Original Article

Early Evolution of Hepatitis B Virus Quasispecies During IFN- α Treatment

Yu-lian Ren and Yao Xie

Objective To investigate the dynamic change of hepatitis B virus quasispecies within complete genome during the early stage of IFN- α treatment and its impact on virological response.

Methods Sixteen patients with chronic hepatitis B receiving IFN- α treatment were investigated. HBV DNA was extracted from serum sample at baseline and week 12. The complete genome of HBV was amplified, then cloned and sequenced. The quasispecies heterogeneity of HBV complete genome was depicted at baseline and week 12.

Results The quasispecies heterogeneity of the genome except for C-ORF were comparable in three groups at baseline and week 12. The quasispecies diversity at amino acid levels of responders within C-ORF were higher than that of non-responders at baseline. The quasispecies diversity within the C-ORF of partial responders was reduced in the early stage of IFN- α treatment. Furthermore, the mean genetic distance at amino acid levels of partial responders was significantly higher than that of the non-responders at week 12. The evolutionary rate was not different between non-responders and partial responders.

Conclusions In the immune clearance phase, the patients who had greater viral quasispecies diversity within C-ORF at amino acid level had more chance to obtain the early virological response during IFN- α treatment.

Key words: Hepatitis B virus; IFN- α ; Quasispecies; Evolution

Hepatitis B virus (HBV) is a major human pathogen which can cause severe hepatic diseases, including chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). According to the World Health Organization, approximately two billion people have been infected with HBV, and more than 350 million are chronic hepatitis patients.¹ Quasispecies comprise a complex and dynamic distribution of non-identical but related genomes.² The evolution of viral quasispecies has been reported important in the pathogenesis of RNA viruses such as hepatitis C virus³ and human immunodeficiency virus.⁴ HBV is a hepatotropic DNA virus replicated by an error-prone polymerase through an RNA intermediate. Because of this feature, the replication of HBV lacks fidelity, which results in

a complex distributions of genomes due to mutations. The high mutation rate of HBV, which is $3.2-7.9 \times 10^{-5}$ nucleotides substitutions per replication cycle⁵ may result in the persistent infection of the virus. Recently, HBV quasispecies have been subjected to detailed investigation. Several studies have depicted the dynamic evolution of HBV quasispecies during nucleos(t)ide analogs treatment.^{6,7} But this study focus on the dynamic evolution of HBV quasispecies during the treatment of IFN- α .

Treatment of chronic hepatitis B with IFN- α has been shown to reduce progression of liver disease and HCC. Undetectable HBV DNA is one of the key points of therapy. IFN- α is a better antiviral drug than nucleos(t)ide analogs referring to its dual antiviral mechanism by antiviral and immunomodulatory properties,⁸ which can offer superior efficacy over nucleos(t)ide analogs, on the basis of HBeAg seroconversion, HBV DNA suppression, and

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HBsAg seroconversion. But some patients can not achieve ideal therapeutic efficacy, only 42% patients had HBeAg seroconversion after 48 weeks of peginterferon alfa-2a treatment⁹ and the mechanism and action sites of IFN- α to HBV is not yet clear. The purpose of this study was to elucidate the change of HBV quasiespecies during the early stage of IFN- α treatment. The results might help us to understand the action mechanism of IFN- α better.

PATIENTS AND METHODS

Patients

Sixteen chronic hepatitis B patients were enrolled in the present study. All patients were HBsAg and HBeAg positive and had elevated HBV DNA level at least six months before IFN- α treatment. All the patients had come into the immune clearance phase with elevated serum transaminase level. None of them had antiviral treatment before. Exclusion criteria included hepatitis C virus (HCV) or human immunodeficiency virus (HIV) co-infection, liver cirrhosis and alcohol or drug abuse. All patients received IFN- α without interruption for 12 weeks. Antiviral efficacy was evaluated at week 12. Virological response was defined as undetectable HBV DNA level can not been measured by PCR assay at the week 12, non-response was defined as serum HBV DNA levels had a drop of no more than 2 log₁₀ copies/ml or was greater than the baseline of antiviral treatment, partial responders was defined as serum HBV DNA levels had a drop of at least 2 log₁₀ copies/ml but was still detectable at week 12.

Liver biochemistry, HBV serology and HBV DNA tests

The liver biochemistry was determined with the routine automated analysis system (Hitachi 7170). HBV serological markers, including HBsAg, the antibody to HBsAg, HBeAg and antibody to hepatitis B e antigen (anti-HBe) were determined using a chemiluminescent microparticle enzyme immunoassay (Abbott Laboratories, Abbott Park, Illinois, USA). HBV DNA levels were determined by HBV Monitor Test (Fosun, Shanghai, China), with a

low detection limit of 500 copies/ml.

Molecular cloning and sequencing

HBV DNA was extracted from 200 μ l serum samples at baseline and week 12 using the QIAamp blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The PCR primers were 5'-TGTCTACTGTTCAAGCCTCCAA-3' (forward, nt 1851 - nt 1875) and 5'-AGTTGCATGGTGCTGGTGMRCASA-3' (reverse, nt 1821-nt 1798). Amplification was performed with Platinum DNA Polymerase (Invitrogen, Carlsbad, CA) to ensure the high-fidelity PCR. The PCR was carried out using the amplification programme of initial 95°C denaturation for 3 min, then 94°C denaturation for 30 s, 63°C annealing for 30 s and 72°C extension for 3 min and 30 s, for a total of 50 cycles, then extension for 10 min. The fragment of nt 1822-nt 1850 was not amplified in view of achieving higher amplification efficiency. An approximately 3186 bp fragment was amplified. PCR products were purified using the TianGen Gel extraction kit (TianGen, Beijing, China), cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA) and transformed into Trans1-T1 Phage Resistant competent *Escherichia coli* cells (TransGen Biotech, Beijing, China) growing on ampicillin plates. An average of 19 (range: 13 to 22) clones per sample were sequenced randomly using an ABI 3730 automated sequencer (Applied Biosystems, Foster City, California, US). We got 502 clones in total finally.

Sequence analysis

Multiple alignments were carried out on all sequences using CLUSTAL X, version 2.0.¹⁰ Genotypes of each sequence were determined using the HBV STAR program online.¹⁰ The HBV genome has four highly overlapping open reading frames (ORFs)-S, P, C and X. The viral quasiespecies heterogeneity of each ORF and the full genome was evaluated with two parameters: complexity and diversity.⁶ The quasiespecies complexity means the total number of viral variants identified in a single sample, which can be calculated as Shannon entropy by the formula:

$Sn = (\pi \ln \pi) / \ln N$,¹² where N is the total number of clones, and π is the frequency of each clone in the viral quasispecies population.¹² The quasispecies complexity was calculated at both the nucleotide and amino acid levels. Quasispecies diversity was evaluated by three parameters: the mean genetic distance (d), the number of synonymous substitutions per synonymous site (dS) and the number of non-synonymous substitutions per non-synonymous site (dN). The mean genetic distance, also termed the Hamming distance, was defined as the number of mutations that distinguished any two sequences from the population. The dS represents 'silent' nucleotide changes that did not alter the amino acid encoded, whereas the dN represents 'replacement' nucleotide changes that resulted in a different amino acid. All parameters were calculated using MEGA 5.0 software¹³ under proper nucleotide or amino acid substitution models.

PHYML version 3.0¹⁴ was used to construct phylogenetic trees for baseline and week 12 using the maximum likelihood method under GTR + I + G model, which was permitted nonuniform equilibrium frequencies of nucleotides and 6 different relative instantaneous rates of substitutions estimated by jModelTest.¹⁵ Relative rates of nucleotide substitution, the shape parameter of the γ distribution (G) and the proportion of invariant sites (I) were also estimated using jModelTest. The viral evolutionary rate during the 12 weeks of four ORFs was calculated using PEBBLE 1.0 software.¹⁶ The serial sample unweighted pair group method with arithmetic mean (sUPGMA) algorithm under the appropriate substitution model for each patient, which was also estimated by jModelTest.

Statistical analysis

All data were presented as median and range. Wilcoxon signed ranks and Mann-Whitney U tests were used to compare variables between two related and independent groups, respectively. *Kruskal-Wallis* tests were performed for comparison of variables among multiple independent groups. The ranks were transformed into the normal scores by which the positive results were compared between each two groups. The correlation analysis was carried out using

Spearman's rho test (rs). Categorical variables were tested by χ^2 analysis and *Fisher's* exact test. In all tests, $P < 0.05$ was considered statistically significant. All analyses were carried out using SPSS 17.0.

RESULTS

The demographic, clinical and laboratory data

Sixteen patients with CHB were enrolled in this study. There were 11 males and 5 females. Based on serum HBV DNA levels at week 12, the 16 patients were classified into the responder group ($n = 6$), partial responder group ($n = 3$) and the non-responder group ($n = 7$). The demographic and clinical features at both baseline and week 12 were shown in Table 1. Age, gender, genotype, HBeAg levels, ALT levels, HBV DNA levels in three groups were comparable at baseline ($P > 0.05$). However the HBV DNA and HBeAg levels in three groups had significant difference at week 12 ($P = 0.01$ and 0.004 , respectively).

The dynamic change of viral quasispecies

The quasispecies complexity and quasispecies diversity of four ORFs and complete genome, including the nucleotide and amino acid levels, were calculated for each patient at baseline and week 12. The quasispecies heterogeneity of the genome except for C-ORF were comparable in three groups at baseline and week 12 ($P > 0.05$). Analysis of genetic distance showed that the viral sequence diversity of responders was significantly greater than that of non-responder group.

The mean genetic distance at amino acid levels and the dN within C-ORF had significant difference at baseline in three groups, $P = 0.039$ and 0.004 , respectively. The ranks were transformed into the normal scores by which the positive results were compared between each two groups. The mean genetic distance at amino acid levels of responders was greater than non-responders, partial responders was greater than that of non-responders, $P < 0.001$ and $P = 0.006$, respectively. The dN of responders was greater than non-responders, $P = 0.008$. All data was shown in Table 2 and Figure 1.

In order to analyse the dynamic change of quasispecies heterogeneity within four ORFs during the early 12 weeks in partial responders and non-responders, Wilcoxon signed ranks was used to compare the data at baseline and week 12. The quasispecies heterogeneity of non-responders had no dynamic change during IFN- α treatment ($P > 0.05$). However, in partial responder group the mean genetic distance at both the nucleotide and amino acid levels, the dN were significantly reduced at week 12, $P = 0.028$, 0.043 and 0.028 , respectively. The dynamic change of viral quasispecies was shown in Table 2.

Mann-Whitney U tests was used to analyze the quasispecies heterogeneity between partial responders and non-responders at week 12. The mean genetic distance at amino acid levels of partial responders was significantly higher than that of the non-responders ($P = 0.047$). All data were shown in Table 2 and Figure 1.

Correlation analysis

Correlation between quasispecies heterogeneity and HBV DNA load as well as HBeAg were analysed using *Spearman's rho* test. There was no correlations between quasispecies heterogeneity and HBV DNA load as well as HBeAg (Table 3). However, HBV DNA and HBeAg had significant positive correlation, $r_s = -0.641$, $P < 0.001$ (Figure 2).

The viral evolutionary rate in the early stage of IFN- α treatment

The viral evolutionary rate in the first 12 weeks of IFN- α treatment was calculated for each patient. The viral evolutionary rate of partial responder and non-responder groups was $0.7 (0.1-13.94) \times 10^{-4}$ substitution/site/month and $1.41 (0.04-13.3) \times 10^{-4}$ substitution/site/month, respectively. There was no significant differences between them ($P = 0.53$).

Phylogenetic analysis

The phylogenetic trees of complete genome were constructed under the maximum likelihood algorithm for baseline and week 12, respectively. HBV genotype A sequence was used as the outgroup (Accession No. AF090842). Identical sequences, except one

representative sequence for each patient, were discarded to ensure that the phylogenetic tree was more concise. As expected, the sequences for each patient were clustered together except for the patients with mixed genotype infection. However, no clusters were seen to be supported by robust bootstrap values for any group, but the sequences of non-responders seemed to mostly clustered in the middle of the phylogenetic trees at week 12 expect for the sequences of genotype B. This indicates that HBV genome does not contain patterns of variability sufficient for robust phylogenetic relation reconstruction.

DISCUSSION

In summary, we researched the quasispecies change of the HBV complete genome and four ORFs in chronic hepatitis B, which showed that the dynamic change of viral quasispecies within C-ORF was different during the early stage of IFN- α treatment. In the immune clearance phase at baseline, the patients who had greater viral quasispecies diversity at amino acid level had the more chance to obtain the early virological response during IFN- α treatment. We did not had the quasispecies information at week 12 in responders because of the HBV DNA load had a striking decline. However, the partial responders had higher mean genetic distance at amino acid level at week 12 compared to the non-responders.

The dynamic change in viral diversity of HBV appears to be focused on the precore/core region. It is implied that this greater diversity in responders seems due to specific immune pressure on the precore/core protein. Increased immune responses are accompanied by the stronger immune pressure inducing the selection of escape mutations, which leads to greater viral diversity.¹⁷ According to this scenario, in our study, non-responders had low quasispecies diversity and they obviously had a weak immune response at baseline. Lim et al¹⁸ reported that viral genetic diversity in genotype B CHB patients was greater in HBeAg seroconverters than in non-seroconverters at immune clearance phase. Wu et al¹⁹ obtained the same conclusion in genotype C patients. Our study focused on the virological response rather than HBeAg

Table 1. Clinical features of hepatitis B patients at baseline and week 12

	Responders (n = 6)	Partial responders (n = 3)	Non-responders (n = 7)	P value
Age (Years)	29.00 (22.00-3.006)	31.00 (22.00-32.00)	29.00 (25.00-38.00)	0.930
Sex (M/F)	4/2	2/1	5/2	1.000
Genotypes (B/C/B+C)	1/4/1	0/2/1	1/6/0	0.746
ALT (U/L)				
Baseline	307.20 (148.40-666.60)	138.60 (115.6-328.6)	79.00 (42.10-349.80)	0.089
Week 12	45.35 (32.70-123.70)	247.60 (32.40-273.50)	57.70 (37.60-314.10)	0.542
HBeAg (S/CO)				
Baseline	390.86 (79.22-1258.98)	1118.79 (680.54-1406.22)	1052.89 (16.20-1611.76)	0.551
Week 12	4.66 (1.04-24.11)	662.42 (377.53-893.94)	858.00 (9.73-1223.81)	0.010
HBV DNA (log ₁₀ copies/ml)				
Baseline	6.85 (5.12-7.85)	8.31 (7.32-8.58)	6.96 (6.00-7.90)	0.076
Week 12	0	5.80 (5.53-6.44)	6.02 (5.39-7.20)	0.004

Table 2. The dynamic change of quasispecies heterogeneity during the early stage of IFN- α treatment

Quasispecies heterogeneity	Responders (n = 6)	Partial responders (n = 3)	Non-responders (n = 7)	P value
Quasispecies complexity				
(nucleotide level)				
Baseline	0.927 (0.856-0.954)	0.876 (0.852-0.977)	0.907 (0.589-0.773)	0.431
Week 12		0.797 (0.715-0.819)	0.820 (0.259-0.945)	0.667
Quasispecies complexity				
(amino acid level)				
Baseline	0.780 (0.741-0.843)	0.731 (0.629-0.797)	0.685 (0.380-0.772)	0.200
Week 12		0.578 (0.537-0.742)	0.615 (0.196-0.699)	0.667
d(10 ⁻² substitution/site)				
(nucleotide level)				
Baseline	1.738 (0.851-3.812)	0.953 (0.587-2.340)	0.515 (0.336-2.715)	0.200
Week 12		0.841 (0.465-1.044)	0.518 (0.127-0.992)	0.267
d(10 ⁻² substitution/site)				
(amino acid level)				
Baseline	2.868 (1.727-5.411)	1.904 (1.272-2.349)	0.917 (0.449-3.695)	0.039
Week 1		1.755 (0.974-2.172)	0.869 (0.276-1.475)	0.047
dS (10 ⁻² substitution/site)				
Baseline	2.229 (0.791-4.736)	0.987 (0.638-0.569)	1.002 (0.214-3.925)	0.891
Week 12		0.941 (0.550-0.945)	0.574 (0.142-1.145)	0.383
dN (10 ⁻² substitution/site)				
Baseline	1.410 (0.804-3.188)	0.864 (0.570-1.143)	0.293 (0.205-0.651)	0.004
Week 12		0.789 (0.436-0.961)	0.449 (0.124-0.788)	0.183

Table 3. The correlations between quasispecies heterogeneity and HBV DNA as well as HBeAg

		Quasispecies complexity		d		dS	dN
		Nucleotide level	Amino acid level	Nucleotide level	Amino acid level		
DNA	r _s	0.127	0.157	0.267	0.262	0.377	0.247
	P	0.538	0.444	0.188	0.197	0.058	0.223
HBeAg	r _s	-0.063	-0.100	-0.096	-0.036	0.047	-0.045
	P	0.760	0.629	0.641	0.862	0.819	0.828

Figure 1. The quasispecies complexity (A and B), viral genetic distance (C and D), dS (the number of synonymous substitutions per synonymous site) and dN (the number of non-synonymous substitutions per non-synonymous site) (E and F) within C-ORF at baseline and week 12.

Notes: The mean genetic distance at amino acid levels of responders was greater than non-responders, partial responders was greater than that of non-responders, too, $P < 0.001$ and $P = 0.006$, respectively. The dN of responders was greater than non-responders, $P = 0.008$. At week 12, the mean genetic distance at amino acid levels of partial responders was significantly higher than that of the non-responders ($P = 0.047$).

Figure 2. The correlations between HBV DNA and HBeAg.

Figure 3. The maximum likelihood phylogenetic trees constructed for the sequences of baseline (left) and week 12 (right).
Notes: A triangle represent all strains of the same patient in the same genotype. The green lines represent responders, the red lines represent partial responders and the black lines represent non-responders. Five hundred replicates bootstrap analysis was performed. The bootstrap value higher than 70% was shown. The sequences for each patient were clustered together except for the patients with mixed genotype infection. However, no clusters were seen to be supported by robust bootstrap values for any group, but the sequences of non-responders seemed to mostly clustered in the middle of the phylogenetic trees at week 12 expect for the sequences of genotype B.

seroconverter but had the similar conclusion at baseline, too.

Cheng et al²⁰ observed the dynamic change of HBV quasispecies over 10 years in spontaneous HBeAg seroconverters, they found that in the period of immune tolerance, HBV quasispecies viral diversity was identical in seroconverters and non-seroconverters 10 years before seroconversion but started to increase approximately 3 years later, some 2 years later, HBV DNA then declined. Wu et al¹⁹ also found that interferon induced seroconversion increases viral diversity concurrent with a reduction in viral load, too. This phenomenon means that there is a gradual increase in viral evolution with HBV DNA decline, suggesting that immunological pressure had focused on HBV and caused immune escape. Our study found that responders have high quasispecies diversity at baseline, but the level declined after 12 weeks of IFN- α treatment, which is contradictory to the previous studies. The discrepancy might be explained as follows. In the early stage of IFN- α treatment, the main mechanism focus on direct inhibition of virus production rather than immunomodulatory properties.²¹ Antiviral effect produces a devastating blow to the virus quasispecies, while the immune escape has not yet formed or can not react immediately in 12 weeks, so that the quasispecies heterogeneity rapidly decline at early stage. The previous study had a longer observation time, the interval of two time points can reach as long as 12 months, so that the virus had enough time to escape, if we extend the observation time, it may also increase of quasispecies diversity. Correlation analysis revealed a significant negative correlation between the quasispecies complexity and HBeAg at week 12, which is consistent with the previous study.²⁰ Remarkably, the responders had a higher quasispecies diversity than non-responders at baseline in the mean genetic distance at amino acid level and dN, rather than the mean genetic distance at nucleotide level and dS. The dS represents 'silent' nucleotide changes that do not alter the amino acid encoded, whereas the dN represents 'replacement' nucleotide changes that do result in a different amino acid, which means that the mutation resulting in altering the amino acid encoded

was different between two groups. This phenomenon reveals that in the case of non-drug intervention, virus in the responders have more amino acid substitution, suggesting the host immune can cause immune escape, and the blow that the host immune to virus is weaker than IFN- α treatment so that virus has enough time to escape. In general, beneficial viral mutations may provide virus selective advantages in immune escape, resistance to anti-viral treatment, and virus replication²². Nearly all mutations associated with immune evasion are nonsynonymous, which indicates that these may weaken the binding of the viral epitope to immunoglobulin or the T cell receptor.²³

The limitations of this study were the lack of quasispecies heterogeneity from immune tolerance phase and the small size of study group, only 3 patients in partial responder group. In addition, there were only 12 weeks of observation time referring to some patients had combined nucleos(t)ide analogs because of the HBV DNA had not a satisfying decline at week 12. To extend the time of investigation may help us better understand the evolution of quasispecies of HBV during IFN- α treatment.

The novel findings of our study are that virological responders have distinctly higher viral diversity than non-responders at the early stage of IFN- α treatment, future studies are needed to address this issue. Long-term follow-up is required to determine whether hepatitis B viral diversity decreases or remains at a high level. Further study will be needed to elucidate the relationship between virological response and viral quasispecies in relation to antiviral therapy.

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