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Short Communication

Complexity and diversity of hepatitis B virus quasispecies: Correlation with long-term entecavir antiviral efficacy

Jin Tong [a], Qing-Ling Li [b], Ai-Long Huang [c,*], Jin-Jun Guo [a,*]

[a] Department of Gastroenterology and Hepatology, The Second Affiliated Hospital of Chongqing Medical University, Chongqing, China
[b] Institute of Life Sciences, Chongqing Medical University, Chongqing, China
[c] Key Laboratory of Molecular Biology on Infectious Diseases, Ministry of Education, Chongqing Medical University, Chongqing, China

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ABSTRACT

This study was undertaken to determine the complexity and diversity of hepatitis B virus (HBV) quasispecies during long-term antiviral therapy and examine their impacts on therapeutic outcome. Six chronic hepatitis B patients receiving entecavir monotherapy (0.5 mg/day) for 3 years were enrolled. The reverse transcriptase region of the HBV polymerase gene was sequenced and HBV quasispecies complexity and diversity were calculated. Sustained virological response (SVR) was defined as serum HBV DNA <57 IU/ml from 48 weeks after treatment to the end of follow up. Four patients achieved a SVR and the other two had a virological breakthrough at week 24. Despite comparable baseline levels, the complexity and diversity of HBV quasispecies were significantly ($p < 0.05$) reduced in sustained responders versus the patients with a virological breakthrough 48 weeks after treatment. Thus, reduction in HBV quasispecies complexity and diversity may predict an SVR to long-term entecavir monotherapy.

Hepatitis B virus (HBV) replicates via reverse transcription of a 3.5-kb pregenomic RNA intermediate by the virally encoded DNA polymerase. Nucleos(t)ide analogs such as lamivudine and entecavir are effective in suppressing HBV replication (Jafri and Lok, 2010) and commonly used to treat chronic hepatitis B (CHB). Due to greater potency (Lok and McMahon, 2007) and lower drug resistance rates (Colonno et al., 2006), entecavir instead of lamivudine has been recommended as the first-line therapy for nucleoside-naive CHB patients (Lok and McMahon, 2009).

HBV reverse transcriptase (RT) lacks a domain corresponding to 3′-5′exonucleolytic activity, thus resulting in an estimated 10^{10} base-pairing errors daily (Doo and Liang, 2001). Like other viruses with error-prone polymerases, such as human immunodeficiency virus (HIV) and hepatitis C virus (HCV), HBV has a quasispecies distribution in infected individuals (Günther et al., 1999). Due to genomic constraints, the diversity of HBV quasispecies is pronouncedly lower than that of HIV or HCV quasispecies (Margeridon-Thermet and Shafer, 2010).

The quasispecies distribution of HBV continuously changes during antiviral therapy (Duarte et al., 1994). Several studies have established a link between the evolution of HBV quasispecies and virological responses (Chen et al., 2009). This study was done to measure the complexity and diversity of HBV quasispecies in CHB patients during 3-year entecavir monotherapy and investigate their associations with therapeutic efficacy.

In total, we enrolled 6 nucleoside/nucleotide-naïve patients with CHB who received 3-year entecavir monotherapy (0.5 mg/day) at the Second Affiliated Hospital of Chongqing Medical University (Chongqing, China). They presented no signs of decompensated liver diseases. Exclusion criteria were HCV- or HIV-coninfection and evidence of nonviral hepatitis or liver cirrhosis. Written informed consent was obtained from each patient, and this study was approved by the Ethics Committee of Chongqing Medical University. The therapeutic efficacy was monitored for 156 weeks by measuring serum alanine aminotransferase (ALT) and HBV DNA levels. Initial response was characterized by a decrease in viral load by at least one log10 IU/ml at week 12 after therapy. Sustained virological response (SVR) was defined as serum HBV DNA <57 IU/ml from 48 weeks after treatment to the end of follow up, and biochemical response as normalization of serum ALT levels (Hoofnagle et al., 2007). Virological breakthrough was defined as $\geqslant 1.0 \times \log_{10}$ IU/ml above a nadir at least 1 month after achieving an initial response in a compliant patient (Lok and McMahon, 2007).

Serum samples were taken from each patient at different time points and HBV DNA was extracted. A 1050-bp fragment encompassing domains A to F of the HBV RT was amplified by PCR, using

* Corresponding authors. Address: Key Laboratory of Molecular Biology on Infectious Diseases, Ministry of Education, Chongqing Medical University, 1 Yixueyuan Road, Yuzhong District, Chongqing 400016, China. Tel.: +86 23 68485230; fax: +86 23 68486780 (A.-L. Huang), Department of Gastroenterology and Hepatology, The Second Affiliated Hospital of Chongqing Medical University, 76 Linjianglu Road, Yuzhong District, Chongqing 400010, China. Tel.: +86 23 63693326; fax: +86 23 68486780 (J.-J. Guo).

E-mail addresses: ahuang1964@yahoo.com.cn (A.-L. Huang), guojinjun1972@163.com (J.-J. Guo).

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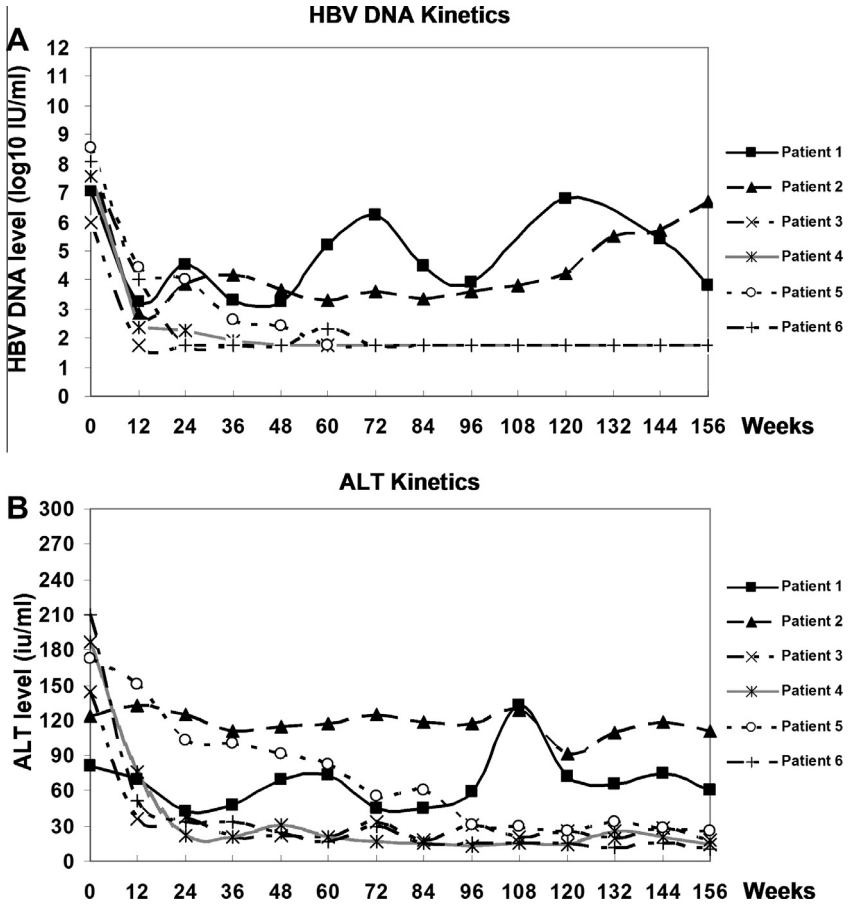

Fig. 1. Kinetics of serum HBV DNA (A) and ALT (B) in the 6 patients with chronic hepatitis B receiving the entecavir treatment.

Table 1
Evolution of HBV quasispecies in six patients receiving ETV therapy.

Patients	Weeks	WT (%)	L180 M ± M204 V/I	L180 M ± M204 V/I + S202G	Total number of clones
Patient 1	0	100	–	–	29
	24	100	–	–	29
	48	87	13%	–	30
	96	71	29%	–	28
	144	50	50%	–	30
Patient 2	0	100	–	–	30
	24	100	–	–	28
	48	88	12%	–	26
	96	92	8%	–	26
	144	21	–	79%	28
Patient 3	0	100	–	–	30
	24	100	–	–	29
	48	100	–	–	30
	96	100	–	–	30
	144	100	–	–	30
Patient 4	0	100	–	–	29
	24	100	–	–	25
	48	100	–	–	30
	96	100	–	–	30
	144	100	–	–	28
Patient 5	0	100	–	–	30
	24	100	–	–	30
	48	100	–	–	30
	96	100	–	–	30
	144	100	–	–	28
Patient 6	0	100	–	–	30
	24	100	–	–	29
	48	100	–	–	30
	96	100	–	–	30
	144	100	–	–	30

Abbreviations: G, glycine; I, isoleucine; L, leucine; M, methionine; S, serine; V, valine; WT, wild type.

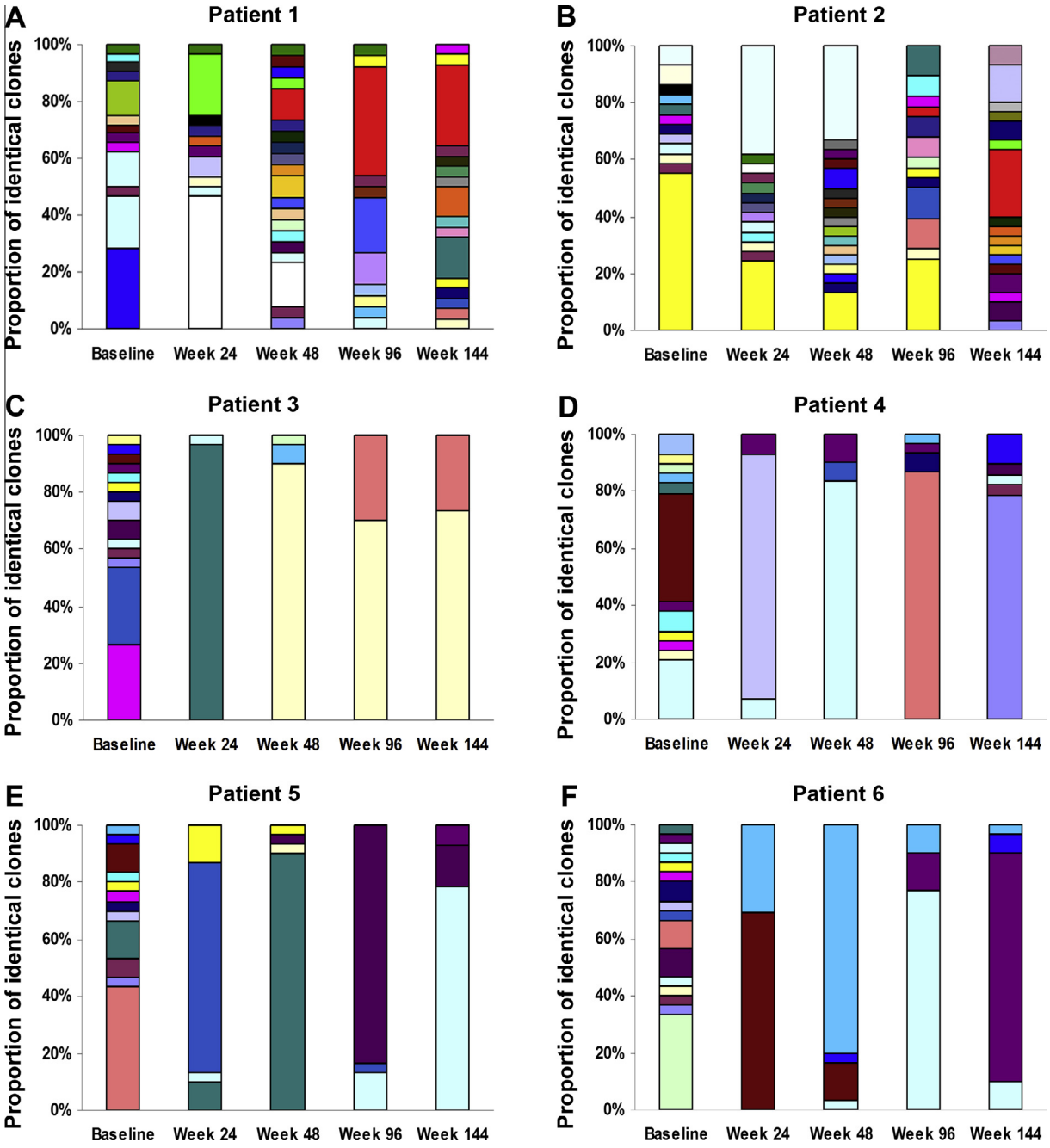

Fig. 2. Dynamic changes of HBV quasispecies within the reverse transcriptase region during the entecavir treatment. Vertical bars indicate the number and the proportion of viral quasispecies within each sample. Each color represents one kind of quasispecies. The same color in different time point means identical quasispecies.

the primers: forward, 5′-GTTCAGGAACAGTAAGCCC-3′ and reverse, 5′-GAAAGGCCTTGTAAGTTGGCG-3′. Nested PCR was done to amplify the RT region (domains A–E; 808 bp in size) when serum HBV DNA level was <19,000 IU/ml. Second-round primers were as follows: forward 5′-GACTCGTGGTGGACTTCTCTCA-3′ and reverse 5′-GGCATTAAAGCAGGATAACCACATTG-3′. PCR products were cloned and at least 25 positive clones per sample were sequenced. HBV quasispecies complexity expressed as normalized Shannon entropy (Sn) was measured by counting the total number of viral variants in a single sample (Domingo et al., 2006). The formula to calculate the Sn is $Sn = -\sum i(pi*lnpi)/lnN$, where N is the total number of clones and pi represents the frequency of each clone in viral quasispecies. Sn was analyzed at both the nucleotide and amino acid levels. HBV quasispecies diversity was assessed by calculating the mean genetic distance (d), number of synonymous substitutions per synonymous site (dS), and number of non-synonymous substitutions per non-synonymous site (dN). The genetic distance at the nucleotide level was calculated using the Maximum Composite Likelihood model and at the amino acid level using the JTT model. The dS and dN were determined using the modified Nei-Gojobori model with Jukes-Cantor corrections. Significant differences in the quasispecies diversity and complexity were determined using unpaired T-test. A p value <0.05 was considered statistically significant.

Fig. 1 presents the kinetics of serum HBV DNA and ALT during the entecavir treatment. All patients had an initial response at

12 weeks after treatment. Four patients (No. 3–6) had undetectable HBV DNA from 72 weeks post-treatment to the end of follow-up, indicative of an SVR. Moreover, a sustained biochemical response was achieved in the four patients. In contrast, the other two patients (Nos. 1 and 2) developed a virological breakthrough and showed no persistent reduction in the serum ALT level during treatment. At week 48, hallmark lamivudine resistance mutations (L180 M ± rtM204 V/I) were detected in patients Nos. 1 and 2 (Table 1). At week 144, circulating HBV variants harboring the rtM204 V/I + rtL180 M + S202G substitution became the dominant virus population (79%) in patient No. 2. The proportion of L180 M ± rtM204 V/I mutants in patient No. 1 increased to 50% at week 144, without emergence of rtM204 V/I + rtL180 M + S202G mutation. Additionally, no drug-resistant mutations tested were found in the 4 sustained responders during the follow-up period. The quasispecies composition in the sustained responders became less complex after treatment versus before treatment, while that in the patients with a virological breakthrough appeared to be more complex after therapy (Fig. 2). At baseline, the HBV quasispecies complexity showed no difference between the sustained responders and patients with a virological breakthrough, either at the nucleotide or amino acid level ($p > 0.05$; Table 2 and Fig. 3). After treatment, however, the four sustained responders had significantly lower quasispecies complexity than the other two patients, at both the nucleotide and amino acid levels ($p < 0.05$). All the measurements (d, dS, and dN) for quasispecies diversity were significantly reduced in the sustained responders from 24 or 48 weeks post-treatment ($p < 0.05$).

The heterogeneity of HBV quasispecies affects the outcome of antiviral therapy. Chen et al. (2009) reported that the HBV quasispecies complexity and diversity are significantly lower in responders than in non-responders at week 4 during the treatment with lamivudine. The HBV quasispecies complexity of responders is significantly lower than that of partial responders at week 4 during the entecavir therapy, whereas the quasispecies diversity of responders is higher than that of the partial responders (Liu et al., 2011). Our data revealed that after entecavir treatment, the responders showed a significant decrease in the HBV quasispecies complexity and diversity compared to the patients with a virological breakthrough. The evolution of HBV quasispecies may be modulated largely by selective pressure from antiviral drugs, which leads to emergence of drug-resistant variants (Sheldon et al., 2006). Since antiviral resistance mutations often have detrimental effects on the replication of HBV, acquisition of additional compensatory mutations is important to restore viral fitness (Sheldon et al., 2006). Under long-term selection with entecavir, the increased complexity and diversity of HBV quasispecies would raise the possibility to acquire drug resistance without reducing virus replication, which may explain virological rebounds in patients No. 1 and 2. Host immune pressure also contributes to the development of quasispecies complexity and diversity during HBV infection (Desmond et al., 2012). Hepatitis B surface antigen (HBsAg) is an important immunomodulator. It is involved in not only inducing the humoral immune response, but suppressing toll-like receptor-mediated innate immune response (Ait-Goughoulte et al., 2010). Thus, quantification of serum HBsAg and examination of

Table 2
Quasispecies complexity and diversity at baseline, week 24, week 48, week 96 and week 144.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Quasispecies complexity (nucleotide level)						
Baseline	0.5921	0.5173	0.6468	0.5914	0.5730	0.6961
Week 24	0.5125	0.5904	0.0445	0.1710	0.2469	0.1839
Week 48	0.8793	0.7064	0.1143	0.1655	0.1279	0.1648
Week 96	0.5943	0.7035	0.1796	0.1562	0.1570	0.1276
Week 144	0.7269	0.7578	0.1705	0.2358	0.1969	0.1202
Quasispecies complexity (amino acid level)						
Baseline	0.5199	0.3561	0.3260	0.2885	0.5668	0.3899
Week 24	0.3755	0.5552	0.0445	0.0866	0.1155	0.1839
Week 48	0.8232	0.6547	0.0430	0.0720	0.0720	0.1325
Week 96	0.5453	0.5105	0.1796	0.0856	0.1155	0.1276
Week 144	0.6933	0.6385	0.1705	0.1375	0.0772	0.1325
d (10–3 substitution/site) (nucleotide level)						
Baseline	1.2776	2.5968	6.4361	3.4566	3.2200	5.6462
Week 24	2.1407	2.1965	0.6912	0.9564	0.7699	1.4732
Week 48	9.2525	13.1085	0.2133	1.0310	0.7596	0.9511
Week 96	2.8362	4.0266	0.7186	0.4745	0.5386	0.9230
Week 144	15.9035	11.0714	0.6691	1.5463	0.9437	0.7828
d (10–3 substitution/site) (amino acid)						
Baseline	5.4083	4.8787	5.3076	2.3178	7.3367	5.8990
Week 24	4.1258	6.6003	1.3907	0.6840	1.0659	2.2143
Week 48	15.8610	11.2262	0.0010	0.5744	1.1560	1.4590
Week 96	6.2846	5.6230	2.2039	0.8873	1.0748	2.8302
Week 144	16.7136	23.1994	2.0524	1.8247	0.6077	1.4579
dS (10–3 substitution/site)						
Baseline	2.3412	2.6136	15.6095	9.0638	2.6872	12.1838
Week 24	2.6203	2.6571	0.7602	2.4616	1.4120	2.3326
Week 48	13.4184	27.9719	0.7019	2.8335	1.2667	1.5623
Week 96	2.5709	5.9621	0.0000	0.6315	0.6050	0.0000
Week 144	31.5205	11.7086	0.0000	2.5243	2.5237	1.0116
dN (10–3 substitution/site)						
Baseline	2.5181	2.2889	2.4814	1.0900	3.4433	2.7628
Week 24	1.9336	3.0368	0.6559	0.3200	0.4989	1.0693
Week 48	7.3967	6.0680	0.0000	0.2686	0.5382	0.6829
Week 96	2.9389	2.6083	1.0330	0.4085	0.5086	1.3232
Week 144	8.9890	10.5180	0.9620	1.1368	0.2861	0.6832

Abbreviations: d, genetic distance; dS, the number of synonymous substitutions per synonymous site; dN, the number of non-synonymous substitutions per non-synonymous site.

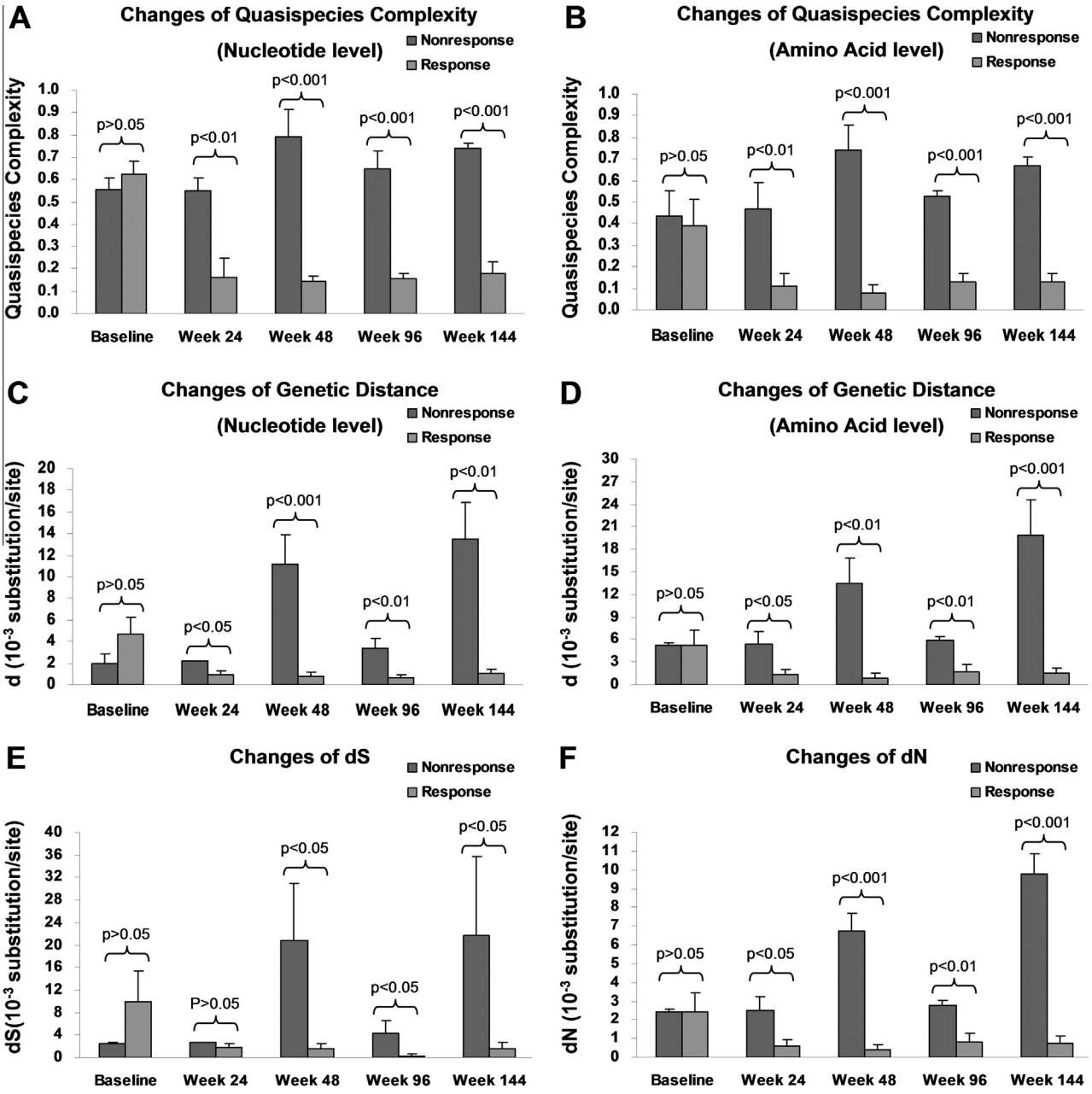

Fig. 3. Measurements of the HBV quasispecies complexity (A and B), viral genetic distance (C and D), dS (the number of synonymous substitutions per synonymous site; E) and dN (the number of non-synonymous substitutions per non-synonymous site; F) within the reverse transcriptase region at indicated time points. Black bars represent patients with a virological breakthrough and grey bars represent responders. The HBV quasispecies complexity and diversity was significantly reduced in responders during the ETV treatment.

mutations in this gene should provide more insight into the evolution of HBV quasispecies during antiviral therapy.

Liu et al. (2011) have proposed that monitoring the HBV quasispecies complexity could be more useful and reliable in predicting antiviral therapy outcome than examination of HBV DNA levels. Our results also show a significant association of the HBV quasispecies heterogeneity with therapeutic efficacy of entecavir, underscoring its implication in predicting virological response to long-term treatment with entecavir. However, our findings were promising but inconclusive due to small sample size and lack of validation in a separate cohort of patients.

In conclusion, evolutionary patterns of HBV quasispecies during long-term entecavir treatment are quite different between sustained responders and patients with a virological breakthrough. A reduction in the HBV quasispecies complexity and diversity is associated with a better virological response, which may be translated to improve antiviral treatment strategies.

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