (PT-INR), and prothrombin time activity (PTA). These markers were measured using standard methodologies in the Department of Laboratory Medicine, Qilu Hospital, Shandong University.

***Statistical analysis***

Quantitative variables were expressed as the mean (centile 25; centile 75). Categorical variables were expressed as number (percentage). Statistical analyses of the data were performed with SPSS version 16.0 (Chicago, IL, United States). Categorical variables were compared by *χ*2 test. Quantitative variables were compared by Student’s *t* test or Mann–Whitney *U* test. Spearman correlation coefficients were calculated to evaluate correlations between TACE methylation status and clinicopathological parameters. All statistical analyses were two-sided and *P* < 0.05 was considered statistically significant. The statistical methods of this study were reviewed by Dr. Shu-Mei Wang from Department of Epidemiology and Biostatistics, School of Public Health, Shandong University.

**RESULTS**

***Basic characteristics***

From June 2012 to July 2014, 269 participants (112 HBeAg-positive CHB patients, 117 HBeAg-negative CHB patients, and 40 HCs) were screened in the Department of Hepatology, Qilu Hospital. Six HBeAg-positive CHB patients and four HBeAg-negative patients were excluded for not giving consent to the study. Twenty-six HBeAg-positive CHB patients were excluded for co-infection with other liver diseases (*n =* 20) and incomplete clinical data (*n =* 6). Thirty-three HBeAg-negative CHB patients were excluded for co-infection with other liver diseases (*n =* 25) and incomplete clinical data (*n =* 8), which left 200 participants. Within the enrolled participants, there were 80 patients in the HBeAg-positive group, 80 in the HBeAg-negative group, and 40 in HC group (Figure 2). The basic characteristics of the enrolled subjects are summarized in Table 2.

***Methylation status of TACE promoter in CHB and HCs***

Methylation status of TACE promoter was detected in PBMCs of all participants (Figure 3). One hundred and thirty of 160 patients with CHB (81.25%) and 38 of 40 HCs (95%) displayed aberrant TACE promoter methylation and the difference was significant (*χ*2 = 4.501, *P* < 0.05). TACE promoter methylation frequency in the HBeAg-positive CHB group (58/80, 72.5%) was significantly lower than in the HBeAg-negative CHB (72/80, 90%; *χ*2 = 8.041, *P* < 0.01) and HC (*χ*2 = 8.438, *P* < 0.01) groups. However, no significant difference could be observed in the methylation frequency between the HBeAg-negative CHB and HC (*χ*2 = 0.873, *P* > 0.05) groups.

***Correlation between******TACE promoter methylation and clinicopathological features***

In the HBeAg-positive CHB group, there was a negative correlation between TACE promoter methylation and HBeAg (*r* = –0.602, *P* < 0.01), ALT (*r* = –0.461, *P* < 0.01) and AST (*r* = –0.329, *P* < 0.01) (Figure 4). There was no significant correlation between TACE promoter methylation and sex (*r* = 0.011, *P* = 0.922), age (*r* = 0.119, *P* = 0.294), log10 HBV DNA (*r* = 0.203, *P* = 0.07), albumin (*r* = –0.045, *P* = 0.689), TBIL (*r* = –0.128, *P* = 0.258), PT-INR (*r* = 0.027, *P* = 0.810), PTA (*r* = –0.138, *P* = 0.223) and creatinine (*r* = 0.079, *P* = 0.484).

In the HBeAg-negative CHB group, there was no significant correlation between TACE promoter methylation and sex (*r* = –0.009, *P* = 0.934), age (*r* = 0.023, *P* = 0.842), HBeAg (*r* = 0.076, *P* = 0.500), log10 HBV DNA (*r* = 0.060, *P* = 0.596), ALT (*r* = –0.111, *P* = 0.327), AST (*r* = –0.073, *P* = 0.519), albumin (*r* = 0.016, *P* = 0.886), TBIL (*r* = –0.144, *P* = 0.201), PT-INR (*r* = –0.195, *P* = 0.084), PTA (*r* = 0.156, *P* = 0.167) and creatinine (*r* = –0.023, *P* = 0.836).

**DISCUSSION**

We investigated the methylation status of TACE gene in PBMCs of 80 patients with HBeAg-positive CHB, 80 patients with HBeAg-negative CHB, and 40 HCs. TACE promoter methylation frequency in HBeAg-positive CHB patients (58/80, 72.5%) was significantly lower than that in HBeAg-negative CHB patients (72/80, 90%; *χ*2 = 8.041, *P* < 0.01) and HCs (*χ*2 = 8.438, *P* < 0.01). However, no significant difference could be observed in the methylation frequency between the HBeAg-negative CHB patients and HCs (*χ*2 = 0.873, *P* > 0.05). In the HBeAg-positive group, the TACE methylation frequency was significantly negatively correlated with HBeAg (*r* = –0.602, *P* < 0.01), ALT (*r* = –0.461, *P* < 0.01) and AST (*r* = –0.329, *P* < 0.01).

The prevalence of hepatitis B is a major concern worldwide. Approximately one-third of the global population has serological evidence of past or present infection with HBV, among which 350–400 million people are chronic HBsAg carriers. Once chronically infected, the covalently closed circular DNA of HBV is hard to eradicate from the nucleus of hepatocytes[20,21]. People with hepatitis B are at an increased risk of developing hepatic decompensation, cirrhosis, and HCC[1]. HBeAg seroconversion is a crucial step during the progression of HBV infection. Until now, no sensitive and effective model for predicting the occurrence of HBeAg seroconversion has been proposed.

DNA methylation and demethylation are important epigenetic mechanisms. Demethylation of promoter regions often links to long-term gene overexpression and is associated with many diseases[22-24]. DNA methylation status is usually stable and suitable for use as a biomarker for disease detection and prognosis prediction[25]. The present study revealed that TACE methylation frequency was significantly lower in HBeAg-positive CHB than in HBeAg-negative CHB and HCs. Also, TACE methylation frequency was negatively correlated with serum ALT and AST. Aberrant TACE methylation status might participate in the progression of HBV infection and induced liver damage.

Most importantly, this study revealed that TACE demethylation was significantly associated with HBeAg level in HBeAg-positive CHB, which indicates that it might possess a potential value for predicting HBeAg seroconversion. Further studies are needed to prove its usefulness. The prediction of HBeAg seroconversion is essential for the management of HBV infection. This finding might help clinicians initiate the correct treatment strategy at an early stage and prevent many patients from developing fatal complications such as hepatic decompensation, cirrhosis or HCC.

There were several limitations in this study. The sample size was relatively small and we cannot confirm our results in a second cohort. Therefore, our findings need further validation with large studies prior to clinical application, and the follow-up of the HBeAg-positive CHB patients should also be performed in a further study. Prospective studies with long-term follow-up would be useful for proving the predictive values for HBeAg seroconversion. This is an exploratory study and further studies are needed to reveal the mechanisms involved.

In conclusion, this study demonstrated demethylation of TACE promoter in HBeAg-positive CHB, which was associated with HBeAg level. It might potentially serve as a marker for HBeAg seroconversion.

**COMMENTS**

***Background***

Hepatitis B e antigen (HBeAg) seroconversion is a crucial step during the progression of hepatitis B virus (HBV) infection. Nevertheless, there have been no sensitive and effective markers for predicting the occurrence of HBeAg seroconversion until now. Tumor necrosis factor (TNF)-α converting enzyme (TACE) have been demonstrated to be involved in liver inflammation. GenBank indicates that there are four CpG islands located on the TACE promoter sequence. However, the methylation status of TACE promoter in chronic hepatitis B (CHB) and its significance for HBeAg seroconversion have not been demonstrated.

***Research frontiers***

HBeAg seroconversion is a crucial step during the progression of HBV infection. The current research hotspot is to find effective biomarkers for predicting the occurrence of HBeAg seroconversion. DNA methylation is one of the most important epigenetic mechanisms. Aberrant methylation or demethylation of several genes is associated with many diseases and many methylation biomarkers have been proposed.

***Innovations and breakthroughs***

This study demonstrated aberrant demethylation of TACE promoter in HBeAg-positive CHB, which was associated with high HBeAg level, and indicated that TACE demethylation might potentially serve as a biomarker for HBeAg seroconversion.

***Applications***

Aberrant demethylation of TACE promoter might be used as a biomarker for predicting the occurrence of HBeAg seroconversion.

***Terminology***

TACE is a modular transmembrane protein with a zinc-dependent catalytic domain. Its main function is to mediate cleavage of substrates like TNF-α, TNF receptor I and TNF receptor II. DNA methylation is one of the most important epigenetic mechanisms, which donates a methyl group to DNA. A biomarker is a substance used as an indicator of a biological state.

***Peer-review***

This manuscript presented some useful data showing that aberrant demethylation of TACE promoter existed in HBeAg-positive CHB, which was associated with high HBeAg level. This might potentially serve as a biomarker for HBeAg seroconversion. The research design, and the data analysis and statistics are reasonable.

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**L-Editor: E-Editor:**

**Table 1** **Primers for methylation-specific polymerase chain reactionof the tumor necrosis factor-α converting enzymegene**

|  |  |  |  |
| --- | --- | --- | --- |
| **Primer name** | **Primer sequence (5’-3’)** | **Product size (bp)** | **Annealing temp (°C)** |
| M | F: *GGAGTTTGAGATTAGTTTGGTTAAC* | 146 | 56 |
|  | R: *TAAACACCTCCTAAATTTAAACGAT* |  |  |
| U | F: *AGGAGTTTGAGATTAGTTTGGTTAATG* | 147 | 56 |
|  | R: *TAAACACCTCCTAAATTTAAACAAT* |  |  |

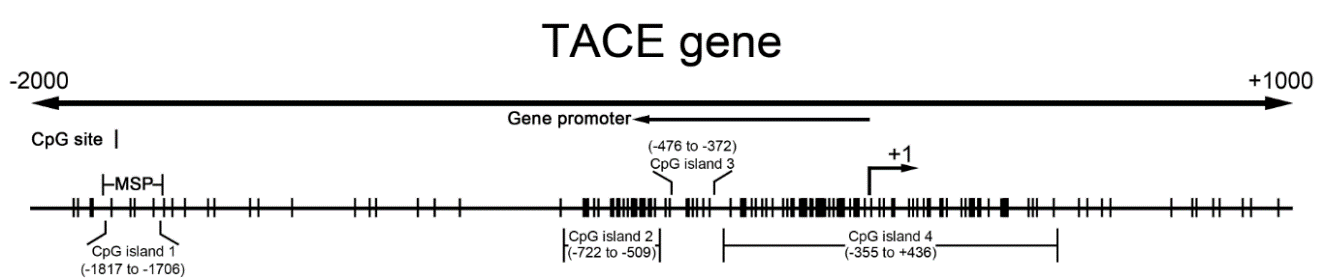
M: Methylated sequence; U: Unmethylated sequence; F: Forward; R: Reverse.

**Table 2 Baseline characteristics of the enrolled participants**

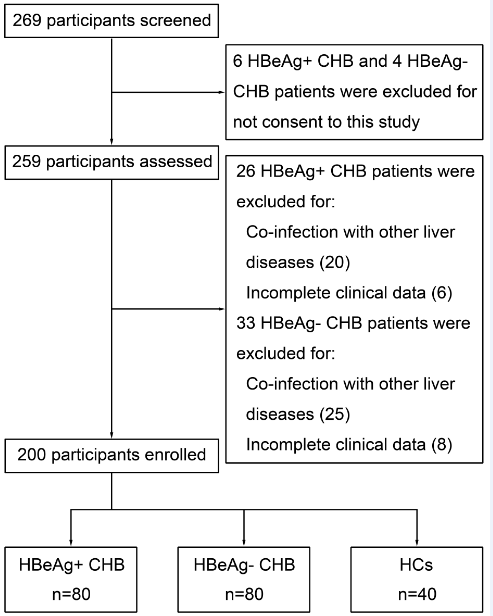
|  |  |  |  |
| --- | --- | --- | --- |
| Variable | HBeAg+ CHB group | HBeAg- CHB group | HC group |
| No. | 80 | 80 | 40 |
| Age (yr) | 41.50 (33.25-51.0) | 45.00 (33.25-52.0) | 36.50 (31.0-44.50) |
| Male sex, *n* (%) | 57 (71.25) | 53 (66.25) | 28 (70.00) |
| HBeAg | 132.85 (24.30-512.12) | NA | NA |
| Log10(HBV DNA) | 5.23 (3.90-6.41) | 3.08 (0.0-5.43) | NA |
| ALT (U/L) | 189.50 (78.25-441.75) | 91.50(44.0-236.50) | NA |
| AST (U/L) | 106.0 (46.0-287.75) | 56.50 (30.0-108.75) | NA |
| TBIL (μmol/L) | 16.95 (11.10-20.0) | 18.75 (15.00-21.18) | NA |
| ALB (g/L) | 44.85 (42.83-48.48) | 45.56 (42.05-49.38) | NA |
| Cr (μmol/L) | 63.0 (53.0-75.50) | 64.00 (56.0-77.75) | NA |
| PT-INR | 1.02 (0.99-1.09) | 1.01 (0.96-1.05) | NA |
| PTA (%) | 81.50 (71.25-93.0) | 87.0 (80.0-102.0) | NA |
| Methylation, *n* (%) | 58 (72.5) | 72 (90) | 38 (95) |

Quantitative variables were expressed as the median (centile 25; centile 75). Categorical variables were expressed as number (percentage). HBeAg: Hepatitis B e antigen; +: Positive; -: Negative; CHB: Chronic hepatitis B; HC: Healthy control; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TBIL: Total bilirubin; ALB: Albumin; Cr: Creatinine; PT-INR: Prothrombin time-international normalized ratio; PTA: Prothrombin activity; No: Number; NA: Not available.

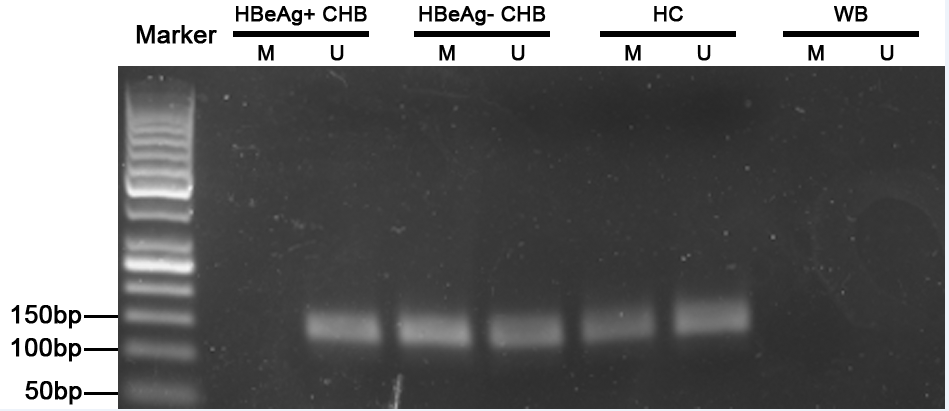
**Figure 1 Gene structure and methylation-specific polymerase primer set for *TACE* gene.**TACE: tumor necrosis factor-α converting enzyme.



**Figure 2 Flow diagram depicting the participant selection process.**



**Figure 3** **Typical methylation-specific polymerase analysis results of *TACE* gene promoter.** M: Methylated sequence; U: Unmethylated sequence; WB: Water blank.



**Figure 4 Comparison of hepatitis B e antigen, alanine alanine aminotransferase, aspartate aminotransferase between hepatitis B e antigen -positive chronic hepatitis B patients with methylated and unmethylated tumor necrosis factor-α converting enzyme promoters.** Significant difference (b*P* < 0.05, Methylated *vs* Unmethylated). HBeAg: Hepatitis B e antigen.

