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Predictive value of interferon-gamma inducible protein 10 kD for hepatitis B e antigen clearance and hepatitis B surface antigen decline during pegylated interferon alpha therapy in chronic hepatitis B patients

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ABSTRACT

Chronic hepatitis B (CHB) is an immune-mediated infectious disease caused by the hepatitis B virus (HBV). No ideal immunological markers are available at present. In this study, the expression level of interferon-gamma inducible protein 10 kD (IP-10) in chronic asymptomatic HBV carriers (AsC), patients with CHB, and patients with HBV-related acute-on-chronic liver failure (ACLF) was detected. Serum IP-10 level changes were evaluated during the pre-, on- and post-treatment periods for CHB patients receiving Peg IFN-α therapy. The correlation between the IP-10 level and the inflammation activity (IA) score, alanine aminotransferase (ALT) level, HBV DNA load, and hepatitis B surface antigen (HBsAg) quantification were also evaluated. The IP-10 expression gradually increased from AsC to patients with CHB and was highest in patients with ACLF. Serum IP-10 levels were positively correlated with the hepatic IA score and ALT level, but negatively with the HBV DNA load and HBsAg quantification. The CHB patients achieved hepatitis B e antigen (HBeAg) clearance or HBsAg decline >1 $\log_{10}$ IU/ml had higher pre-treatment IP-10 levels and more obvious on-treatment reduction of the IP-10 level than did patients with HBeAg persistent-positive or HBsAg decline <1 $\log_{10}$ IU/ml. Multivariate logistic-regression analysis revealed that the serum IP-10 level was an independent predictor of HBeAg clearance and HBsAg decline. In conclusion, IP-10 expression distinctly varies at different clinical stages of HBV infection. Higher pre-treatment serum IP-10 expression and dynamic down-regulation might be associated with an increased probability of HBeAg clearance and HBsAg decline in CHB patients during Peg IFN-α therapy.

1. Introduction

Hepatitis B is a potentially life-threatening liver disease caused by the hepatitis B virus (HBV). An estimated 2 billion people have been infected with HBV worldwide, and more than 240 million have chronic liver infection (WHO, 2012). Although the precise mechanisms of immune activation and viral clearance in patients with HBV infection are unclear, the immune status varies among patients at different clinical stages and may affect disease progression. In particular, the immunoregulatory imbalance of Th1/Th2 cytokines drifting into Th1 domination plays an important role in transformation from the immune-tolerant to the immune-active phase (Jiang et al., 2002). Interferon (IFN), one representative cytokine of Th1 cells, is crucial in regulating immune activation and hepatic inflammation activity in HBV-infected patients (Chen et al., 2012). One important mechanism of IFN mediating the antiviral response involves up-regulation of IFN-gamma inducible protein 10 kD (IP-10), a chemokine of CXC family (also termed CXCL-10). IP-10 can enhance Th1-type immunocompetence by recruiting activated T lymphocytes, mononuclear macrophages, natural killer cells, and dendritic cells. To date, research on the relationship between IP-10 and viral hepatitis has mainly focused on chronic hepatitis C (Fattovich et al., 2011; Lagging et al., 2011; Payer et al., 2012; Reiberger et al., 2008; Romero et al., 2006; Zeremski et al., 2011). IP-10 is closely correlated with hepatic inflammation and fibrosis (Reiberger et al., 2008; Zeremski et al., 2011). In particular, serum IP-10 combined with interleukin-28B-related single-nucleotide polymorphisms may predict an antiviral response in patients with hepatitis C (Fattovich et al.,

Abbreviations: ACLF, acute-on-chronic liver failure; ALT, alanine aminotransferase; AsC, asymptomatic hepatitis B virus carriers; CHB, chronic hepatitis B; CI, confidence interval; GAPDH, glyceraldehydes 3-phosphate dehydrogenase; HAI, histological activity index; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; IA, inflammation activity; IFN, interferon; IFN-γ, interferon gamma; IP-10, interferon-gamma inducible protein 10 kD; NAs, nucleoside/nucleotide analogues; NC, normal controls; OR, odds ratio; Peg IFN-α, pegylated interferon alpha; PCR, polymerase chain reaction.

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[1] Accepts full responsibility for the conduct of the study.

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2011; Lagging et al., 2011; Payer et al., 2012). A few pilot studies have indicated that IP-10 expression is increased in patients with CHB (Wang et al., 2008; Yang et al., 2012), severe hepatitis (Luo et al., 2008), and hepatocellular carcinoma (Cui et al., 2010). In addition, IP-10 expression in peripheral blood mononuclear cells is positively correlated with the HBV DNA load in patients with cirrhosis (Wang et al., 2010).

Recent studies have revealed that effective anti-HBV therapy arrests or slows the progression of CHB, especially in patients who achieve hepatitis B e antigen (HBeAg) (or further hepatitis B surface antigen [HBsAg]) clearance or seroconversion. Many investigators have performed candidate molecule screens or transcriptional profiling to identify correlates of the antiviral response to pegylated IFN-α (Peg IFN-α) therapy. Unfortunately, no ideal immunological indicators have been confirmed. The serum IP-10 level is reportedly correlated with HBsAg loss during antiviral treatment with nucleoside/nucleotide analogues (Jaroszewicz et al., 2011). Similarly, a higher pre-treatment serum IP-10 level, which is associated with a higher HBV DNA loader and greater HBeAg and HBsAg decline, might predict HBeAg loss in HBeAg-positive CHB patients (Sonneveld et al., 2013). However, a multivariate analysis revealed that although the serum IP-10 level differs between patients with and without HBeAg seroconversion, it is an independent predictive marker for HBeAg seroconversion (Bae et al., 2012).

Therefore, further study is necessary to elucidate (1) the variation in intrahepatic expression of IP-10 mRNA and protein at different stages of HBV infection, (2) the relationship between the IP-10 level and immune activity and inflammation aggravation, (3) dynamic changes of IP-10 level during Peg IFN-α therapy, and (4) the baseline and/or on-treatment predictive value of the IP-10 level for HBsAg/HBeAg clearance and seroconversion. In this study, we systemically examined the intrahepatic expression of IP-10 mRNA and protein and the serum IP-10 concentration in chronic asymptomatic HBV carriers (AsC), CHB patients, and HBV-related acute-on-chronic liver failure (ACLF) patients to evaluate the relationships between IP-10 expression with immune activation and disease aggravation in patients with CHB. Most importantly, based on dynamic detection of serum IP-10 changes in a cohort of 60 CHB patients, we identified the predictive value of serum IP-10 changes for HBeAg clearance and HBsAg decline during Peg IFN-α antiviral therapy.

2. Materials and methods

2.1. Patients and study design

This was an open-label, observational, clinical controlled study. A cohort of 60 CHB patients, 15 AsC, and 15 HBV-related ACLF patients were recruited from January 2010 to December 2011. Serum samples from 15 healthy blood donors and normal tissues trimmed from 15 donor livers during liver transplantation were collected as the normal controls (NC). The recruited AsC and patients with CHB were diagnosed according to the Asia-Pacific consensus statement on the management of chronic hepatitis B (a 2008 update) by the Asian Pacific Association for the Study of the Liver (APASL) (Liaw et al., 2008). The ACLF patients were diagnosed according to the consensus recommendation for ACLF criteria, also developed by APASL (Sarin et al., 2009). Sixty CHB patients undergoing 48-week therapy with Peg IFN-α at 180 to 90 μg/week (Pegasys®; Roche, Shanghai, China) met the following additional criteria: HBV DNA load of $\geqslant 10^4$ copies per milliliter (copies/ml); elevated serum alanine aminotransferase (ALT) level of 2–10 times the upper limit of normal (ULN) and no immunotherapy or HBV vaccine treatment for 6 months prior to enrollment. Exclusion criteria for all recruited patients were co-infected with hepatitis A virus (HAV), hepatitis C virus (HCV), hepatitis E virus (HEV) or human immunodeficiency virus (HIV); evidence of liver comorbidities of other etiologies (e.g., alcohol-related, drug-related, or autoimmune disease); serious concurrent medical illnesses (e.g., malignancy, severe cardiopulmonary disease, psychiatric disorders, uncontrolled diabetes mellitus, or hyper- or hypothyroidism); and pregnant or nursing. The study protocol was approved by the Ethics Committee of the Third Affiliated Hospital of Hebei Medical University, and written informed consent was obtained from each subject during the screening period.

2.2. Sample collection and preparation

Fasting serum samples were collected from all recruited patients during the screening period. Samples were then continuously collected during on-treatment (at 12, 24, and 48 weeks) and follow-up (at 72 and 96 weeks) periods for all 60 CHB patients. All samples were stored at −80 °C before analysis. Liver specimens were collected by routine liver biopsy from AsC ($n = 15$), CHB patients ($n = 42$), donor/receptor livers of NC by liver transplantation ($n = 15$) and ACLF patients ($n = 15$). Secondary liver biopsies were performed for 28 CHB patients (66.67%) within 6 months after treatment termination. One-third of each specimen was immediately stored in RNA fixer at −80 °C for RNA extraction. The remainder was fixed in 10% formalin for pathological and immunohistochemical analyses.

2.3. Laboratory assays and assessment criteria

The serum ALT level was measured by a fully automatic biochemical analyzer (AU2700; Olympus, Tokyo, Japan) with a 40 IU/L upper limit of normal. HBV DNA was quantified by real-time quantitative polymerase chain reaction (PCR) assay (ABI PRISM 7000; Roche Molecular Systems Inc., Alameda, CA, USA) and a diagnostic kit (Daan Genetech, Guangzhou, China) with a linear range from 5.0×10^2 to 1.0×10^9 copies/ml. HBV serological markers, including HBsAg, HBeAg, and anti-HBe were detected using an auto-electrochemiluminescence technique (E170; Roche Modular Systems Inc, Basel, Switzerland), with a normal detection limit of <0.05 IU/ml for HBsAg, <1.0 S/CO for HBeAg and >1.0 S/CO for anti-HBe.

2.4. RNA extraction and real-time PCR analysis

Total RNA was extracted from hepatic tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according the manufacturer's instructions. cDNA was synthesized using the PrimeScript™ RT reagent kit (Fermentas, Ontario, Canada). IP-10 mRNA levels were determined by real-time quantitative PCR (ABI PRISM 7500; Roche Molecular Systems, Inc., Alameda, CA, USA) and GoTaq Green Master Mix (Promega Biotech, Beijing, China). Real-time PCR (20 μl amplification mixtures) was performed as follows: 94 °C for 5 min followed by 30 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 45 s. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the endogenous control. The primers (Sangon Biotech, Shanghai, China) were designed as follows: IP-10 forward: 5′-GCC TCT CCC ATC ACT TCC CTA C-3′; IP-10 reverse: 5′-GAA GCA GGG TCA GAA CAT CCA C-3′; GAPDH forward: 5′-ACC ACA GTC CAT GCC ATC ACT-3′; GAPDH reverse: 5′-TCC ACC ACC CTG TTG CTG TA-3′. Relative mRNA quantifications were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.5. Pathological evaluation and immunohistochemistry staining

Liver tissues were fixed in 10% formalin, paraffin-embedded, and serially sectioned (5 μm). The tissue sections were stained

with hematoxylin and eosin (H&E). Hepatic inflammation activity (IA) scores were assessed by a hepato-pathologist in a blinded fashion according to the improved Ishak histological activity index (HAI) system (Ishak et al., 1995). IA scores ranged from 0 (no disease) to 18 (severe disease), including periportal or periseptal interface hepatitis (piecemeal necrosis) (0–4), confluent necrosis (0–6), focal (spotty) lytic necrosis, apoptosis and focal inflammation (0–4), and portal inflammation (0–4).

Briefly, the immunohistochemistry (IHC) staining was performed as follows: antigen retrieval via pressure cooking for 15 min in citrate buffer (pH 6.0), blocking endogenous peroxidase activity with 3% H_2O_2 for 15 min; incubation with polyclonal rabbit-anti-human IP-10 antibody (1:250 dilution; Abcam Inc., Cambridge, MA, USA) overnight at 4 °C, incubation with goat-anti-rabbit secondary antibody (Zhongshan Biotech, Beijing, China) for 40 min at 37 °C, after washing with phosphate-buffered saline, the sections were visualized using a 3,3′-diaminobenzidine substrate kit (Zhongshan Biotech, Beijing, China) followed by counterstaining with haematoxylin. Brown-yellow cellular staining indicated positive expression. Immunoreactivity was quantified by outlining the whole section using Image Pro Plus software (Media Cybernetics, Silver Spring, MD, USA) to determine the integral optical density (IOD) score.

2.6. Enzyme-linked immunosorbent assay

Serum IP-10 concentrations were measured with sandwich enzyme-linked immunosorbent assay (ELISA) using a commercial kit and the manufacturer's recommended protocol (R&D Systems, Minneapolis, MD, USA). The optical density at 450 nm was measured on an automated microplate reader (VersaMax, Molecular Devices, Palo Alto, CA, USA). A standard curve was drawn by plotting the optical density versus the IP-10 concentration.

2.7. Statistical analysis

HBV DNA loads were logarithmically transformed. Continuous variables between groups were compared using the Mann–Whitney *U* test, and categorical variables were compared using the χ^2 and Fisher's exact tests. Spearman linear correlation tests were performed to evaluate the correlation between the IP-10 and the IA score, HBV DNA loads, HBsAg quantifications, and ALT level. Stepwise multiple logistic regression analysis was performed to identify independent factors significantly associated with HBeAg clearance and HBsAg decline. The following covariates were analyzed: age, sex, duration of infection, antiviral therapy history, HBV DNA load, ALT level, HBsAg quantification, and serum IP-10 level. A p value of <0.05 was considered statistically significant. Statistical analyses were performed using SAS software for Windows (version 8.2; SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Demographic and clinical characteristics

A total of 90 chronic HBV-infected patients and 15 blood/liver donors (63 males and 42 females) were enrolled in this study. Their mean age was 37 ± 12 years (range: 16–65 years). Sixty CHB patients (38 HBeAg-positive and 22 HBeAg-negative) received antiviral therapy with Peg IFN-α for 48 weeks and were followed up for another 48 weeks. The baseline demographic, clinical, biochemical and virological characteristics are shown in Table 1. At the end of follow-up, 60.53% (23/38) of HBeAg-positive CHB patients achieved HBeAg clearance. Of these patients, 86.96% (20/23) further achieved HBeAg-to-anti-HBe seroconversion. Meanwhile, HBsAg decline >1 $\log_{10}$ IU/ml occurred in 58.33% (35/60) of patients, of whom 23 were HBeAg-positive and 12 were HBeAg-negative. A total of 8.33% (5/60) of patients achieved HBsAg disappearance at the end of follow-up. During the treatment and follow-up periods, none of the CHB patients who received Peg IFN-α therapy withdrew because of adverse events. The Peg IFN-α dose was reduced to 135 μg or 90 μg/week in nine patients because of granulocytopenia, but none of them terminated therapy.

3.2. Baseline levels of serum IP-10, intrahepatic IP-10 mRNA and protein

The baseline levels of serum IP-10 and intrahepatic IP-10 mRNA and protein were measured in each group (NC, AsC, CHB and ACLF). As shown in Fig. 1a–c, all were significantly higher in the CHB and especially ACLF groups than in the AsC and NC groups (all $p < 0.001$). The baseline levels of serum IP-10 and intrahepatic

Table 1
Demographic, clinical, and serum characteristics of patients with chronic HBV infection.

Characteristic	NC group (n = 15)	AsC group (n = 15)	CHB group (n = 60*)					ACLF group (n = 15)
			HBeAg(+) (n = 38)		HBeAg(−) (n = 22)	HBsAg decline >1 log (n = 35)	HBsAg decline >1 log (n = 25)	
			HBeAg clearance(Y) (n = 23)	HBeAg clearance(N) (n = 15)				
Mean age, years	38 ± 12	26 ± 6	36 ± 8	35 ± 12	40 ± 15	37 ± 12	36 ± 12	45 ± 16
Male/female, n	8/7	9/6	13/10	9/6	14/8	21/14	15/10	10/5
Serum ALT, U/L	22 ± 6	28 ± 6	225 ± 67	170 ± 64[a]	186 ± 67	223 ± 65	160 ± 59[c]	251 ± 52
HBV DNA, $\log_{10}$ copies/ml	–	7.17 ± 0.87	5.36 ± 0.97	6.31 ± 0.80[a]	5.44 ± 0.85	5.21 ± 0.87	6.21 ± 0.75[c]	4.72 ± 0.71
Serum HBsAg, IU/ml	–	6135.59 ± 3542.15	5806.92 ± 2679.87	7235.46 ± 3299.66	5068.01 ± 2442.38	6018.22 ± 2341.71	5717.98 ± 3476.80	3468.45 ± 2698.48
IA score of Ishak HAI	0.43 ± 0.63	0.80 ± 0.77	8.82 ± 2.13	6.30 ± 2.45[b]	5.80 ± 1.82	8.70 ± 2.32	5.73 ± 1.70[c]	15.00 ± 2.27
Genotype(B/C/B + C/other)	–	5/8/2/0	4/16/2/1	5/8/2/0	5/14/3/0	10/17/7/1	12/12/1/0	2/10/3/0

ALT, alanine aminotransferase; HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; IA, inflammation activity; HAI, histological activity index; NC, normal controls; AsC, asymptomatic chronic HBV carriers; CHB, chronic hepatitis B; ACLF, acute-on-chronic liver failure. Compared with the HBeAg clearance sub-group in the HBeAg(+) CHB group. Compared with the HBsAg decline >1 log IU/ml sub-group in the CHB group.

[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$.

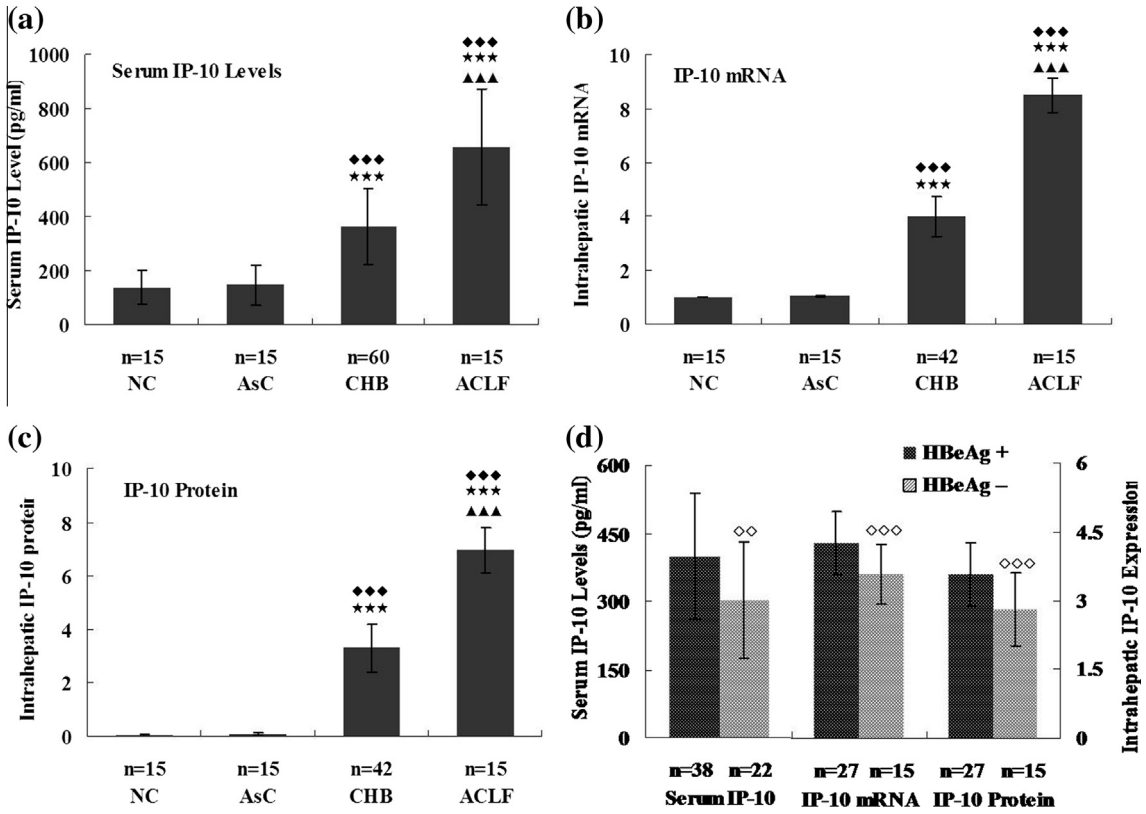

Fig. 1. Baseline levels of the serum IP-10 concentration (a), the intrahepatic IP-10 mRNA (b) and protein (c) in each group and sub-group (d). NC, normal controls; AsC, asymptomatic chronic HBV carriers; CHB, chronic hepatitis B; ACLF, acute-on-chronic liver failure. Compared with NC group, ♦♦♦$p < 0.01$; compared with AsC group, ★★★$p < 0.01$; compared with CHB group, ▲▲▲$p < 0.01$; compared with HBeAg positive CHB group (pre-treatment), ◇◇◇$p < 0.01$, ◇◇$p < 0.05$.

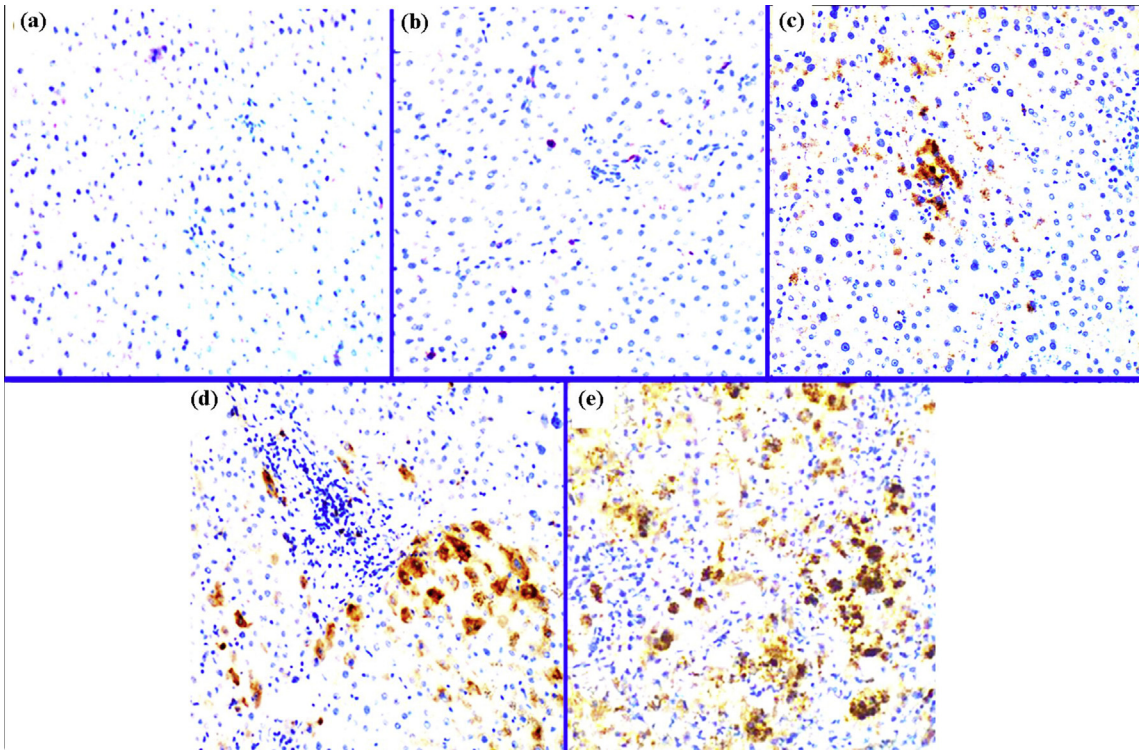

Fig. 2. Expressions of intrahepatic IP-10 protein (IHC 200×), in (a) normal controls (NC); (b) chronic asymptomatic HBV carriers (AsC group); (c) HBeAg negative chronic hepatitis B patients (HBeAg− CHB group); (d) HBeAg positive chronic hepatitis B patients (HBeAg+ CHB group); (e) HBV-related acute-on-chronic liver failure patients (ACLF group).

IP-10 mRNA and protein were then compared between the HBeAg-positive and HBeAg-negative CHB groups. All were significantly higher in the HBeAg-positive group than in the HBeAg-negative group ($p = 0.006$, $p = 0.004$ and $p = 0.002$ respectively) (Fig. 1d).

The expression of intrahepatic IP-10 protein was quantified by semi-quantitative analysis of IOD score from IP-10 IHC staining. Typical figures for analysis are shown in Fig. 2. Intrahepatic IP-10 protein was mainly expressed in hepatocytes around portal areas and necro-inflammatory regions. It was also expressed in interstitial cells in perisinusoidal spaces and in some infiltrated lymphocytes. Quantitative analysis showed that expression of intrahepatic IP-10 protein in the ACLF group was significantly higher than that in the CHB and AsC groups ($p < 0.001$) (Figs. 1c, 2d and e). Similarly, there was a significant difference in the intrahepatic IP-10 protein expression between the HBeAg-positive and HBeAg-negative CHB groups ($p = 0.002$) (Fig. 1d).

Retrospective analysis of the 38 HBeAg-positive CHB patients showed that baseline serum IP-10 levels were significantly higher in patients who achieved HBeAg clearance than in HBeAg persistent-positive patients ($p = 0.004$) (Fig. 3a, week 0) as well as in patients with HBsAg decline >1 $\log_{10}$ IU/ml than in those with HBsAg decline <1 $\log_{10}$ IU/ml ($p < 0.001$) (Fig. 3b, week 0).

3.3. Kinetics of serum IP-10 levels during and after treatment in CHB patients

Serum IP-10 levels decreased more obviously in the HBeAg clearance group and the HBsAg decreased >1 $\log_{10}$ IU/ml group compared with the HBeAg persistent-positive group and the HBsAg decreased <1 $\log_{10}$ IU/ml group (Fig. 3a and b). Although there was no statistically significant difference after stopping treatment between the HBeAg clearance and HBeAg persistent-positive groups, the levels of serum IP-10 in the HBeAg clearance group were slightly lower than those in the HBeAg persistent-positive group (weeks 48, 72, and 96). A similar regularity was found between the groups of HBsAg decline >1 $\log_{10}$ IU/ml and HBsAg decline <1 $\log_{10}$ IU/ml (only week 96). Moreover, serum IP-10 levels gradually decreased and were well consistent with HBsAg decline in HBsAg decline >1 $\log_{10}$ IU/ml patients, especially during the on-treatment period (Fig. 3b and c). In contrast, the changes of serum IP-10 levels and HBsAg were not consistent in HBsAg decline <1 $\log_{10}$ IU/ml patients, HBsAg was only mildly decreased, and serum IP-10 levels were nearly steady (Fig. 3b and c).

3.4. Changes of IA scores, intrahepatic IP-10 mRNA and protein expression in CHB patients

In this section, we only statistically analyzed the 28 patients (19 HBeAg-positive and 9 HBeAg-negative) who consented to both pre- and post-treatment liver biopsy (Fig. 4). These patients were divided into two groups according to HBeAg clearance or HBsAg decline. IA score, intrahepatic IP-10 mRNA and protein levels were compared between pre- and post-treatment. The changes (degree of decline) of these factors between two groups were also statistically analyzed. The results showed no statistical pre-treatment differences in IA score and IP-10 mRNA between HBeAg clearance and persistent positive patients ($p = 0.092$ and 0.058 respectively), but a slight difference in IP-10 protein expression was shown ($p = 0.026$). However, because the HBeAg clearance group had a relatively lower post-treatment IA scores and IP-10 mRNA and protein expression levels, the degree of decline was slightly obvious in HBeAg clearance patients than in HBeAg persistent-positive patients ($p = 0.014$, 0.044, and 0.020 respectively) (Fig. 4a–c). Similarly, there was a significant difference in and declining trend for the IA score and intrahepatic IP-10 mRNA and protein expression between patients with HBsAg decline >1 $\log_{10}$ IU/ml and HBsAg decline <1 $\log_{10}$ IU/ml (Fig. 4d–f).

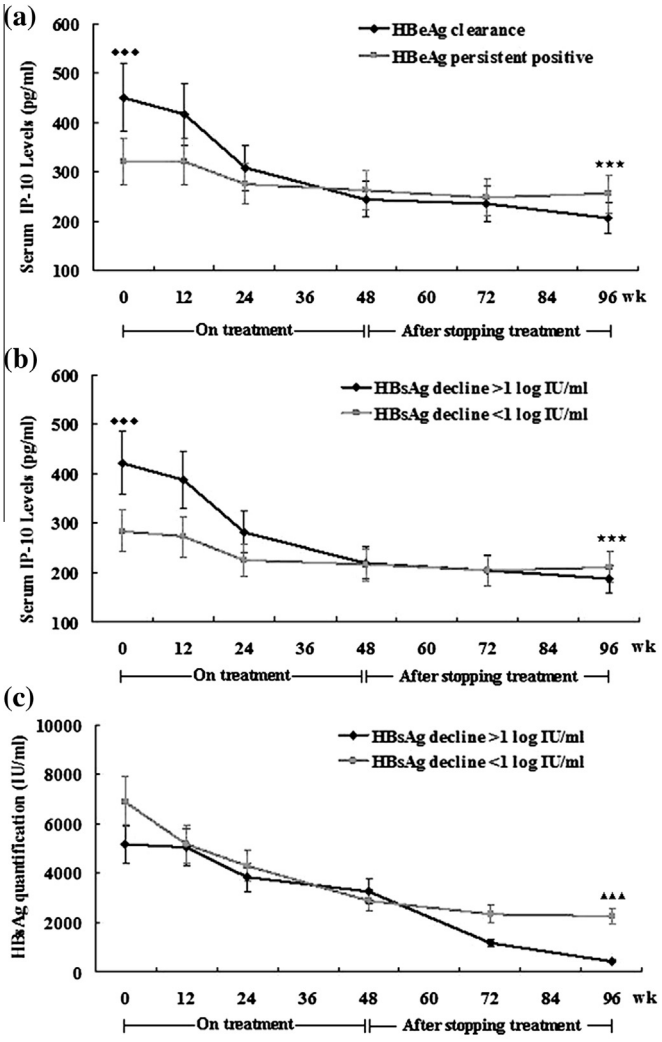

Fig. 3. Dynamic decline in serum IP-10 levels during the treatment and follow-up periods. (a) Changes in IP-10 were compared between the HBeAg clearance group and HBeAg persistent-positive group. (b and c) Changes in IP-10 and HBsAg were compared between the HBsAg decline >1 $\log_{10}$ IU/ml group and <1 $\log_{10}$ IU/ml group. Comparison of the IP-10 decline between the HBeAg clearance and the HBeAg persistent-positive groups, and between the HBsAg decline >1 $\log_{10}$ IU/ml and the HBsAg decline <1 $\log_{10}$ IU/ml groups, ♦♦♦$p < 0.01$; comparison of serum IP-10 levels between post-treatment and pre-treatment in HBeAg clearance and HBsAg decline >1 $\log_{10}$ IU/ml groups, ★★★$p < 0.01$; comparison of HBsAg quantification between post-treatment and pre-treatment in HBsAg decline >1 $\log_{10}$ IU/ml and <1 $\log_{10}$ IU/ml groups, ▲▲▲$p < 0.01$.

3.5. Pre-treatment IP-10 level was positively correlated with IA score and ALT level, but negatively correlated with HBV DNA load and HBsAg quantification in CHB patients

To better understand the clinical significance of the baseline IP-10 level in CHB patients, we analyzed the relationship between the baseline IP-10 level with the following clinical characteristics: IA score, ALT level, HBV DNA load and HBsAg quantification. Spearman linear correlation analysis revealed that the pre-treatment serum IP-10 level was positively correlated with intrahepatic expression of IP-10 mRNA and protein (data not shown); therefore, only the serum IP-10 level was included in the subsequent statistical analysis. The results showed that

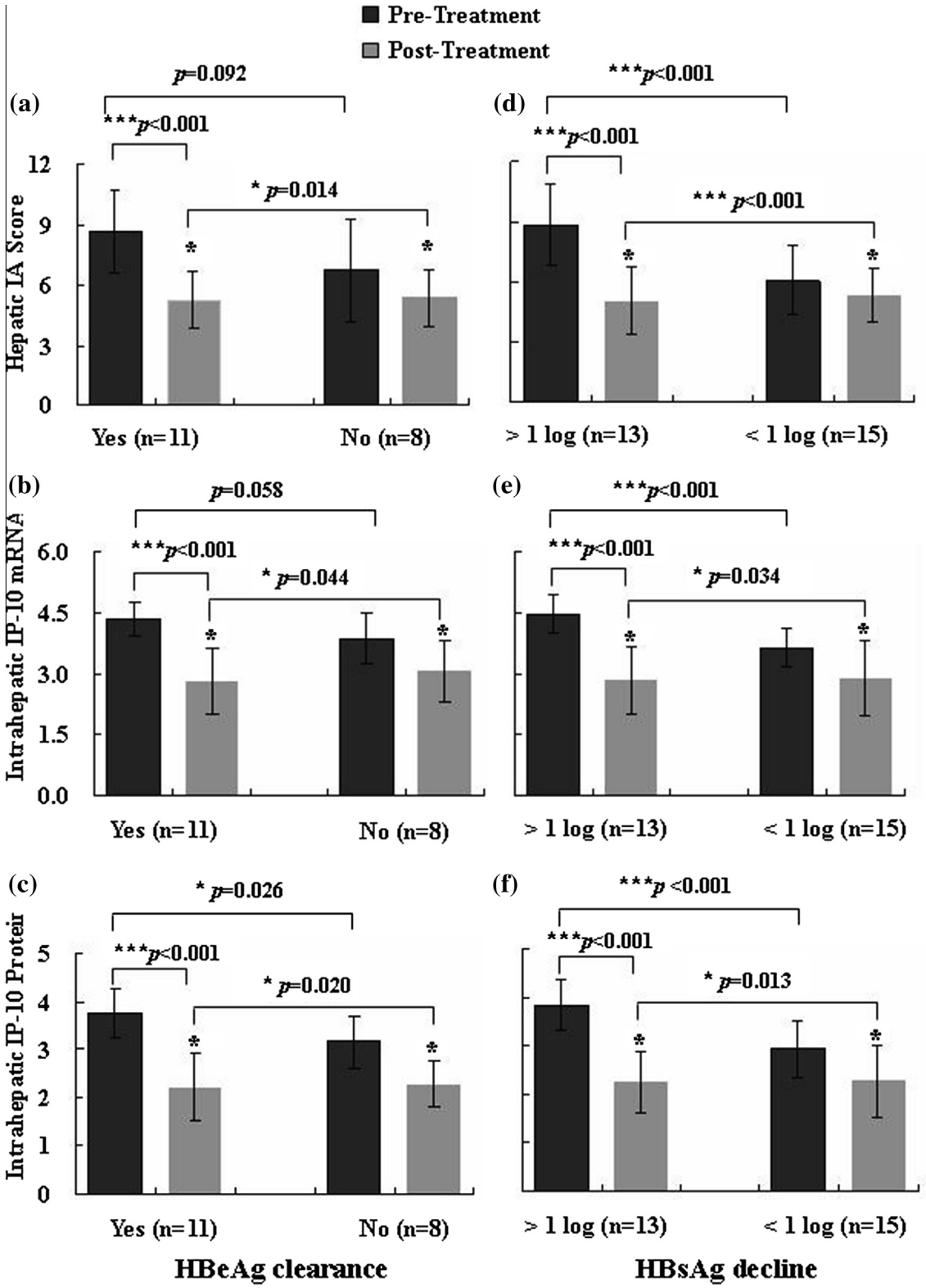

Fig. 4. Comparison of hepatic IA score, IP-10 mRNA and protein expression between patients with HBeAg clearance and HBeAg persistence-positive (a–c) and patients with HBsAg decline >1 $\log_{10}$ copies/ml and HBsAg decline <1 $\log_{10}$ copies/ml (d–f). *Degree of pre-treatment and post-treatment decline in the same group.

the serum IP-10 level was positively correlated with the hepatic IA scores and ALT level ($r = 0.771$, 0.651, $p < 0.001$) (Fig. 5a and b), but negatively correlated with the $\log_{10}$ HBV DNA load and HBsAg quantification in all the CHB patients ($r = -0.691$, -0.587, $p < 0.001$) (Fig. 5c and d).

3.6. High pre-treatment IP-10 levels (>350 pg/ml) were independently associated with post-treatment HBeAg clearance and HBsAg decline

To identify predictors of post-treatment HBeAg clearance and HBsAg decline, clinical characteristics together with the baseline

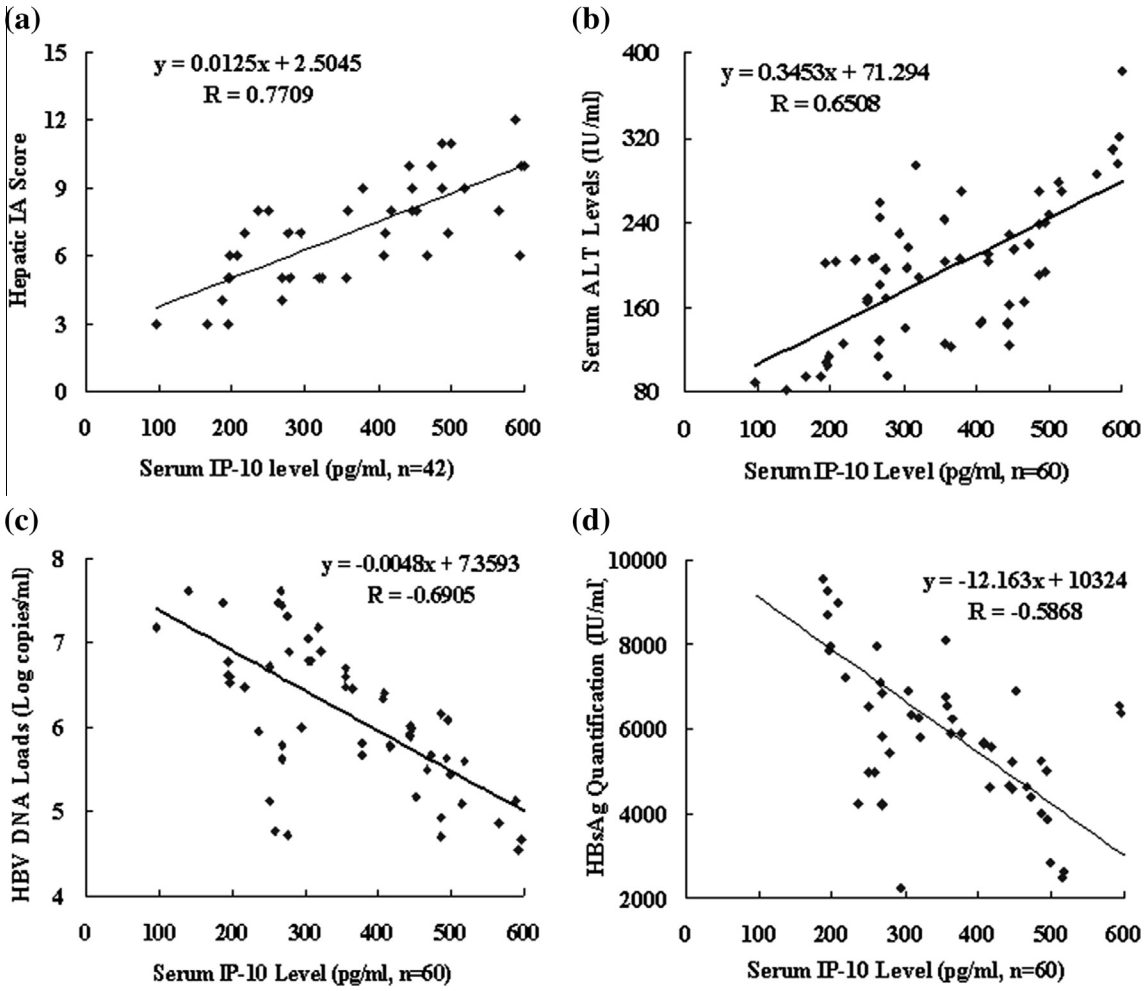

Fig. 5. Spearman univariate linear correlation analysis of serum IP-10 levels with hepatic IA score (a), serum ALT level (b), log HBV DNA load (c), and HBsAg quantification (d) in CHB patients in the pre-treatment period.

Table 2
Multiple logistic-regression analysis of independent factors affecting HBeAg clearance or HBsAg decline.

	Variables	*B*	Wald	*P* value	Odds ratio	95% confidence interval	
						Lower	Upper
Factors affecting HBeAg clearance	ALT levels (IU/L) (⩽200 = 0; >200 = 1)	1.321	2.931	0.087	3.745	0.826	16.983
	Serum IP-10 levels (pg/ml) (⩽350 = 0; >350 = 1)	1.803	5.125	0.024	6.068	1.274	28.902
	Constant	−1.393	3.582	0.058	0.248		
Factors affecting HBsAg decline	ALT levels (IU/L) (⩽200 = 0; >200 = 1)	1.159	2.915	0.088	3.186	0.842	12.048
	HBV DNA loads (Log cp/ml) (⩽5 = 0; 5–6 = 1; >6 = 2)	−1.010	3.745	0.053	0.364	0.131	1.013
	Serum IP-10 levels (pg/ml) (⩽350 = 0; >350 = 1)	1.543	4.377	0.036	4.677	1.102	19.841
	Constant	0.269	0.079	0.778	1.309		

HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; ALT, alanine aminotransferase; HBV, hepatitis B virus; IP-10, interferon-gamma inducible protein 10 kD; *B*, regression coefficient; Wald, chi-square value; cp/ml, copies/ml.

IP-10 level were evaluated by multivariate logistic regression analysis. When the dependent variable was dichotomized as post-treatment HBeAg clearance or nonclearance, the factors affecting HBeAg clearance were a high pre-treatment IP-10 level (>350 pg/ml; odds ratio [OR] 6.068; 95% confidence interval [CI] 1.274–28.902) and elevated ALT level (>200 IU/L; OR, 3.745; 95% CI, 0.826–16.983). Similarly, a higher IP-10 levels (>350 pg/ml; OR, 4.677; 95% CI, 1.102–19.841), lower HBV DNA load ($\leqslant 5$ $\log_{10}$ copies/ml, OR, 0.364; 95% CI, 0.131–1.013), and elevated ALT level (>200 IU/L; OR, 3.186; 95% CI, 0.842–12.048) were independent predictors of HBsAg decline when the dependent variable was dichotomized as HBsAg decline >1 $\log_{10}$ IU/ml and <1 $\log_{10}$ IU/ml after treatment. Thus, as was the case for HBeAg clearance and HBsAg decline, IP-10 was a stronger predictor with a higher OR than the other markers (Table 2).

4. Discussion

Previous studies have confirmed that IP-10 chemo-attracts activated T-lymphocytes and natural killer cells and then recruits these cells to participate in immune response and inflammation

(Moriai et al., 2009). Therefore, in patients with HBV infection, IP-10 might be an important factor in the regulation of antiviral immunity and may affect disease progression. Recent reports verified that IP-10 was expressed in the livers of CHB patients (Wang et al., 2008), and that this expression varied among patients with different liver diseases (Yang et al., 2012; Luo et al., 2008; Cui et al., 2010). However, whether IP-10 expression level is related with the progression of disease caused by HBV infection and with the biochemical and virological factors in patients with CHB, has not been elucidated yet.

In the present study, we found that IP-10 was mainly derived from hepatocytes around portal areas and necro-inflammatory regions, as well as from interstitial cells in perisinusoidal spaces (Fig. 2). We also found that the serum and intrahepatic IP-10 levels were higher in patients than in NC (Fig. 1). This result indicates that the IP-10 level might be related to the progression of disease. More importantly, the serum IP-10 level was positively correlated with the ALT level and hepatic IA score, but negatively correlated with the log HBV DNA load and HBsAg quantification in CHB patients (Fig. 5).

Recently, baseline serum IP-10 levels were found to be closely correlated with HBsAg loss in CHB patients undergoing nucleotide/nucleotide analogues (NAs) therapy (Jaroszewicz et al., 2011). A higher baseline IP-10 level was also found to be associated with greater declines in the HBV DNA load and HBeAg and HBsAg quantification from week 4 onward (Sonneveld et al., 2013). Based on these preliminary results, we focused on the dynamic changes of IP-10 levels in CHB patients during 48-week antiviral therapy with Peg IFN-α and further 48-week follow-up. Additional detailed analyses were performed to determine the predictive value of the IP-10 level for HBeAg clearance and HBsAg decline. Our data are in accordance with those of previous reports showing that higher expression of baseline IP-10 are notably correlated with HBeAg clearance and HBsAg decline (Jaroszewicz et al., 2011; Sonneveld et al., 2013). These findings suggested that regardless of the therapy applied, IP-10 might be a positive predictive marker for an antiviral response in CHB patient. However, significant on-treatment reduction of IP-10 was also found to be notably correlated with HBeAg clearance and HBsAg decline; this finding is in contrast to that of a previous study (Sonneveld et al., 2013). The difference in these results may be due to the difference in the study duration (96 weeks in our study and only 12 weeks in their study). Moreover, the intrahepatic IP-10 mRNA and protein expression was also found to be correlated with HBeAg clearance and HBsAg decline (Fig. 4). Our data indicate that the higher pretreatment IP-10 level could be a promising predictor of HBeAg clearance and HBsAg decline.

Interestingly, our results are in contrast to those showing that a higher IP-10 level is a negative predictive marker for a response to therapy in patients with chronic HCV infection. The reason for this difference might be that HCV is an RNA virus, while dsRNA, which appears during the HCV life cycle, is an important IFN-inducing factor. A higher IP-10 level might indicate increased activation of the endogenous IFN pathway and thus a poor therapeutic response to exogenous IFN therapy. In addition, IP-10 is reportedly present in an antagonist form in chronic HCV-infected nonresponders and may be related to treatment failure (Casrouge et al., 2011).

Several immunologic factors or biomarkers have been shown to be associated with an antiviral response in CHB patients, such as IL-10, -12, -21 (Ma et al., 2012; Wu et al., 2010), ALT level, HBV DNA load and HBsAg quantification (Lee et al., 2011; Lok et al., 1998; Rijckborst et al., 2010). In this study, we performed logistic multivariate analysis to screen predictive markers for HBeAg clearance and HBsAg decline among in which age, sex, duration of infection, past antiviral therapy history, ALT level, HBV DNA load, HBsAg quantification and serum IP-10 level. A high pre-treatment IP-10 level (>350 pg/ml) and increasing ALT level were independently associated with HBeAg clearance. Meanwhile, a higher IP-10 level, lower HBV DNA load and increasing ALT level remained independent predictors for HBsAg decline. IP-10 was the strongest predictor with the highest OR.

In conclusion, we found that pre-treatment expression and dynamic on-treatment changes of IP-10 may represent predictive markers of HBeAg clearance and HBsAg decline in CHB patients undergoing Peg IFN-α therapy. More data from multi-center, prospective, cohort studies with more patients are needed to confirm our conclusion. Our data indicate that therapy to increase serum IP-10 levels may be an important strategy for successful Peg IFN-α antiviral treatment. This hypothesis will be investigated in future studies of CHB.

Potential competing interests

The authors state no potential competing interests relevant to this article.

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Specific author contributions

Wang Y.D. performed the experiments and statistical analysis and drafted the manuscript. Zhao C.Y. designed the study and revised the manuscript. Zhang L. and Yu W.Y. participated in part of the experiments and helped to draft the figures and tables. Shen C. and Wang W. collected all patients' materials and helped with the statistical analysis. Zhen Z. and Zhou J.Y. were responsible for patients' antiviral treatment and follow-up. All authors had access to the study data and approved the final manuscript.

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