Clinical Immunology 198 (2019) 31–38

Contents lists available at ScienceDirect

Clinical Immunology

journal homepage: www.elsevier.com/locate/yclim

Plasma CXCL13 is a predictive factor for HBsAg loss and clinical relapse after discontinuation of nucleos(t)ide analogue treatment

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ARTICLE INFO

Keywords:
CXCL13
Nucleos(t)ide analogue
Discontinuation
Clinical relapse
HBsAg loss

ABSTRACT

In this study, we investigated whether plasma cytokine/chemokine levels could predict HBsAg loss or clinical relapse (CR) after stopping nucleos(t)ides analogue (NA) treatment. Theplasma cytokines/chemokines levels were measured at 0, 4, 8, 12, 24 and 48 weeks after NA discontinuation by using the enzyme-linked immunoassay (ELISA) kit. Cox regression analysis revealed that CXCL13 level at the end of treatment (EOT) was an independent predictor for CR (HR 0.26, $p < 0.001$) and HBsAg loss (HR 3.01, $p = 0.008$) after treatment cessation. Among the patients with EOT CXCL13 level < 80 pg/*ml*, the cumulative incidences of CR and HBsAg loss were 65% and 0% at 4 years, respectively. As for the patients with EOT CXCL13 level ≥ 1000 pg/*ml*, 47.5% cases had HBsAg loss. Our study showed that EOT CXCL13 level was associated with off-treatment response, which may be used to guide cessation of NA treatment in clinical practice.

1. Introduction

Chronic hepatitis B virus (HBV) infection remains a challenging global health problem with > 350 million carriers worldwide [1]. Oral antiviral agents, nucleos(t)ide analogues (NAs), are the main therapeutic option for the majority of CHB patients [2,3]. The NAs treatment has excellent tolerance and a good safety profile; however, but is difficult to achieve HBV eradication or hepatitis B surface antigen (HBsAg) clearance in the vast majority of cases [4]. Therefore, several studies have reported on the discontinued use of NAs after dissimilar durations of treatment in accordance with different guidelines. Some studies described high rates of relapse and advised against NA cessation [5,6]. However, other studies reported a sustained response and HBsAg seroclearance in treatment cessation's patients and cessation of NA treatment has been recently included in the EASL guidelines [4,7–9].

Recently, several studies have shown the potential role of cytokines and chemokines in chronic hepatitis B (CHB). CXCL9 (monokine induced by IFN-γ [MIG]) and IP-10 (IFN-γ-inducible protein 10, also called CXCL10) have been found during hepatitis flares in CHB [10]. Proinflammatory cytokines interferon-gamma (IFN-γ) and transforming growth factor beta (TGF-β) may also be involved in suppressing HBV replication [11–13]. In addition, hepatitis B e-antigen (HBeAg)-seroconversion has been found to be associated with several cytokines, such as IL-10, IL-12 [14], IL-21 [15], CXCL13 [16] and IP-10 [17,18] in CHB patients receiving NA or peginterferon therapy. A recent study demonstrated that serum IP-10 is associated with HBsAg seroclearance [19]. However, their roles in sustained responders after treatment discontinuation remain to be elucidated.

Therefore, the purpose of this prospective study was to investigate the dynamic changes in the plasma cytokine and chemokine levels in the CHB patients after discontinuing NA treatment and their associations with the sustained off-treatment response and HBsAg loss.

2. Materials and methods

2.1. Patients

This was a prospective observational cohort study of CHB patients discontinuing NA therapy between November 2012 and February 2017 from Nanfang Hospital (Southern Medical University, Guangzhou,

Abbreviations: CR, clinical relapse; NA, Nucleos(t)ide analog; CHB, Chronic hepatitis B; HBV, Hepatitis B virus; ELISA, the enzyme-linked immunoassay; HBsAg, HBV surface antigen; MIG, monokine induced by IFN-γ; IP-10, IFN-γ-inducible protein 10; ALT, normalized alanine aminotransferase; LLOD, a lower limit of detection; ULN, the upper limit of normal; APASL, The Asian Pacific Association for the Study of the Liver; AASLD, The American Association for the Study of the Liver Diseases; EASL, The European Association for the Study of the Liver; CXCR3, C–X–C chemokine receptor type 3; CXCR5, C–X–C chemokine receptor type 5; PBC, the primary biliary cirrhosis; KC, Kupffer cell

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https://doi.org/10.1016/j.clim.2018.11.016
Received 6 April 2018; Received in revised form 27 July 2018; Accepted 29 November 2018
Available online 30 November 2018

Table 1
Cohort characteristics of 105 patients.

Characteristics	Total N = 105	HBeAg + at start of treatment N = 72	HBeAg- at start of treatment N = 33
Baseline (end of treatment)			
Age, *years*[a]	36(29–42)	33(28-40)	41(36–47)
Male sex	90(85.7%)	59(81.9%)	31(93.9)
(PEG-)IFN-experienced	23(21.9%)	17(23.6%)	6(18.2%)
NA therapy [b]	49(46.7%)	34(47.2%)	15(45.5%)
NA therapy duration, *months*[a]	50(34–72)	49(35-69)	63(33–79)
Consolidation therapy duration, *months*[a]	27(17–43)	25(14-41)	32(22–51)
Lab (serum)			
ALT, *U/l*[a]	21.0(17.1–30.8)	19.7(17.0–27.4)	24.0(19.9–33.5)
HBV DNA, log *IU/ml*	UD	UD	UD
HBsAg, *log IU/ml*[a]	2.9(2.3–3.3)	3.0(2.3–3.4)	2.8(2.1–3.2)
Lab (plasma)			
CXCL13, log *pg/ml*[a]	1.9(1.8–2.3)	2.0(1.8–2.3)	1.9(1.8–2.2)
CXCL9, *log pg/ml*[a]	1.8(1.7–1.9)	1.8(1.7–1.9)	1.8(1.6–1.9)
IP-10, *log pg/ml*[a]	2.2(2.0–2.3)	2.2(2.0–2.3)	2.1(2.0–2.2)
CCL5, *log pg/ml*[a]	4.1(3.9–4.4)	4.1(3.9–4.3)	4.2(3.9–4.4)
TGF-β, *log pg/ml*[a]	3.2(3.0–3.4)	3.2(3.0–3.3)	3.2(3.0–3.4)
IL-7, *pg/ml*[a]	0.72(0.56–0.96)	0.70(0.57–0.98)	0.72(0.53–0.94)
IFN-γ, *pg/ml*[a]	15.0(9.3–31.5)	15.0(9.7–32.5)	15.0(8.9–25.9)
Start of treatment (*serum*)			
ALT, *U/l*[a]	199(87–346)	190(84-326)	199(97–438)
HBV DNA, log *IU/ml*[a]	6.3(5.8–7.5)	6.8(6.0–7.6)	5.8(4.5–6.3)

Abbreviations: UD, undetectable (< 20 IU/ml).
[a] Median (interquartile range).
[b] First-line: entecavir, tenofovir. Second-line: lamivudine, adefovir and telbivudine.

Table 2
Factors predictive of clinical relapse.

	Univariable Cox regression			Multivariable Cox regression		
	HR	(95%CI)	p	HR	(95%CI)	p
Start of treatment						
HBeAg positivity	0.69	0.38–1.26	0.224			
HBV DNA, *log IU/ml(serum)*	1.08	0.82–1.42	0.583			
ALT,*U/l(serum)*	1.00	0.999–1.000	0.354			
End of treatment						
Age, *per year*	1.05	1.01–1.08	0.010*	1.06	1.02-1.10	0.003**
Consolidation therapy duration, *months*	0.996	0.98–1.01	0.565			
Female sex	0.86	0.30–1.93	0.569			
HBsAg level, *per* log *IU/ml(serum)*	1.43	0.98–2.07	0.064	1.93	1.24–3.01	0.004**
ALT level, *U/l(serum)*	0.99	0.97–1.01	0.417			
Lab (plasma, after treatment discontinuation)						
CXCL13_0W level, *per* log *pg/ml*	0.61	0.32–1.16	0.131	0.26	0.13–0.52	< 0.001***
CXCL13_4W level, *per* log *pg/ml*	0.72	0.40–1.27	0.253			
CXCL13_8W level, *per log pg/ml*	0.68	0.37–1.23	0.201			
CXCL9_0W level, *per log pg/ml*	0.65	0.18–2.32	0.509			
CXCL9_4W level, *per log pg/ml*	0.93	0.37–2.37	0.881			
CXCL9_8W level, *per log pg/ml*	2.48	1.14–5.39	0.022*	15.27	4.89-47.72	< 0.001***
IP-10_0W level, *per* log *pg/ml*	0.55	0.16–2.67	0.549			
IP-10_4W level, *per log pg/ml*	0.45	0.16–2.23	0.445			
IP-10_8W level, *per log pg/ml*	2.98	0.97–9.10	0.056	1.37	0.38–4.95	0.636
CCL5_0W level, *per log pg/ml*	0.81	0.33–1.97	0.637			
CCL5_4W level, *per log pg/ml*	0.93	0.45–1.92	0.847			
CCL5_8W level, *per log pg/ml*	0.69	0.26–1.78	0.437			
IL-7_0W level, *per log pg/ml*	0.64	0.32–1.28	0.206			
IL-7_4W level, *per log pg/ml*	0.96	0.53–1.76	0.900			
IL-7_8W level, *per log pg/ml*	0.82	0.45–1.51	0.528			
TGF-β_0W level, *per log pg/ml*	1.22	0.45–3.33	0.693			
TGF-β_4W level, *per log pg/ml*	1.28	0.77–2.13	0.342			
TGF-β_8W level, *per log pg/ml*	0.86	0.30–2.49	0.784			
IFN-γ_0W level, *pg/ml*	0.995	0.987–1.003	0.198			
IFN-γ_4W level, *pg/ml*	0.995	0.986–1.003	0.212			
IFN-γ_8W level, *pg/ml*	0.997	0.990–1.004	0.379			

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$ (2-ailed).

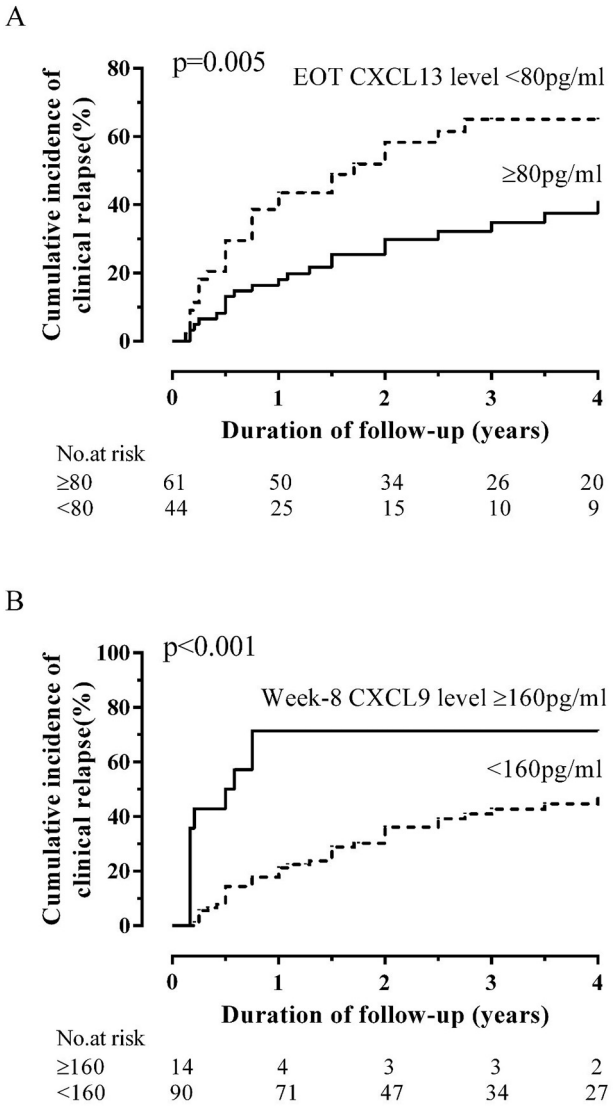

Fig. 1. Cumulative rates of clinical relapse after nucleos(t)ide analogue discontinuation according to end-of-treatment (EOT) plasma CXCL13 level (A) and 8-week plasma CXCL9 level (B).

China). The design and methods of this study were as previously described [20,21]. All start-of-treatment HBeAg-negative or HBeAg-positive patients with CHB who had been treated with NAs and who fulfilled the criteria by the stopping rule of APASL were included. HBeAg-positive patients were required to achieve HBeAg seroconversion and undetectable HBV DNA followed by at least 12 months of consolidation therapy, consistent with the criteria of international guidelines [2,3,22]. HBeAg-negative patients were required to achieve undetectable HBV DNA followed by at least 18 months of consolidation therapy, in line with the Asian-Pacific stopping criteria [3]. Patients combined with HCV or HDV infection, alcoholism, autoimmune hepatitis, and malignancy were excluded.

This study is part of an ongoing effort to prospectively investigate NA therapy discontinuation. All follow-up data through August 2017 was analyzed in this study. The procedures of this study were in accordance with the Helsinki Declaration and were approved by the Ethics Committee (study identifier NFEC-201209-K3).

2.2. Follow-up, retreatment criteria, and definitions

After NA therapy discontinuation, patients were followed monthly during the initial 3 months following cessation. Thereafter, the patients were followed every 3 months. After 2 years of follow-up, the patients were evaluated every 6 months for 5 years. Comprehensive biochemical and virological tests were performed at each follow-up visit. Ultrasonography and liver stiffness measurements were also performed at treatment discontinuation, once per year after off-treatment follow-up.

Sustained responders were defined as an HBV DNA level of < 2000 IU/ml, combined with normal ALT level after treatment discontinuation. CR was defined as an HBV DNA level of > 2000 IU/ml, combined with an ALT level of > 2 times the upper limit of normal (ULN). Patients with CR would restart NA treatment (Entecavir/Tenofovir disoproxil fumarate) and were followed every 3 months for 5 years.

2.3. Biochemistry and laboratory methods

Prior to treatment, HBV DNA level was quantitated using a polymerase chain reaction HBV assay with a lower limit of detection (LLOD) of 1000 copies/ml (5 copies/ml = 1 IU/ml) (Daan Gene Co, Ltd.; Sun Yat-sen University; Guangzhou, China). After NA discontinuation and during the off-treatment follow-up, HBV DNA level was assessed using the Cobas TaqMan polymerase chain reaction HBV assay with a LLOD of 20 IU/ml (Roche Diagnostics, Basel, Switzerland). The HBsAg, HBeAg, and anti-HBe levels were quantitatively determined using ARCHITECT (Abbott Laboratories, Chicago, Il, USA). The HBsAg test had a lower limit of detection of 0.05 IU/ml.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Plasma levels of CXCL13, CXCL9(MIG), IP-10(CXCL10), CCL5(RANTES), IFN-γ, TGF-β, IL-7 were measured at off-treatment at 0, 4, 8, 12, 24, 48 weeks, respectively. The concentrations of CXCL13, CXCL9, IL-7 (R&D Systems, Minneapolis, USA) and IP-10, CCL5 (BioLegend, San Diego, CA, USA) and IFN-γ, TGF-β (eBioscience, San Diego, CA) were quantitated by enzyme-linked immunoassay (ELISA) according to the manufacturer's instructions.

2.5. Statistical analysis

End of treatment (EOT) was defined as the time of discontinuing the NA therapy. Follow-up time was calculated from the EOT to the last follow-up or loss to follow-up. Data are presented as mean ± SD or median (interquartile range). Characteristics of patients with HBsAg loss and without HBsAg loss were compared using the $\chi 2$ test, for categorical variables. The Student *t*-test or Mann–Whitney test for continuous variables were used when appropriate. Kaplan–Meier analysis and the log-rank test were used to analyze follow-up data. Changes in quantitative chemokines/cytokines levels were calculated in both the sustained responder group and the clinical relapsed group within 48 weeks (from EOT level to the last follow-up level) or relapsed point. Cox proportional hazards regression analysis was used to analyze the association between variables and endpoints. Spearman correlation analysis was used to study the correlation between chemokines/cytokines and other covariates.

All data were analyzed using the statistical package SPSS for Windows (version 22.0; SPSS Inc., Chicago, IL, USA). The analysis used the “survival ROC” package, written using R, for performance assessment in time-dependent ROC curve estimation. A two-tailed p-value < 0.05 was considered statistically significant.

3. Results

3.1. Patient population

A total of 112 patients with informed consent were enrolled. Seven

Table 3
Factors predictive of HBsAg loss.

	Univariable Cox regression			Multivariable Cox regression		
	HR	(95%CI)	p	HR	(95%CI)	p
Start of treatment						
HBeAg positivity	0.73	0.21–2.49	0.611			
HBV DNA, *log IU/ml(serum)*	1.01	0.58–1.75	0.987			
ALT,*U/l(serum)*	1.00	0.999–1.002	0.590			
End of treatment						
Age, *per year*	0.99	0.92–1.07	0.883			
Consolidation therapy duration, *months*	1.01	0.98–1.03	0.522			
Female sex	2.60	0.68–9.9	0.161			
HBsAg level, *per log IU/ml(serum)*	0.27	0.15–0.47	< 0.001***	0.25	0.14-0.45	< 0.001***
ALT level, *U/l(serum)*	1.01	0.97–1.05	0.645			
Lab (plasma, after treatment discontinuation))						
CXCL13_0W level, *per* log *pg/ml*	2.04	1.09–3.81	0.025*	3.01	1.33-6.81	0.008**
CXCL13_4W level, *per log pg/ml*	1.89	0.995–3.01	0.052			
CXCL13_8W level, *per log pg/ml*	1.95	1.04–3.64	0.037			
CXCL9_0W level, *per log pg/ml*	3.20	0.76–13.52	0.114			
CXCL9_4W level, *per log pg/ml*	2.12	0.50–8.96	0.306			
CXCL9_8W level, *per log pg/ml*	3.43	0.995–11.83	0.051			
IP-10_0W level, *per log pg/ml*	0.88	0.04–19.63	0.936			
IP-10_4W level, *per log pg/ml*	2.20	0.12–38.94	0.591			
IP-10_8W level, *per log pg/ml*	1.62	0.19–14.20	0.663			
CCL5_0W level, *per log pg/ml*	0.78	0.17–4.85	0.791			
CCL5_4W level, *per log pg/ml*	0.57	0.13–2.43	0.442			
CCL5_8W level, *per log pg/ml*	1.21	0.20–7.47	0.834			
IL-7_0W level, *per log pg/ml*	1.67	0.46–6.08	0.437			
IL-7_4W level, *per log pg/ml*	1.72	0.50–5.94	0.392			
IL-7_8W level, *per log pg/ml*	1.41	0.35–5.71	0.834			
TGF-β_0W level, *per log pg/ml*	0.57	0.11–2.99	0.510			
TGF-β_4W level, *per log pg/ml*	1.55	0.44–5.40	0.495			
TGF-β_8W level, *per log pg/ml*	0.78	0.14–3.99	0.759			
IFN-γ_0W level, *pg/ml*	0.998	0.99–1.01	0.705			
IFN-γ_4W level, *pg/ml*	0.998	0.99–1.01	0.728			
IFN-γ_8W level, *pg/ml*	0.996	0.98–1.01	0.573			

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$ (2-tailed).

patients were negative for HBsAg at end of treatment and were excluded from this analysis. Therefore, 105 patients with HBsAg positive and HBV DNA negative at the moment of NA discontinuation were included in this analysis. Among the 105 HBV patients, HBV genotype was determined in 51 cases, including 33 cases of subtype B, and 18 cases of subtype C. Seventy-three patients were positive for pre-treatment HBeAg while 33 patients were negative. Seven patients were lost to follow-up (at week 8, 24, 36, 36, 48, 72, and 96, respectively). All patients were followed for a median of 3 years (Interquartile range [IQR]: 1.5–4.5 years). The characteristics of the patients are shown in Table 1.

3.2. Factors associated with clinical relapse

Of the 105 patients, 47 experienced clinical relapses after stopping NA treatment during the 5-year follow-up (median, 3.1 years). The cumulative incidences of CR at year 1, 2, 3 and 5 were 28.7%, 41.6%, 47.3%, and 50.8%, respectively (HBeAg-positive: 28.0%, 34.5%, 42.3% and 47.0% respectively; HBeAg-negative: 30.3%, 61.0%, 61.0% and 61.0% respectively). No patient developed decompensated liver disease after treatment discontinuation.

To investigate whether cytokines/chemokine expression was associated with CR after treatment discontinuation, the early plasma cytokines/chemokine expression levels were analyzed at time points (EOT, at 4 and 8 weeks of treatment discontinuation) in CHB patients who stopped NA treatment (Table 2). In univariate analysis, the 8-week CXCL9 level (HR: 2.48, 95%CI 1.14–5.39, p = 0.022) and age (HR: 1.05, 95% CI 1.01–1.08, p = 0.010) were significantly associated with CR (Table 2). In multivariate analysis, age (HR:1.06, 95%CI 1.02–1.10, p = 0.003), EOT HBsAg level (HR: 1.93, 95%CI 1.24–3.01, p = 0.004), plasma EOT CXCL13 level (HR: 0.26, 95%CI 0.13–0.52, p < 0.001), and 8-week CXCL9 level (HR:15.27, 95%CI 4.89–47.22, p < 0.001) were independently associated with CR rate (Table 2). Using the Kaplan–Meier method, the clinical relapse rates were compared between EOT CXCL13 level ≥ 80 pg/ml and < 80 pg/ml in 105 patients as well as between 8-week CXCL9 level ≥ 160 pg/ml and < 160 pg/ml in 104 patients (one patient had developed clinical relapse at 6 weeks; Fig. 1). The 4-year rates of clinical relapse were 41% and 65% in patients with plasma EOT CXCL13 level ≥ 80 and < 80 pg/ml, respectively (p = 0.005, Fig. 1A). The cumulative 4-year CR rates were 71% and 47% in patients with 8-week CXCL9 ≥ 160 pg/ml and < 160 pg/ml, respectively (P < 0.001, Fig. 1B). All of the 10 patients with a CXCL9 level ≥ 160.0 pg/ml had relapsed within one year since discontinuation.

3.3. Predictors for HBsAg loss after stopping NA therapy

Of the 105 patients, 11 experienced HBsAg loss after stopping NA treatment. Among the 58 patients who achieved a sustained response, 9 (15.5%) cleared HBsAg; 2 patients (4.3%) cleared HBsAg in 47 patients who were re-treated after CR. The characteristics of the patients with HBsAg loss are shown in Supplementary Table 1. For all 105 patients, Cox regression analysis revealed that lower HBsAg and higher CXCL13 levels at EOT were independent predictors for the development of HBsAg loss (HR: 0.25, 95%CI 0.14–0.45, p < 0.001; HR: 3.01, 95%CI 1.33–6.81, p = 0.008, respectively; Table 3).

HBsAg loss rates were compared with EOT HBsAg level and CXCL13 level in 105 patients using the Kaplan–Meier method (Fig. 2). The 4-

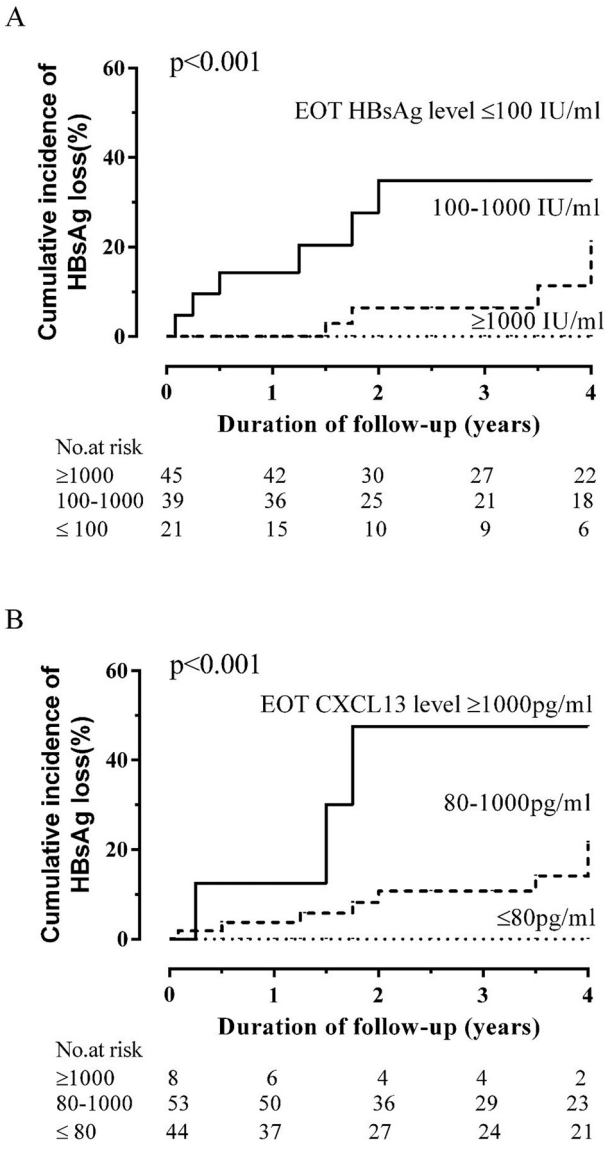

Fig. 2. Cumulative rates of HBsAg loss after nucleos(t)ide analogue discontinuation according to serum HBsAg(A) and plasma CXCL13(B) level at the end of treatment.

year rates of HBsAg loss were 34.9% and 21.2% in patients with serum EOT HBsAg level ≤ 100 and 100–1000 IU/ml, respectively ($p < 0.001$; Fig. 2A). No patients developed HBsAg loss when serum EOT HBsAg ≥1000 IU/ml. The plasma EOT CXCL13 level was categorized into ≤80 pg/ml, 80–1000 pg/ml, and ≥ 1000 pg/ml. 47.5% of patients had plasma EOT CXCL13 level ≥ 1000 pg/ml. These studies also showed that 21.6% of patients with plasma EOT CXCL13 level 80–1000 pg/ml developed HBsAg loss after 192 weeks of follow-up ($p < 0.001$; Fig. 2B). No patient developed HBsAg loss when plasma EOT CXCL13 level ≤ 80 pg/ml.

3.4. Dynamic profile of cytokines/chemokines after stopping NA treatment

After stopping NA treatment, plasma CXCL13, CXCL9 and IP-10 levels showed significant changes in patients with CR as compared to patients with sustained response (−0.9 vs. −1.2 log pg/ml per year, $p = 0.025$; +1.6 vs. +0.2 log pg/ml per year, $p < 0.001$; +1.9 vs. +0.4 log pg/ml per year, $p < 0.001$; Fig. 3). Increasing trends in the level of CXCL9 and IP-10 were observed in both groups, while a trend toward decreasing level of CXCL13 was observed exclusively in patients with sustained response.

3.5. Correlation between cytokines/chemokines levels and other factors

All EOT cytokines/chemokines levels were not significantly correlated with age, gender, start-of-treatment HBeAg status, consolidation therapy duration (all $p > 0.05$, data was not shown). Several clinical parameters at EOT, and at off-treatment at 4, 8, 12, 24 and 48 weeks, were examined for correlation with plasma cytokines/chemokines in patients who discontinued treatment. The cytokines/chemokine levels were not significantly correlated with any virological (HBsAg, HBV DNA) or biochemical (ALT) parameters except TGF-β (Table 4 and Supplementary Table 2). The CXCL9 level was significantly correlated with HBV DNA level (at off-treatment week 8, 48) and ALT level (at off-treatment week 8, 12, 24), while CXCL9 was only significantly correlated with HBsAg level at off-treatment at 48 weeks (Supplementary Table 2). Additionally, IP-10 was significantly correlated with ALT levels (at off-treatment week 4, 8, 12, 24, 48) and HBV DNA level (at off-treatment 8 weeks) (Supplementary Table 2). However, no significant correlation was observed between CXCL13 level and any virological or biochemical parameters (Table 4).

4. Discussion

According to the recent AASLD and EASL guidelines, discontinuation of NA therapy could be considered if the patient is confirmed to be without advanced liver disease and has had appropriate preceding long-term NA treatment [2,3]. Earlier studies show that the rate of durable virological relapse was approximately 46%, with most of the relapse occurring within the first or second year after NA discontinuation [23]. A recent study from Japan suggests that the estimated annual incidence of HBsAg seroclearance (1.78%) after stopping NAs is much higher than that during therapy (0.15%) [24]. In our study, 10.5% of patients with long-term nucleosi(t)de treatment discontinuation had HBsAg loss. Therefore, in some patients, long-term antiviral treatment is not necessary if they have the appropriate course of treatment and long-term monitoring after treatment discontinuation. Our study demonstrated that the EOT HBsAg level and age were associated with relapse, which is consistent with the results of our previous work, as well as other studies [20,21,25].

Hu et al. have reported that baseline serum level of anti-HBc and HBsAg is a predictive factor for HBsAg seroclearance in the untreated HBeAg-seronegative carriers [26]. Previous studies have shown that HBsAg level and its early decline can predict HBsAg loss after spontaneous HBeAg seroconversion or during treatment [27–29]. In the current study, we found that EOT serum HBsAg level was the predictor of HBsAg clearance after NA treatment discontinuation ($p < 0.001$), which is consistent with a previous observation that serum EOT HBsAg level < 100 IU/ml was highly predictive of HBsAg loss [30].

Cytokine/chemokines play an important role in immunomodulating HBV infection. Previous studies have reported the association between the control of CHB and cytokine/chemokine. IL-6 and IL-1β regulate NTCP expression and can thus inhibit HBV entry [31]. IFN-γ、TNF-α and TGF-β induce cccDNA deamination and subsequent degradation [31]. CXCL9、RANTENS and IL-17 can contribute to disease progression and chronicity of CHB [10,32,33]. IP-10、CXCL13、IL-21、IL-10 and IL-12 can predict for HBeAg seroconversion or HBsAg clearance of CHB [17,18]. Therefore, in this study, 14 cytokines and chemokines (IL-6、IL-1β、IFN-γ、TNF-α、TGF-β、IP-10、CXCL9、CXCL13、RANTENS、IL-7、IL-17F、IL-21、IL-10 and IL-12p70) were originally selected for analysis, but only 7 cytokines/chemokines expression levels were detectable and further analyzed because the levels of 7 cytokines/chemokines were below and above the limit of detection of the assay kit (data not shown).

In this study, multivariate analysis confirmed that plasma EOT CXCL13 level and 8-week plasma CXCL9 level were the independent

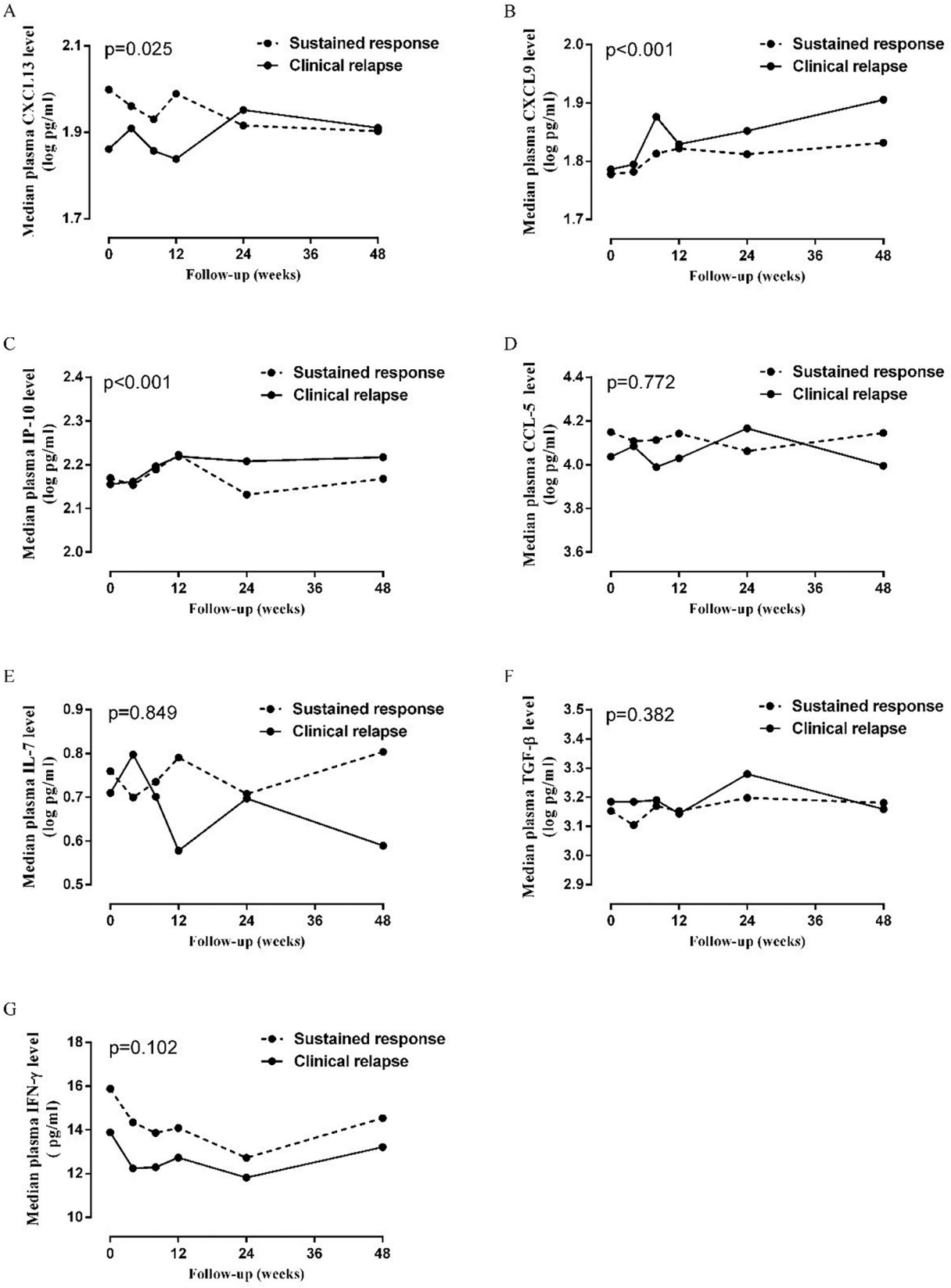

Fig. 3. Dynamic changes of plasma cytokines/chemokines in patients with CHB after NA treatment discontinuation.
The changes in quantitative plasma chemokines/cytokine levels were calculated within 48w from end-of-treatment levels to the level of the last follow-up or the level of the clinical relapsed time. Data are presented as compared between groups by Mann–Whitney *U* test.

Table 4
Spearman correlation between CXCL13 level and other virological and biochemical parameters at the end of treatment and after treatment discontinuation.

Spearman correlation	*r*	p	*r*	p	*r*	p
	CXC13 vs. HBsAg		CXC13 vs. HBV DNA		CXC13 vs. ALT	
Baseline	−0.04	0.658	–[a]	–[a]	0.13	0.201
4-Week	−0.08	0.407	0.01	0.951	0.12	0.226
8-Week	−0.16	0.116	−0.07	0.488	0.15	0.129
12-Week	−0.19	0.074	−0.07	0.483	0.07	0.485
24-Week	−0.05	0.632	−0.02	0.862	0.17	0.122
48-Week	−0.14	0.246	−0.08	0.515	−0.14	0.246

[a] Not computed as HBV DNA was undetectable at the end of treatment in all patients.

prognostic factors for CR of the patients with NA treatment discontinuation. CXCL9 and IP-10 are chemokines binding to the chemokine receptor CXCR3 on the cell surface, which was highly expressed in effector T cells regulating T cell trafficking and function [34,35]. Previous studies demonstrated that plasma IP-10 was a useful predictive marker for HBsAg loss or HBeAg loss during antiviral treatment [18,19]. However, our multivariate analysis did not demonstrate an association between IP-10 and CR or HBsAg loss. It has been shown that the pre-treatment CXCL9 level was associated with sustained virological response in CHB patients receiving peginterferon-α-2a therapy [36]. Likewise, our study also indicated that plasma levels of CXCL9 and IP-10 were positively correlated with hepatitis B viral load and ALT following therapy withdrawal. A lower 8-week CXCL9 level was associated with the sustained response after NA treatment cessation. After stopping NA therapy, the increase in CXCL9 level was reduced and reached a plateau in patients with a sustained response, whereas the CXCL9 level was markedly increased in the patients with a CR. However, the molecular mechanism underlying CXCL9 as a predictor of clinic relapse remains to be investigated.

CXCL13 was the ligand for C–X–C chemokine receptor type 5 (CXCR5) and is a selectively chemotactic cytokine for B cells. It is also known as a follicular helper T cell marker involved in germinal center formation [37,38]. It was reported that intrahepatic CXCL13 mRNA was significantly higher in primary biliary cirrhosis (PBC) patients compared to healthy control [39]. Hence, it was suggested that CXCL13 could promote aggregation of CD19 + B cells and CXCR5 + CD4 + T cells to the liver [37]. Our previous study shows that the HBV-related increase of CXCL13 production in Kupffer cell(KC)and plasma CXCL13 level during telbivudine treatment might be associated with immune control of chronic HBV infection [1,16]. The present study showed that at the end of treatment, CXCL13 level was associated with sustained response and HBsAg loss after NA treatment cessation. It has been shown that CXCL13 level at the commencement of therapy is correlated with viral load in CHB patients [16]. However, our results found that CXCL13 level was not correlated with ALT level and HBsAg level at the end of treatment. This discrepancy may be attributed to the fact that our patients had passed the immune clearance phase after experiencing long-term antiviral treatment with complete viral suppression. Therefore, the HBV DNA was negative and EOT ALT level was normal in our cohort. Although CXCL13 decreased in the sustained response group and increased in the CR group in the dynamic of off-treatment follow-up, no correlation between CXCL13 and ALT, HBsAg, HBV DNA in the dynamic change was found. It has been demonstrated that KC cells in the liver of HBV patients have a high CXCL13 expression, and CXCL13 in the liver plays a key role in the recruitment of CXCR5+ lymphocytes [16]. Boltjes et al. demonstrate that a direct interaction between HBsAg and KC results in HBsAg uptake, cytokine production, and the IFN-γ production [40]. Therefore, the CXCL13 level at EOT may reflect the host's immune response. However, the detailed molecular mechanism underlying EOT CXCL13 as a predictive factor for HBsAg loss is still unknown and should be further investigated.

Despite several strengths of our study, including the prospective off-treatment study design and dynamically studying the mechanism of cytokines/chemokines in CHB patients discontinuing NA treatment, this study had several limitations. Due to the low level of cytokine and chemokine expression and limited sample volumes after NA treatment discontinuation, we identified only seven cytokines/chemokines that might have a potential relationship with clinical recurrence or HBsAg clearance. Secondly, we primarily studied the off-treatment expression and dynamics of cytokines and chemokines without on-treatment cytokines and chemokines due to lack of on-treatment samples. Thirdly, we did not investigate the molecular mechanism of CXCL13 on immune control. Hence, further studies are needed to elucidate the mechanism of cytokine/chemokines in NA treatment discontinuation.

5. Conclusion

In conclusion, this study showed that lower plasma EOT CXCL13 level and higher off-treatment 8-week plasma CXCL9 level were correlated with clinical relapse in CHB patients with NA treatment discontinuation. And higher plasma EOT CXCL13 level and lower EOT plasma HBsAg level were correlated with HBsAg loss after treatment cessation.

Acknowledgments

The authors express sincere thanks to all participants of this study.

Conflict of interest statement

The authors declare that they have no competing interests.

Funding

This work was supported by the grants from the Major Science and Technology Special Project of China (2017ZX10302201004008), the National Natural Science Foundation of China (81572000) and the Clinical Research Startup Program of Southern Medical University by High-level UNIVERSITY Construction Funding of Guangdong Provincial Department of Education (LC2016PY003).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2018.11.016.

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