

Retrospective Study

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

Zhen-Li Wang, Shuai Gao, Xin-You Li, Feng-Kai Sun, Feng Li, Yu-Chen Fan, Kai Wang

Zhen-Li Wang, Shuai Gao, Xin-You Li, Feng-Kai Sun, Feng Li, Yu-Chen Fan, Kai Wang, Department of Hepatology, Qilu Hospital of Shandong University, Jinan 250012, Shandong Province, China

Yu-Chen Fan, Kai Wang, Institute of Hepatology, Shandong University, Jinan 250012, Shandong Province, China

Author contributions: Wang ZL and Gao S performed the majority of the experiments and wrote the manuscript; Li XY and Li F collected all the human material and revised the manuscript; Sun FK and Fan YC acquired the data and performed statistical analysis; Wang K designed and supervised the study and revised the manuscript.

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Correspondence to: Kai Wang, MD, PhD, Department of Hepatology, Qilu Hospital of Shandong University, No. 107, Wenhuxi Road, Jinan 250012, Shandong Province, China. wangdoc876@126.com
Telephone: +86-531-82169596
Fax: +86-531-86927544

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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

($\chi^2 = 4.501, P < 0.05$). TACE promoter methylation frequency in HBeAg-positive CHB (58/80, 72.5%) was significantly lower than that in HBeAg-negative CHB (72/80, 90%; $\chi^2 = 8.041, P < 0.01$) and HCs ($\chi^2 = 8.438, P < 0.01$). However, no significant difference was observed in the methylation frequency between HBeAg-negative CHB and HCs ($\chi^2 = 0.873, P > 0.05$). 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 the TACE promoter, which may 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 the TACE promoter in HBeAg-positive CHB was associated with high HBeAg, alanine aminotransferase and aspartate aminotransferase levels. These findings imply that demethylation of the TACE promoter may potentially serve as a biomarker for HBeAg seroconversion.

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INTRODUCTION

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 denotes 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 the promoter region of genes occurs in many diseases and may be used as a biomarker^[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 the 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 demethylation of the TACE gene promoter may occur in patients with chronic hepatitis B (CHB) and may be associated with disease progression.

In this study, we determined the methylation status of the TACE promoter in patients with HBeAg-positive CHB, 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; (5) decompensated liver disease or HCC. Prior to sample collection, informed consent was obtained from each participant and the

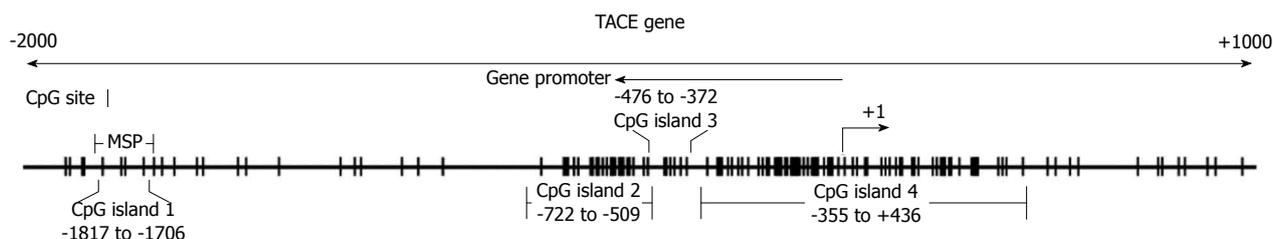


Figure 1 Gene structure and methylation-specific polymerase primer set for *TACE* gene. TACE: Tumor necrosis factor- α converting enzyme.

Table 1 Primers for methylation-specific polymerase chain reaction of the tumor necrosis factor- α converting enzyme gene

Primer name	Primer sequence (5'-3')	Product size (bp)	Annealing temperature (°C)
M	F: GGAGTTTGAGATTAGTTGGTTAAC R: TAAACACCTCCTAAATTTAAACGAT	146	56
U	F: AGGAGTTTGAGATTAGTTGGTTAATG R: TAAACACCTCCTAAATTTAAACAAT	147	56

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

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 3'-end; (2) the primers in the M pair and U pair contained the same CpG sites within their sequence; and (3) two sets of primers had similar product T_m values, which were 68.1°C for the M pair and 67.3°C for the 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 $25\ \mu\text{L}$ containing $1\ \mu\text{L}$ bisulfite-treated DNA, $0.5\ \mu\text{L}$ of each primer ($10\ \mu\text{mol/L}$), $10.5\ \mu\text{L}$ nuclease-free water, and $12.5\ \mu\text{L}$ 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 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). The 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, China.

Statistical analysis

Quantitative variables are expressed as the mean (24^{th} - 75^{th} percentiles). Categorical variables are expressed as number (percentage). Statistical analyses were performed with SPSS version 16.0 (SPSS Inc., 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

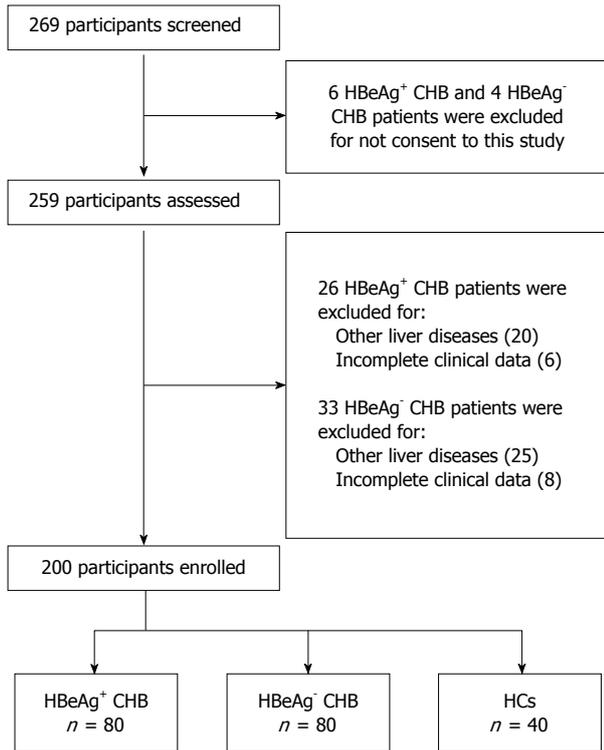


Figure 2 Flow diagram of the participant selection process. HBeAg: Hepatitis B e antigen; CHB: Chronic hepatitis B; HC: Healthy control.

Table 2 Baseline characteristics of the enrolled participants

Variable	HBeAg ⁺ CHB group	HBeAg ⁻ CHB group	HC group
Number	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, <i>n</i> (%)	57 (71.25)	53 (66.25)	28 (70.00)
HBeAg	132.85 (24.30-512.12)	NA	NA
Log ₁₀ (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, <i>n</i> (%)	58 (72.5)	72 (90)	38 (95)

Quantitative variables are expressed as the median (25th-75th percentiles). Categorical variables are expressed as number (percentage). ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TBIL: Total bilirubin; ALB: Albumin; Cr: Creatinine; PT-INR: Prothrombin time-international normalized ratio; PTA: Prothrombin activity; NA: Not available.

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 at 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 because of other liver diseases ($n = 20$) or incomplete clinical data ($n = 6$). Thirty-three HBeAg-negative CHB patients were excluded because of other liver diseases ($n = 25$) or incomplete clinical data ($n = 8$), leaving 200 participants. In the enrolled participants, there were 80 patients in the HBeAg-positive group, 80 in the HBeAg-negative group, and 40 in the 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

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

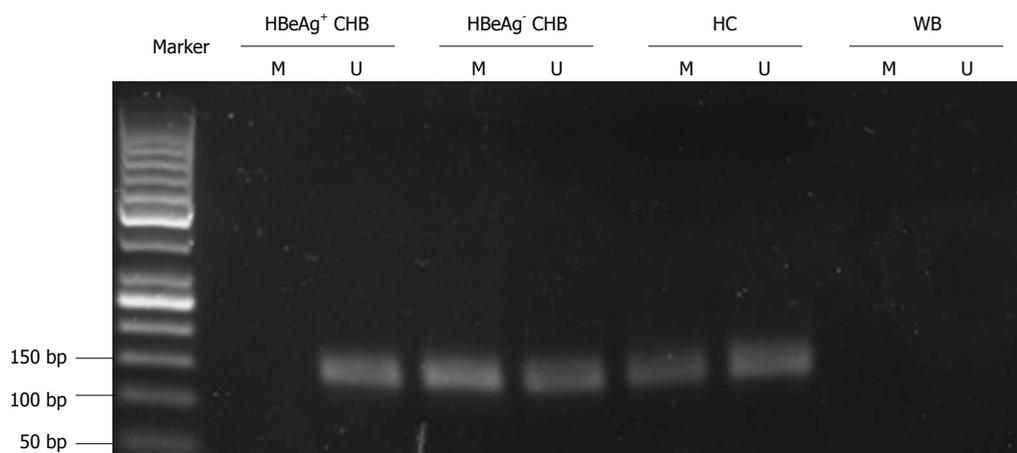


Figure 3 Typical methylation-specific polymerase analysis results of the *TACE* gene promoter. WB: Water blank.

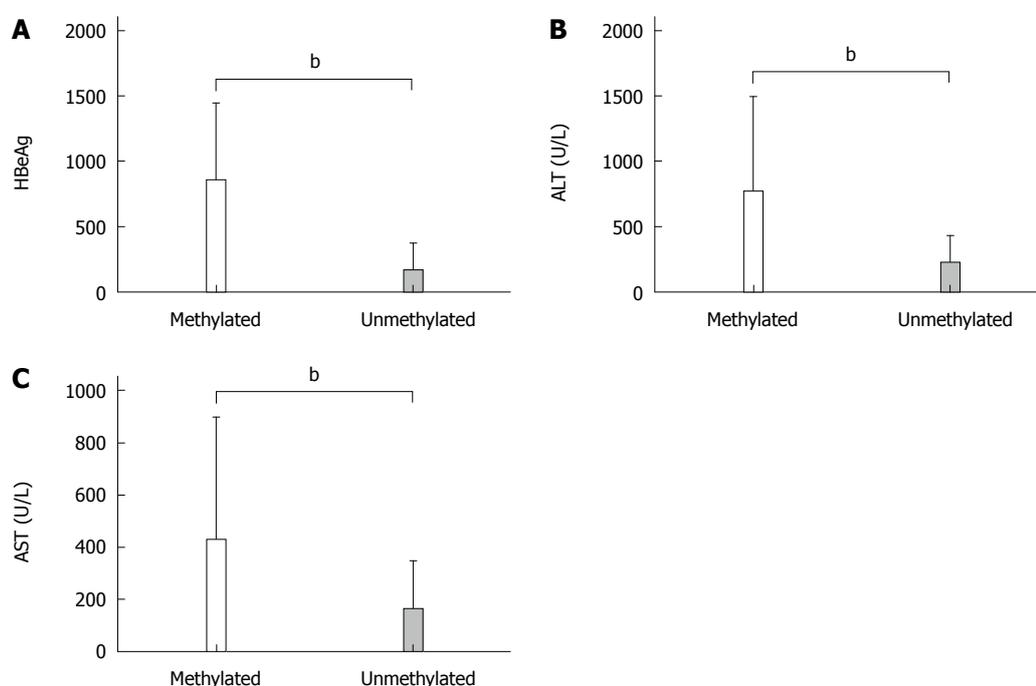


Figure 4 Comparison of HBeAg, ALT, and AST between HBeAg-positive chronic hepatitis B patients with methylated and unmethylated *TACE* promoters. Significant difference (^b $P < 0.05$, methylated vs unmethylated).

-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 the *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%; $\chi^2 = 8.041$, $P < 0.01$) and HCs ($\chi^2 = 8.438$, $P < 0.01$). 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, and 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 a promoter region is often associated with long-term gene over-expression and is linked to 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 the TACE methylation frequency was significantly lower in HBeAg-positive CHB than in HBeAg-negative CHB and HCs. Also, the TACE methylation frequency was negatively correlated with serum ALT and AST. Aberrant TACE methylation status may have a role in the progression of HBV infection and induce liver damage.

Most importantly, this study revealed that TACE demethylation was significantly associated with the HBeAg level in HBeAg-positive CHB, which indicated 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 could not 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 determining 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 the TACE promoter in HBeAg-positive CHB, which was associated with HBeAg level. TACE promoter demethylation may 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 the 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 has been associated with a number of diseases and many methylation biomarkers have been proposed.

Innovations and breakthroughs

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

Applications

Aberrant demethylation of TACE promoter may 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 such as 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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