Enhanced Demethylation of Interferon-γ Gene Promoter in Peripheral Blood Mononuclear Cells Is Associated with Acute-on-Chronic Hepatitis B Liver Failure

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Acute-on-chronic hepatitis B liver failure (ACHBLF) refers to liver failure occurring in patients with chronic hepatitis B (CHB) related liver diseases. Interferon-γ (IFN-γ) plays an important role in the exacerbation of liver function. However, the exact mechanism, by which IFN-γ mediates ACHBLF, is not fully understood. Forty patients with ACHBLF, fifteen patients with CHB and ten healthy controls were included in this present study. ELISA was performed to measure the level of serum IFN-γ. The methylation status of IFN-γ promoter in peripheral blood mononuclear cells (PBMCs) was determined using methylation-specific PCR. Model for End-stage Liver Disease (MELD) scoring was performed for evaluating the severity of liver failure. The serum level of IFN-γ in patients with ACHBLF or CHB was significantly lower than that in healthy controls, while the serum IFN-γ level in ACHBLF patients was significantly higher than that in CHB patients. In ACHBLF patients, the level of IFN-γ was positively correlated with total bilirubin and MELD score, but negatively correlated with prothrombin time activity. These results suggest the involvement of IFN-γ in the pathogenesis of ACHBLF. Importantly, the degree of methylation of the IFN-γ gene promoter in ACHBLF patients (60%, 24/40) was significantly lower than that in CHB patients (93%, 14/15), but was higher than that in the control group (20%, 2/10). Furthermore, in ACHBLF patients, the serum IFN-γ level was significantly higher in unmethylation group than that in methylation group. In conclusion, enhanced demethylation of IFN-γ gene promoter in PBMCs may be associated with the onset of ACHBLF.

Keywords: Acute-on-chronic liver failure; hepatitis B; interferon-gamma; methylation; promoter

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Among these cytokines, interferon-\(\gamma\) is particularly important because it induces cell-mediated immunity (Ando et al. 1993; Ohta et al. 2000). Tanaka et al. (1996) reported that IFN-\(\gamma\) production induced by priming with Propionibacterium acnes is essential for the induction of Propionibacterium acnes and LPS-induced severe liver injury. Although the roles of IFN-\(\gamma\) have been studied extensively in animal models of fulminant hepatitis or fulminant hepatic failure or liver injury (Ando et al. 1993; Tanaka et al. 1996; Nishimura and Ohta 1999), the level of IFN-\(\gamma\) in the patient with ACHBLF remains not fully understood. Furthermore, little is known about the exact mechanisms of the molecular regulation of IFN-\(\gamma\) production. Methylated modification of DNA gene promoter is a common style in regulating gene expression on transcriptional level (Ohta et al. 2000; Lee et al. 2001; Lee et al. 2002). DNA methylation modification often occurs in the CpG islands, which are CpG dinucleotide-rich areas located mainly in the gene promoter regions. In a murine model of acute liver injury, the CpG site of the IFN-\(\gamma\) promoter is hypomethylated in Th1 clones, whereas the same site is methylated in more than 98% of Th2 clones (Ohta et al. 2000).

Therefore, this present study was aimed to: (1) determine the serum level of IFN-\(\gamma\) in ACHBLF patients; and (2) identify whether the change in the IFN-\(\gamma\) profile is associated with its promoter methylation of peripheral blood mononuclear cells.

**Materials and Methods**

**Patients**

This present study included 40 patients with ACHBLF, 15 patients with chronic hepatitis B (CHB), and 10 healthy controls between June 2008 and December 2009 at the Department of Hepatology, Qilu Hospital, Shandong University. The study protocol was approved by the Ethics Committee in Qilu Hospital of Shandong University, and written informed consent was obtained from each subject.

ACHBLF was defined as the following: (a) history of chronic hepatitis B, or chronic asymptomatic HBsAg (+) based on a compatible laboratory data and/or histopathologic diagnosis and ultrasonographic findings with serum HBsAg (+) > 6 months; (b) recent development of increasing jaundice (serum total bilirubin > 171 umol/L) and decreasing plasma prothrombin activity (< 40%); (c) recent development of complications such as ascites, and/or hepatic encephalopathy or hepatorenal syndrome (Chinese Society of Infectious Diseases and Parasitology and Chinese Society of Hepatology of Chinese Medical Association. 2000; Ke et al. 2003; Wasmuth et al. 2005; Shiv et al. 2009). All patients were seronegative for markers of hepatitis A, C, D and E viruses and HIV, no evidence for hepatocellular carcinoma or other metastatic liver tumor, no evidence of autoimmune liver disease; and no immunosuppressive medication within the 3 months before study entry.

**Isolation of Peripheral Blood Mononuclear Cells**

Fifteen milliliters of heparinized venous peripheral blood were collected from each patient and control. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by gradient centrifugation via Ficoll-Paque (Pharmacia Diagnostics, Uppsala, Sweden) according to the manufacturer’s instructions. Cells were washed twice with phosphate-buffered saline (PBS), and then kept at −80°C until use.

**Measurement of IFN-\(\gamma\) by ELISA**

The concentrations of IFN-\(\gamma\) in the serum were measured using a commercial ELISA assay kit according to the manufacturer’s instructions (Bender, Vienna, Austria) Absorbance was read at 450 nm. The minimum detection concentration of the assay was 0.99 pg/mL.

**DNA Isolation, Bisulfite Modification, Methylation specific PCR (MSP)**

DNA was extracted from \(5 \times 10^6\) PBMC using the QIAamp® DNA Blood Mini kit (QIAGEN, Victoria, Australia) according to the manufacturer’s instructions. Extracted DNA was treated with bisulfite using the CpGenome™ DNA Modification Kit (Chemicon® International, Temecula, CA) according to the manufacturer’s instructions. In the chemical modification of cytosine to uracil by bisulfite treatment, all cytosine residues are converted to uracil, but those that methylated are resistant to this modification and remain as cytosine (Shiraishi and Hayatsu 2004). Bisulfite modified DNA was subjected to MSP using primers specific for unmethylated IFN-\(\gamma\) (5′-TGAAAGTTAATTTATTAGGGA-3′) and (5′-TAAAAATCCTTTAAAATCTTTTCA-3′) or methylated IFN-\(\gamma\) (5′- AAGAGTTAATTTATTAGGGA-3′ and 5′-TAAAAATCCTTTAAAATCTTTTCA-3′). PCR reactions were performed in a volume of 25 \(\mu\)l containing 1 × PCR buffer, 0.15 mM dNTP, 0.5 mM of each primer, and 1.5 U of Taq polymeras and 2 \(\mu\)L of bisulfite-treated DNA. The PCR protocol included an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 52°C for 1 min, and primer extension at 72°C for 45 s; PCR cycles were followed by final extension at 72°C for 10 min. PCR samples were then electrophoresed on a 1.8% agarose gel. DNA of placenta which was treated with SspI methylase (New England Biolabs, Beverly, MA) was used as a PCR-positive control. Water was substituted for DNA as a negative control.

**Statistical analysis**

All data were analyzed using SPSS version 13.0 software. Each variable was normally distributed. The data were summarized as the mean ± s.d. Comparison between groups was analyzed by student’s test. One-sample t test was performed when we compared the IFN-\(\gamma\) level from methylation and unmethylation group in CHB patients and healthy controls. Pearson correlation was used between variables. The difference of methylation of the IFN-\(\gamma\) promoter in different groups was assessed using chi-square test. P-values less than 0.05 were considered statistically significant.

**Results**

**Demographic and clinical characteristics of the patients**

The basic demographic characteristics of the enrolled patients are shown in Table 1. There was no significant difference in sex ratio among the three groups. ACHBLF patients displayed higher levels of total serum bilirubin, alanine transaminase (ALT), and prolonged prothrombin
time, but lower levels of albumin and prothrombin activity (PTA) compared with healthy controls and CHB patients. In addition, higher levels of alpha-fetoprotein (AFP) and mortality rate were detected in ACHBLF patients than that in CHB patients.

Concentrations of IFN-γ in the serum

We firstly determined the serum concentrations of IFN-γ using ELISA in all the subjects enrolled in the present study. As shown in Fig. 1, the level of IFN-γ was significantly lower in ACHBLF patients (8.49 ± 1.33 pg/ml) than that in healthy control (14.65 ± 3.65 pg/ml, p < 0.05). Moreover, the serum level of IFN-γ was significantly higher in ACHBLF patients (8.49 ± 1.33 pg/ml) compared with CHB patients (7.53 ± 2.59 pg/ml, p < 0.05).

Correlation of IFN-γ with Clinical and Laboratory Parameters in ACHBLF patients

Total bilirubin (TBIL), PTA, alanine aminotransferase (ALT) and HBV-DNA load are usually considered to serum markers for the severity of liver failure. To investigate the correlation between IFN-γ and disease severity in ACHBLF patients, linear correlation analysis was performed. In ACHBLF patients, we observed that the level of IFN-γ positively correlated with TBIL (r = 0.686, p < 0.01; Fig. 2A) but negatively correlated with PTA (r = −0.613, p < 0.01; Fig. 2B). However, the level of IFN-γ was not correlated with serum ALT level (Fig. 2C) or HBV-DNA load (Fig. 2D).

Correlation of IFN-γ with MELD in ACHBLF patients

The model for end-stage liver disease (MELD) scores is considered as a relatively better mold to assess prognosis in acute liver failure (Malinchoc et al. 2000). MELD is based on serum creatinine, total serum bilirubin, International Normalized Ratio (INR) for prothrombin time, and etiology of cirrhosis (Malinchoc et al. 2000). We analyzed the correlation between IFN-γ level and the MELD score in ACHBLF patients. The results showed that the IFN-γ level was correlated positively with MELD score (r = 0.611, p < 0.01; Fig. 3).

Methylation of the IFN-γ promoter

We compared the percentage of methylation of IFN-γ
promoter in the patients and control group. The results showed that the percentage of methylation of the IFN-γ promoter gene was higher in the ACHBLF group (60%; Fig. 4) than in the healthy control group (20%; p < 0.05; Fig. 4).

However, the degree of IFN-γ promoter methylation was lower in the ACHBLF group (60%) than in CHB (93%, p < 0.05).

Fig. 2. Linear correlation analysis between IFN-γ levels and clinical and laboratory parameters in ACHBLF patients. A. The level of IFN-γ positively correlated much with TBIL (r = 0.686, p < 0.01). B. The level of IFN-γ negatively correlated with PTA (r = −0.613, p < 0.01). C. The level of IFN-γ was not correlated with serum ALT level (r = 0.091, p = 0.577). D. The level of IFN-γ was not correlated with serum HBV-DNA level (r = −0.057, p = 0.725).

Fig. 3. Linear correlation analysis between IFN-γ levels and MELD score in ACHBLF patients. The IFN-γ level was correlated positively with MELD score (r = 0.611, p < 0.01).
IFN-γ level in methylation group and unmethylation group

To investigate the relationship between IFN-γ level and IFN-γ promoter methylation, we divided each group into methylation group and unmethylation group. As shown in Fig. 5, the IFN-γ level was significantly lower in methylation group (7.90 ± 1.05 pg/ml) as compared to unmethylation group (9.37 ± 1.22 pg/ml, p < 0.05) in ACHBLF patients. In CHB patients, the IFN-γ level was significantly lower in methylation group (6.28 ± 1.00 pg/ml) than that of a patient with the unmethylated IFN-γ promoter (8.05 pg/ml, p < 0.05) using one-sample t test. In the healthy control group, there are only two patients with methylation of IFN-γ gene promoter, we performed one-sample t test to compare the difference in IFN-γ level between methylation group and unmethylation group. There was no significant difference between methylation group (n = 2, mean = 15.01 pg/ml) and unmethylation group (14.55 ± 3.90 pg/ml, p > 0.05). Furthermore, no significant difference of HBeAg status or HBV-DNA level was found between methylation and unmethylation groups.

Discussion

In the present study, we firstly described that the levels of IFN-γ in ACHBLF patients were significantly higher than CHB patients, but they were all significantly lower compared with healthy controls. Furthermore, a significant correlation was found between the serum IFN-γ level and severity index such as PTA (p < 0.01) and TBIL (p < 0.01) in ACHBLF patients. We also showed that the serum IFN-γ level was correlated positively with MELD score (p < 0.01) in ACHBLF patients. These results suggest that IFN-γ may participate in the pathogenesis of ACHBLF. In addition, we demonstrated that methylation of IFN-γ promoter in peripheral blood mononuclear cells was associated with decreased level of IFN-γ in the ACHBLF patients. This is the first notation to our knowledge that methylation of IFN-γ promoter may influence the secretion of serum IFN-γ in ACHBLF patients.

Although the mechanisms for the onset of ACHBLF are not fully understood, an abnormality of cellular immunity is believed to be a contributing factor for the onset of liver failure. IFN-γ has a variety of important roles in the activation of a range of Th1-associated cellular immune functions in immunopathology of chronic hepatitis B (Löhr et al. 1998; Kondo et al. 2006). In an IFN-γ receptor-deficient mouse model, IFN-γ has been approved to be indispensable (Ohta et al. 2000). In the present study, our data demonstrated that the serum level of IFN-γ in ACHBLF was significantly higher than CHB, which is consistent with the previous report that the intrahepatic IFN-γ production of ACLF patients was markedly up-regulated compared with CHB and healthy controls (Zou et al. 2009).

TBIL and PTA are commonly used as severity index in ACHBLF patients. In the present study, we demonstrated that the IFN-γ level was positively correlated with TBIL but negatively correlated with PTA. Furthermore, a significant positive correlation between serum IFN-γ level and MELD was found in ACHBLF patients. These results suggest the involvement of IFN-γ in the pathogenesis of ACHBLF. Our data also showed that the serum level of IFN-γ in ACHBLF patients was significantly lower than healthy subjects. However, Ambrosino et al. (2003) reported an inconsistent result. These conflicting observations may be explained that we selected different stages of ACHBLF patients and/or differences in the ethnic and etiological backgrounds of patients.

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**Fig. 4.** The percentage of methylation of IFN-γ promoter in the patients and control group.

The percentage methylation of the IFN-γ promoter was 60% (24/40) of ACHBLF, 93.3% (14/15) of CHB, and 20% (2/10) of healthy controls. *P < 0.05.

**Fig. 5.** Comparison of IFN-γ level between methylation group and unmethylation group.

In ACHBLF patients, the IFN-γ level was significantly lower in methylation group (7.90 ± 1.05 pg/ml) as compared to unmethylation group (9.37 ± 1.22 pg/ml, p < 0.05). In CHB patients, the IFN-γ level was significantly lower in methylation group (6.28 ± 1.00 pg/ml) than that in a patient with the unmethylated IFN-γ promoter (8.05 pg/ml, p < 0.05) using one-sample t test. In the healthy control group, we also performed one-sample t test to compare the difference in IFN-γ level between unmethylation group and methylation group. There was no significant difference between methylation group (n = 2, mean = 15.01 pg/ml) and unmethylation group (14.55 ± 3.90 pg/ml, p > 0.05). *P < 0.05.
individuals. In different stages of ACLF patients, the immune function of monocytes is different (Xing et al. 2006). Besides, the compartmentalization of the IFN-γ from peripheral into liver might partly accounts for reduced peripheral IFN-γ level in ACHBLF patients. In addition, our previous study showed that the frequency of Th17 cells had a negative correlation with Th1 cells, and the imbalance of Th17 and Th1 cells may result in pathogenesis of CHB patients (Ge et al. 2010). A considerably lower level of serum IFN-γ combined with a considerably higher level of intrahepatic IFN-γ could be a characteristic of ACHBLF (Ohta et al. 2000).

Our present study also demonstrated that the percentage of methylation of the IFN-γ promoter gene was higher in the ACHBLF group than healthy control group. Of great interest, the methylation of IFN-γ promoter was associated with decreased level of IFN-γ in ACHBLF. These results suggest that methylation of the IFN-γ promoter may regulate the IFN-γ synthesis in HBV infection. Our results are consistent with published findings that epigenetic methylation status of IFN-γ may play a mechanistic role in the modulation of cytokine secretion in the mucosa (Rivkah et al. 2009) and patients with bronchial asthma (Kwon et al. 2008). The exact mechanisms leading to altered methylation of IFN-γ promoter are not known. The HBV virus itself is considered to be a contributing factor for the methylated alteration of the IFN-γ promoter. Mikovits et al. (1998) reported that human immunodeficiency virus induces methylation of IFN-γ promoter through stimulating DNA methyltransferase activity. Recently, HBV has been found to induce over expression of DNMTs, which might lead to methylation of host CpG islands and inhibition of viral gene replication in natural infection model and temporary transfection model (Vivekanandan et al. 2010). Therefore, according to the present results and previous studies (Mikovits et al. 1998; Ohta et al. 2000; Lee et al. 2001; Lee et al. 2002; Kwon et al. 2008; Rivkah et al. 2009; Vivekanandan et al. 2010), we speculate that hepatitis B virus might have the ability to induce methylation IFN-γ promoter which may down-regulate the IFN-γ secretion. However, further research needs to be performed to confirm this hypothesis. In addition, we did not find any significant difference of IFN-γ in both methylated and unmethylated group in healthy controls. The similar IFN-γ level in both methylated and unmethylated group might be attributed to a small number of methylated cases in the healthy controls. Moreover, these results also suggest that not only methylation status, but also other factors including HBV itself, oxidative stress and so on, may be involved in the regulation of IFN-γ production. Actually, unpublished data from our research group revealed that oxidative stress participates in the methylated modification of some cytokine promoters.

There are also some limitations in this present study. First, the number of the ACHBLF patients is limited. Second, because cytokines represent not only endocrine but also autocrine and paracrine effector molecules, it should be pointed out that elevated serum IFN-γ levels are not representative of their role in the pathophysiology of liver failure. Third, one of the major factors restricting our present study is the availability of human liver samples. Therefore, the intrahepatic IFN-γ level and the methylation of IFN-γ promoter of liver are being studied in our future research.

In conclusion, we have demonstrated that the decreased IFN-γ level is positively correlated with serum TBIL level and MELD scores in ACHBLF patients. These results indicate its potential role in the pathogenesis of ACHBLF. In addition, methylation of IFN-γ promoter is associated with decreased level of IFN-γ in ACHBLF patients, suggesting that enhanced demethylation of the IFN-γ gene promoter in PBMCs is involved in ACHBLF.

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Conflict of Interest

The authors declare no conflict of interest.

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