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Intrahepatic IFN-alpha expression in liver specimens from HBV-infected patients with different outcomes

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Abstract. – BACKGROUND: Interferon-alpha (IFN-α), an active cytokine, plays an important role in antiviral host responses, including protection against hepatitis B virus (HBV) infection. This study was designed to investigate the correlation between intrahepatic IFN-α expression levels and disease severity using liver biopsy specimens from HBV-infected patients with different outcomes.

PATIENTS AND METHODS: Immunohistochemistry (IHC) was performed to detect intrahepatic IFN-α expression in liver biopsy specimens obtained from 69 HBV-infected patients with different outcomes (including 23 cases with chronic hepatitis B [CHB], 18 cases with severe hepatitis B [SHB], and 28 cases with liver cirrhosis [LC]). In situ hybridization (ISH) was carried out to measure the levels of HBV DNA in liver samples. In addition, the liver specimens of 33 healthy liver transplant donors without detectable liver diseases comprised a normal control (NC) group.

RESULTS: The intrahepatic expression levels of IFN-α were higher in the HBV-infected patients than the NC group ($p = 0.001$). Intrahepatic IFN-α expression was also significantly higher in the SHB and CHB groups compared to the NC group ($p = 0.001$ and $p = 0.001$, respectively), while the intrahepatic HBV DNA levels of the SHB patients were higher than those of LC patients ($p = 0.013$). Furthermore, intrahepatic IFN-α expression was positively correlated with serum alanine aminotransferase (ALT) levels in CHB patients; no significant correlations were discovered between intrahepatic IFN-α expression and intrahepatic HBV DNA levels in all other subgroups.

CONLUSIONS: Intrahepatic IFN-α expression may correlate with liver inflammation after hepatitis B virus infection, and IFN-α may play a vital role in the occurrence of SHB.

Key Words:
Hepatitis virus B, Interferon-alpha, Chronic hepatitis B, Severe hepatitis B, Liver cirrhosis.

Introduction

Hepatitis B virus (HBV) is a double-stranded enveloped DNA virus, that contains a relaxed-circular, partially duplex 3.2 kb genome within its core[1,2]. This hepatotropic and non-cytopathic virus has caused about 350 million people to become chronically infected, and approximately 1 million people die from liver failure, cirrhosis and hepatocellular carcinoma (HCC) each year[3,4]. The mechanisms responsible for the liver damage caused by HBV infection are complicated, and both viral and host factors can influence the outcome of HBV infection[5-9]. Inflammation and apoptotic processes induced by the immune system are the key mechanisms of virus elimination. Previous studies have shown that a large amount of the hepatocyte damage in patients with severe hepatitis B (SHB) may be caused by necrosis and apoptosis of hepatocytes; however, which one is the main cause has not been determined[10-13].

IFN-α is an important cytokine, can modulate the immune response, induce antiviral protein expression, and inhibit HBV replication[14]. *In vitro* experiments, IFN-α could suppress the level of HBV replication in human hepatoma cell lines[15]. It has also been shown that intrahepatic IFN-α expression in patients with HBV infection can activate NK cells, and mediate hepatocyte apoptosis via the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) pathway[16].

This study focused on the relationship between intrahepatic IFN-α expression and disease severity in patients with different outcomes of HBV infection. The intrahepatic expression levels of IFN-α in biopsy samples were examined by immunohistochemistry (IHC), while the replication levels of HBV DNA in liver samples were detected by in situ hybridization (ISH).

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Patients and Methods

Study Subjects

The liver biopsy specimens were collected from 69 patients with HBV infection, including 23 chronic hepatitis B (CHB) cases, 18 severe hepatitis B (SHB) cases, and 28 cases of liver cirrhosis (LC). All patients were positive for hepatitis B surface antigen (HBsAg) for more than 6 months. Exclusion criteria included the following: co-infection of human immunodeficiency virus (HIV) or other hepatitis viruses (hepatitis A, C and E viruses), autoimmune hepatitis, drug-induced hepatitis and liver injury caused by other etiologies, HCC, portal vein thrombosis or cardiovascular comorbidities. In addition, 33 liver specimens were collected from healthy liver transplant donors with undetectable liver diseases to serve as the normal control (NC group). The detailed characteristics of these subjects are described in Table I.

Patients were informed of the aims of the study and written consent was acquired. This study was approved by the West China Hospital Ethics Committee and conformed to the ethical guidelines of the 1975 Declaration of Helsinki.

Preparation of Liver Biopsy Samples and Integrated Tissue Slice Slides and Histologic Scoring System

Percutaneous liver biopsies were performed using a 16-gauge biopsy needle under ultrasound guidance. Biopsy specimens were fixed in 10% phosphate-buffered formalin, embedded in paraffin, and sectioned at 3 μm thickness. The slices were stretched at 40°C and mounted on a glass slide at a marked region. Then the integrated tissue slide was accomplished with 12 tissue slices on one slide.

Finally, positive IHC signals were indicated by yellow, brown or tan staining; positive ISH signals were indicated by purple-blue or blue-black staining. Two experienced pathologists examined the integrated slides without information regarding patients. The percentage of positive cells and the intensity of positive staining were scored according to the Axiotis score standard based on evaluation of five randomly chosen fields at 400-fold magnification[17]. If the histology scores of the two pathologists were different for the same sample, we chose the mean as the final result.

Detection of Intrahepatic IFN-α Expression by Immunohistochemistry

Sections of formalin-fixed and paraffin-embedded liver samples were incubated with a goat anti-human IFN-α polyclonal antibody at a dilution of 1:200 (Santa Cruz Biotechnology; CA, USA) at 4°C overnight. This step was followed by three washes with phosphate-buffered saline (PBS), and staining with biotin-labelled rabbit anti-goat IgG at a dilution of 1:100 (Zhongshan Goldenbridge Biotechnology; Beijing, China) at room temperature for 1 h. Finally samples were stained with a 3-3'-Diaminobenzidine kit (Wuhan Boster Biological Technology; Wuhan, China), and counterstained with hematoxylin. Otherwise, samples were incubated with PBS instead of IFN-α polyclonal antibody as a negative control.

Detection of HBV DNA in Liver Tissue by in Situ Hybridization (ISH)

ISH was carried out as previously described by Long et al[18]. The levels of HBV DNA in the liver biopsy samples were detected with a 3.2 kb HBV DNA probe labelled with digoxin with the use of a DIG-High prime kit (Roche; Basel, Switzerland). Briefly, paraffin sections were deparaffinised, rehydrated, incubated with H_2O_2, digested with protease K and repaired with super-

Table I. Characteristics of normal controls and patients with HBV infection.

	CHB	SHB	LC	NC	*p*-value
Total number	23	18	28	33	–
Age (year)	35.4 ± 8.3	44.1 ± 10.9	43.6 ± 9.3	38.1 ± 7.7	0.002
ALT (IU/L)	29.0 (13-101)	79.5 (19-2448)	46.0 (6-381)	33.0 (6-61)	< 0.001
Serum HBV DNA (copies/mL)	4.70E+06 (BLQ-5.70E+07)	6.20E+05 (BLQ-5.60E+07)	6.14E+04 (BLQ-4.34E+07)	NA	0.065
IFN-α$^{\Psi}$	1.67 ± 0.65	1.86 ± 0.80	1.32 ± 0.76	1.02 ± 0.72	0.001
HBV DNA$^{\Psi}$	0.67 ± 0.58	0.86 ± 0.74	0.41 ± 0.68	NA	0.027

ALT: alanine aminotransferase; BLQ: below limit of quantification; NA: not applicable. Ψ: ISH scores of intrahepatic IFN-α expression and HBV DNA in liver samples.

sonic waves. After being denatured, the slices were put into -20°C anhydrous ethanol and the hybridization solution containing the DIG-HBV DNA probe was put into 0°C ice water; then these were incubated together at 56 °C overnight. This step was followed by three washes and an incubation with anti-Dig-alkaline phosphatase Fab fragments at a dilution of 1:200 (Roche; Basel, Switzerland) at 37°C for 1 h. Finally, the hybridized signal was detected with a NBT/BCIP kit (Boehringer Mannheim GmbH; Mannheim, Germany) . Each sample was counterstained with 1% methyl green before being mounted.

Statistical Analysis

The histological scores of intrahepatic IFN-α expression and HBV DNA level in liver tissue were presented as the mean ± standard deviation of each sample. The Kruskal-Wallis test was used to evaluate the differences among more than 2 groups, while the Mann-Whitney *U* test were used to compare variables between 2 groups. The correlation analysis was performed by using non-parametric correlation analysis (Spearman). Data were analyzed with SPSS statistical software package, version 16.0 (SPSS Inc., Chicago, IL, USA). A difference with a *p* value less than 0.05 was considered statistically significant.

Results

The data reported in the study were obtained from CHB, SHB, LC patients and healthy liver transplant donors. Detail characteristics of these subjects are presented in Table I.

Intrahepatic Expression of IFN-α in Liver Samples from HBV-infected Patients and Normal Controls

Intrahepatic IFN-α expression was detected by IHC in all 69 samples from HBV-infected patients as well as 33 samples from healthy liver transplant donors. Expression of IFN-α in liver samples was apparent in the cytoplasm, and representative images are showed in Figure 1.

As presented in Figure 2A, the expression level of IFN-α was higher in the HBV-infected group (n=69) than that in the NC group (n=33; $p = 0.001$) (Figure 2A). According to the statistical

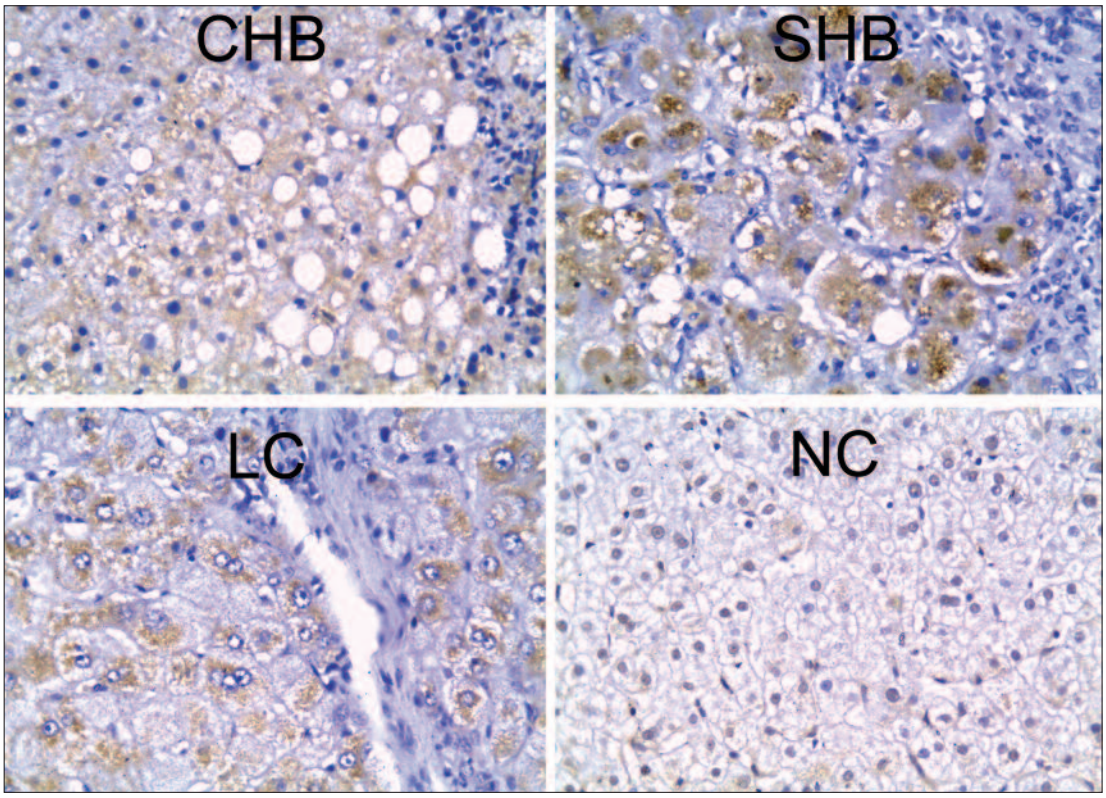

Figure 1. Intrahepatic IFN-α expression in liver samples from HBV-infected patients with different outcomes and normal controls (200-fold magnification, yellow and brown staining in the nucleus and cytoplasm indicate positive IFN-α expression).

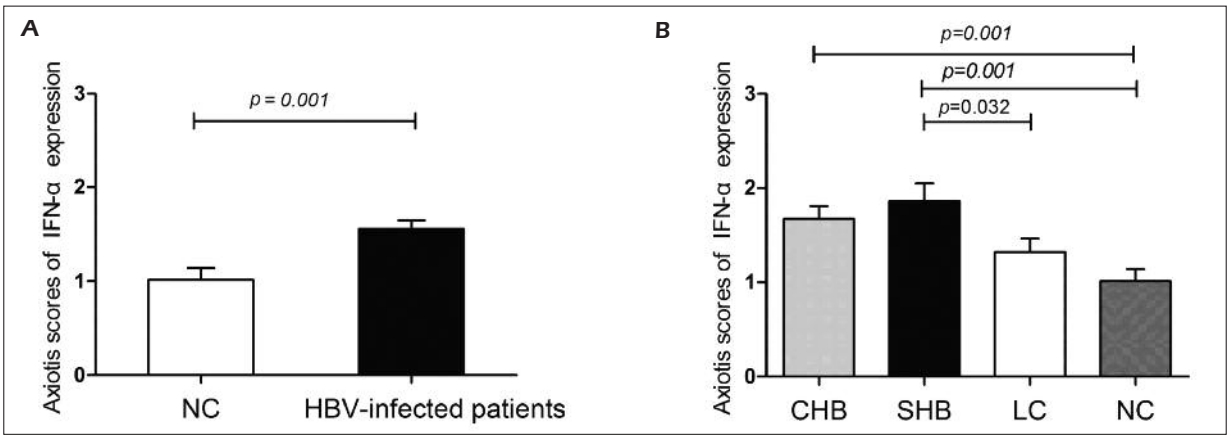

Figure 2. Intrahepatic IFN-α expression in liver samples. ***A,*** Intrahepatic IFN-α expression in HBV-infected patients (n=69) and normal controls (n=33). ***B,*** Intrahepatic IFN-α expression in HBV-infected patients with different outcomes.

analysis presented in Figure 2B, the IHC scores of intrahepatic IFN-α expression in the SHB group were significantly higher than those observed in the LC and NC groups ($p = 0.032$ and $p = 0.001$, respectively). However, while the intrahepatic IFN-α expression levels in the SHB group were higher than those of the CHB group, the difference was not statistically significant ($p = 0.349$). Moreover, there was no statistically significant difference in IFN-α expression between the LC and CHB groups ($p = 0.098$).

Levels of HBV DNA in Liver Samples from HBV-infected Patients with Different Outcomes

The presence of HBV DNA in liver biopsy samples was observed in the nucleus/cytoplasm (Figure 3A, B, C). There was significant difference in the levels of HBV DNA in the 69 HBV-infected patients ($\chi^2 = 7.198$, $p = 0.027$). The levels of HBV DNA in the SHB group were higher than those observed in the LC group ($p = 0.013$) (Figure 3D); however there was no statistically significant difference when comparing with the CHB group ($p = 0.489$), and this result was similar to the intrahepatic IFN-α expression of the SHB group. Moreover, we observed a statistically significant difference between expression levels of HBV DNA between the CHB and LC groups ($p = 0.043$) (Figure 3D).

Correlation of Intrahepatic IFN-α Expression with Serum ALT Levels, and with the Levels of HBV DNA in Liver Biopsy Samples

In this study, it was observed that there was a positive correlation between the intrahepatic expression of IFN-α and serum ALT levels in CHB patients ($r = 0.508$, $p = 0.014$) (Figure 4), but not in SHB, LC or NC patients ($r = -0.298$, $p = 0.229$; $r = 0.105$, $p = 0.594$; and $r = -0234$, $p = 0.189$; respectively). In addition, correlation analysis showed that there was no significant correlation between intrahepatic IFN-α expression and HBV DNA levels in liver biopsy samples in the CHB, SHB or LC groups ($r = -0.129$, $p = 0.558$; $r = -0.095$, $p = 0.708$; and $r = -0.013$, $p = 0.946$, respectively).

Discussion

The mechanism of HBV infection is complicated, as both the virus and the host immune response can have a direct impact on the natural course of HBV infection and the clinical outcome of liver disease[4,8,19]. As an active cytokine, IFN-α can coordinate the immune response against viruses and other intracellular infections. Previous studies have shown that IFN-α can active the expression of hundreds of genes, and generate some IFN-inducible proteins to establish the "antiviral state"[20-22]. IFN-α also directly enhances NK cell-mediated cytotoxicity through activating NK cell receptors, and also regulates cytotoxic T cells differentiation[23-26]. Both IFN-α and IFN-γ are required to maintain NK cell function[27]. At present IFN-α is a candidate for the first-line treatment of patients with chronic hepatitis B, chronic hepatitis C and other viral infection.

Many studies have shown that IFN-α expression can directly inhibit HBV DNA replication and intracellular hepatitis B surface antigen se-

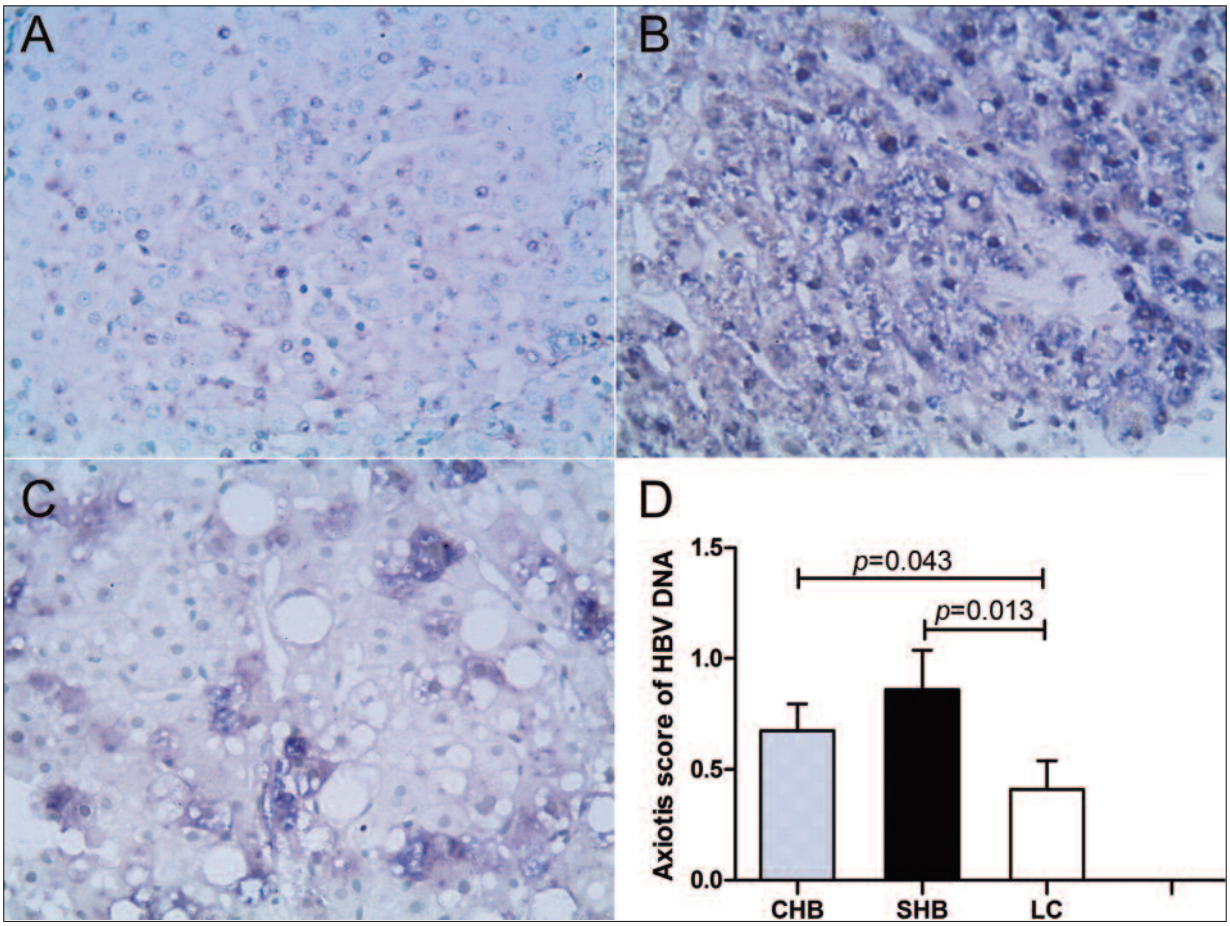

Figure 3. Levels of HBV DNA in liver samples from HBV-infected patients. ***A,*** HBV DNA detected by ISH in liver samples from CHB patients. ***B,*** HBV DNA detected by ISH in liver samples from SHB patients. ***C,*** HBV DNA detected by ISH in liver samples from LC patients (200-fold magnification, blue and purple-blue staining inside the nucleus/cytoplasm indicate presence of HBV DNA). ***D,*** Axiotis scores of HBV DNA in liver samples from HBV-infected patients.

cretion[15,28]. Intrahepatic IFN-α expression in liver tissue from patients with acute hepatitis B infection was significantly higher than patients with chronic hepatitis B infection[29]. IFN-α also can induce apoptosis in hepatocytes through stimulating tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) pathway or non-TRAIL-induced apoptosis pathways in the liver[16]. On the other hand, severe hepatitis caused by HBV infection can lead to large areas of necrosis of hepatocyte, and this process may involve IFN-α-mediated immune clearance response and hepatocyte apoptosis[10-13,30]. Leifeld, et al[31] reported that intrahepatic IFN-α expression in patients with fulminant liver failure caused by a variety of etiologies was significantly higher than that observed in chronic liver disease cases (including chronic hepatitis B, chronic hepatitis C, primary

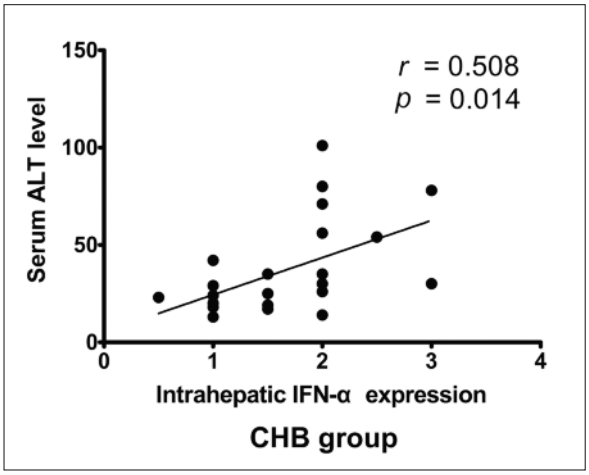

Figure 4. Correlation between intrahepatic IFN-α expression and serum ALT level in CHB patients (Pearson's correlation coefficient, r = 0.508, $p = 0.014$).

biliary cirrhosis and chronic alcoholic hepatitis) and normal subjects. Our data showed that compared with normal subjects, intrahepatic IFN-α expression was significantly higher in HBV-infected patients, and intrahepatic IFN-α expression was higher in the CHB group (or SHB group) than in the NC group. It has been suggested that a modest rise in IFN-α expression may contribute to controlling the progression of HBV infection-related diseases, the immune response and preventing chronicity of HBV infection. IFN-α also has a potential role in promoting the inflammatory response through the tumor necrosis factor (TNF) signal pathway[32]. In this study, there was a positive correlation between the expression levels of IFN-α in liver tissue from CHB patients and serum ALT levels. It is possible that the antiviral process accompanied by elevated intrahepatic IFN-α overexpression after HBV infection, promoted active inflammation and apoptosis mechanisms, and this led to the hepatocyte injury favouring the clearance of HBV infection. Moreover, the intrahepatic IFN-α expression levels in the SHB group was higher compared to the CHB group, demonstrating that elevated intrahepatic IFN-α expression may be involved in severe liver injury and occurrence of SHB; however, there was no statistically significant difference between the two group.

Kupffer cells, plasmacytoid dendritic cells, fibroblasts, and endothelial cells are the major source of intrahepatic IFN-α/β[22,29,33,34]. In patients with liver cirrhosis, disruption of the normal liver architecture, the formation of pseudolobuli, encircled by fibrosis, and decreased numbers of IFN-α expression cells (such as Kupffer cells) are observed by liver histology. These changes may lead to reduced IFN-α expression levels in liver samples from patients with liver cirrhosis, and no statistically significant difference was observed in intrahepatic IFN-α expression between the LC and NC groups, this was also the case for the LC and CHB groups. Moreover, normally or slightly elevated ALT levels could be found inconspicuously in cirrhosis patients at times, and bilirubin enzyme separation phenomenon could be observed in SHB patients with the recurrence of condition. It is possible this is the reason why no correlation was found between intrahepatic IFN-α expression and serum ALT levels in the LC and SHB groups. Further work is required to understand the underline mechanisms of how IFN-α regulates the immune response after HBV infection.

Conclusions

Our study suggests that intrahepatic IFN-α expression may correlate with liver inflammation after hepatitis B virus infection, and plays an important role in the occurrence of SHB.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Ganem D. Assembly of hepadnaviral virions and subviral particles. Curr Top Microbiol Immunol 1991; 168: 61-83.
2) Summers J, O'Connell A, Millman I. Genome of hepatitis B virus: restriction enzyme cleavage and structure of DNA extracted from Dane particles. Proc Natl Acad Sci 1975; 72: 4597-4601.
3) World Health Organization. Hepatitis B. Available at: http://www.who.int/mediacentre/factsheets/fs204/en/.
4) Ganem D, Prince AM. Hepatitis B virus infection–natural history and clinical consequences. N Engl J Med 2004; 350: 1118-1129.
5) Huang Y, Lok AS. Viral factors and outcomes of chronic HBV infection. Am J Gastroenterol 2011; 106: 93-95.
6) Hui CK, Leung N, Yuen ST, Zhang HY, Leung KW, Lu L, Cheung SK, Wong WM, Lau GK. Natural history and disease progression in Chinese chronic hepatitis B patients in immune-tolerant phase. Hepatology 2007; 46: 395-401.
7) Museru O, Franco-Paredes C. Epidemiology and clinical outcomes of hepatitis B virus infection among refugees seen at a US travel medicine clinic: 2005-2008. Travel Med Infect Dis 2009; 7: 171-174.
8) Pungpapong S, Kim WR, Poterucha JJ. Natural history of hepatitis B virus infection: an update for clinicians. Mayo Clin Proc 2007; 82: 967-975.
9) Pan CQ, Zhang JX. Natural history and clinical consequences of hepatitis B virus infection. Int J Med Sci 2005; 2: 36-40.
10) Guicciardi M, Gores G. Apoptosis: a mechanism of acute and chronic liver injury. Gut 2005; 54: 1024.
11) Malhi H, Gores GJ, Lemasters JJ. Apoptosis and necrosis in the liver: a tale of two deaths? Hepatology 2006; 43(S1): S31-S44.
12) Ryo K, Kamogawa Y, Ikeda I, Yamauchi K, Yonehara S, Nagata S, Hayashi N. Significance of Fas antigen-mediated apoptosis in human fulminant hepatic failure. Am J Gastroenterol 2000; 95: 2047-2055.

13) Schwabe RF, Brenner DA. Mechanisms of liver injury. I. TNF-alpha-induced liver injury: role of IKK, JNK, and ROS pathways. Am J Physiol-Gastrointest Liver Physiol 2006; 290: G583.

14) Wu J, Lu M, Meng Z, Trippler M, Broering R, Szczeponek A, Krux F, Dittmer U, Roggendorf M, Gerken G. Toll-like receptor-mediated control of HBV replication by nonparenchymal liver cells in mice. Hepatology 2007; 46: 1769-1778.

15) Mao R, Zhang J, Jiang D, Cai D, Levy JM, Cuconati A, Block TM, Guo JT, Guo H. Indoleamine 2,3-dioxygenase mediates the antiviral effect of gamma interferon against hepatitis B virus in human hepatocyte-derived cells. J Virol 2011; 85: 1048-1057.

16) Yanase N, Kanetaka Y, Mizuguchi J. Interferon-alpha-induced apoptosis via tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-dependent and -independent manner. Oncol Rep 2007; 18: 1031-1038.

17) Axiotis C, Monteagudo C, Merino M, LaPorte N, Neumann R. Immunohistochemical detection of P-glycoprotein in endometrial adenocarcinoma. Am J Pathol 1991; 138: 799-806.

18) Long Y, Chen E, Liu C, Huang F, Zhou T, He F, Liu L, Liu F, Tang H. The correlation of hepatocyte nuclear factor 4 alpha and 3 beta with hepatitis B virus replication in the liver of chronic hepatitis B patients. J Viral Hepat 2009; 16: 537-546.

19) Sun C, Fu B, Gao Y, Liao X, Sun R, Tian Z, Wei H. TGF-β1 down-regulation of NKG2D/DAP10 and 2B4/SAP expression on human NK cells contributes to HBV persistence. PLoS Pathog 2012; 8: e1002594.

20) van Boxel-Dezaire AH, Rani M, Stark GR. Complex modulation of cell type-specific signaling in response to type I interferons. Immunity 2006; 25: 361-372.

21) Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD. How cells respond to interferons. Annu Rev Biochem 1998; 67: 227-264.

22) Stetson DB, Medzhitov R. Type I interferons in host defense. Immunity 2006; 25: 373-381.

23) Nguyen KB, Watford WT, Salomon R, Hofmann SR, Pien GC, Morinobu A, Gadina M, O'Shea JJ, Biron CA. Critical role for STAT4 activation by type 1 interferons in the interferon-gamma response to viral infection. Sci Signal 2002; 297: 2063-2066.

24) Kolumam GA, Thomas S, Thompson LJ, Sprent J, Murali-Krishna K. Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. J Exp Med 2005; 202: 637-650.

25) Nguyen KB, Salazar-Mather TP, Dalod MY, Van Deusen JB, Wei X-Q, Liew FY, Caligiuri MA, Durbin JE, Biron CA. Coordinated and distinct roles for IFN-αβ, IL-12, and IL-15 regulation of NK cell responses to viral infection. J Immunol 2002; 169: 4279-4287.

26) Perrella A, Perrella O, Conca P, Tarantino G. CD8+/CD38+: immune activity and clinical significance in HCV patients with and without interferon therapy. Eur Rev Med Pharmacol Sci 2000; 4: 127-131.

27) Lee C-K, Rao DT, Gertner R, Gimeno R, Frey AB, Levy DE. Distinct requirements for IFNs and STAT1 in NK cell function. J Immunol 2000; 165: 3571-3577.

28) Zhijian Y, Zhen H, Fan Z, Jin Y, Qiwen D, Zhongming Z. Hepatitis B virus core protein with hot-spot mutations inhibit MxA gene transcription but has no effect on inhibition of virus replication by interferon alpha. Virol J 2010; 7: 278.

29) Nouri-Aria KT, Arnold J, Davison F, Portmann BC, Meager A, Morris AG, Alexander GJM, Eddleston ALWF, Williams R. Hepatic interferon-α gene transcripts and products in liver specimens from acute and chronic hepatitis B virus infection. Hepatology 1991; 13: 1029-1034.

30) Schulze-Bergkamen H, Schuchmann M, Fleischer B, Galle PR. The role of apoptosis versus oncotic necrosis in liver injury: facts or faith? J Hepatol 2006; 44: 984-993.

31) Leifeld L, Ramakers J, Schneiders AM, Dumoulin FL, Sterneck M, Muller A, Sauerbruch T, Spengler U. Intrahepatic MxA expression is correlated with interferon-alpha expression in chronic and fulminant hepatitis. J Pathol 2001; 194: 478-483.

32) Huys L, Van Hauwermeiren F, Dejager L, Dejonckheere E, Lienenklaus S, Weiss S, Leclercq G, Libert C. Type I interferon drives tumor necrosis factor-induced lethal shock. J Exp Med 2009; 206: 1873-1882.

33) Fang JW, Wu PC, Lai CL, Lo CK, Meager A, Lau JY. Hepatic expression of interferon-alpha in chronic hepatitis B virus infection. Dig Dis Sci 1994; 39: 2014-2021.

34) Jilbert AR, Burrell CJ, Gowans EJ, Hertzog PJ, Linnane AW, Marmion BP. Cellular localization of α-interferon in hepatitis B virus-infected liver tissue. Hepatology 1986; 6: 957-961.