Early changes in quasispecies variant after antiviral therapy for chronic hepatitis B

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Abstract. Hepatitis B virus (HBV) polymerase gene is targeted by nucleos(t)ide analogues (NUC), but it is unclear how HBV quasispecies of whole genome changes during early period of NUC treatment. To understand the unknown region of drug sensitivity and treatment resistance, HBV quasispecies of whole genome during early period of NUC treatment was examined using ultra-deep sequencing. Eleven patients with chronic HBV infection who received NUC treatment were enrolled in the current study. Viral DNA was extracted from serum samples before and early period of NUC treatment. Polymerase chain reaction analysis was subsequently performed on the DNA products. The viral quasispecies of the entire genome was analyzed by ultra-deep sequencing. The regions and positions corresponding to the changes in the quasispecies were investigated before and early period of NUC treatment. The secondary structure changes were predicted by mutations/substitutions detected using Lasergene Protean v14.1 software. The frequency of quasispecies variants increased significantly in the polymerase domain from before to early period of NUC treatment (3.08±1.28 vs. 3.51±1.47%, P<0.008), particularly the reverse transcription (RT) domain (3.76±1.25 vs. 4.52±1.37%, P<0.012). In addition, increased variation detected from HBsAg domain showed statistically significant during NUC treatment (6.81±3.26 vs. 7.81±3.26%, P<0.040). The amino acid (aa) mutations/substitutions were detected and compared from before to early period of treatment.

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Interestingly, most of them were located in the RT region (RT1 motif: aa21-aa51) and small S region in the early duration of NUC treatment. Furthermore, several mutation patterns, such as cI97L and cP130T showed alterations in the secondary structure and predicted antigenicity of HBV protein. Although the HBV whole genome can be affected by NUC treatment, RT 1 motif region and small S region are more sensitive to the early period of NUC treatment. This study suggested the initial changes of HBV quasispecies might affect the long-term drug sensitivity and resistance to NUC treatment.

Introduction

Hepatitis B virus (HBV) infection is a public health problem that causes liver diseases such as chronic hepatitis B (CHB) and progressive liver fibrosis, and may lead to cirrhosis and hepatocellular carcinoma (HCC). Approximately 2 billion people have ever been infected with HBV worldwidely, an estimated 257 million people are chronic carriers of HBV surface antigen (HBsAg), and an estimated 650,000 people will die due to CHB every year (1,2). HBV is a small enveloped virus, with a partially double-stranded DNA genome approximately 3.2 kb in length. The genome replicates by the reverse transcription (RT) activity of polymerase. The genome has four genes, the polymerase, surface, core, and X genes with overlapping open reading frames. The most commonly used drugs to treat CHB are nucleos(t)ide analogues (NUC), which inhibit HBV DNA polymerase activity and suppress HBV replication. Drugs available to treat hepatitis B include lamivudine, telbivudine, entecavir, adefovir, and tenofovir. Although all NUC are effective inhibitors of HBV replication, they are rarely curative, and few patients achieve HBsAg clearance. Therefore, long-term NUC therapy is necessary for most patients. However, long-term treatment increases the possibility of HBV genomic changes that confer drug or multidrug resistance, and reduce the treatment efficacy in CHB patients, and could progress to severe liver disease. Drug-resistance mutations were found in approximately 70% of patients treated with lamivudine for up to 5 years, 29% of patients treated with adefovir for up to 5 years, 34% of patients treated with telbivudine for up to 3 years, 1.2% of patients

treated with entecavir for up to 6 years (3), and no resistance to tenofovir for up to 7 years (4). The well-known drug resistances (e.g., rtL80I/V, rtI169T, rtV173L, rtL180M, rtA181T, rtT184A, rtV191I, rtS202G, rtM204V, rtM204I, rtV207I, rtN236T, rtM250L) have been reported during long-term NUC treatment (5-7). In addition, C gene deletion mutants and preS gene deletion mutants in patients during NUC therapy may promote vital immune escape resulting in severe liver disease and even hepatic failure (8-10). Cases of severe viral reactivation, viral recurrence, and exacerbation of hepatitis have been reported after discontinuation of NUC treatment (11).

Identification of HBV genotypic NUC resistance is mainly performed by PCR amplification and Sanger sequencing. New technologies, including ultra-deep sequencing methods, that enable high-throughput analysis of thousands of amplified regions were recently developed to increase sequencing capacity while generating clonal sequences (12). Ultra-deep sequencing method is an appealing feature for clinical virologists that are involved in the diagnosis of emerging viral infections, molecular epidemiology of viral pathogens, drug-resistance testing, and also do some basic and clinical research, especially the studies of HBV and HCV (7,12-15). Sanger Sequencing method was widely used for many years and ultra-deep sequencing replaced it recently due to its advantages what is high-throughput sequencing and more sensitive and efficient in detecting minor variants, which can identify mutants that constitute as little as 1% of the total viral population (7,14,16). Detecting minor population by ultra-deep sequencing assay would be particularly useful for HBV as emerging mutations may be obscured by the ongoing production of mutated variants for a prolonged period (7). In addition, more than one major population in one site can be detected by this assay while Sanger sequencing only can detected one major population in one site. Analysis of the HBV quasispecies in early period of NUC treatment will help us to clarify the substitutions related to genomic sensitivity or resistance to NUC treatment.

So far, although NUC treatment commonly treat patients with hepatitis B infection, the cost of treatment remains economic burden to the patients. Therefore, it is important to choose the appropriate medication and monitor changes in the medication, especially in the early period of treatment. The aim of this study is to understand the unknown region of drug sensitivity and treatment resistance during early period of NUC treatment.

In this study, HBV quasispecies of whole genome during early period of NUC treatment was examined using ultra-deep sequencing. we examined the initial changes in viral quasispecies in relation to drug sensitivity and treatment resistance following NUC therapy. Viral quasispecies were determined by ultra-deep sequencing.

Materials and methods

Patients and serum samples. Eleven CHB patients without advanced liver disease who received NUC treatment were enrolled in this study. The inclusion criteria were: i) HBV DNA titer >4 log copies/ml; ii) ALT ≥31 IU/l; iii) HBsAg positive for more than 6 months without antiviral therapy; iv) receiving NUC treatment after diagnosis; and v) without advanced liver disease such as liver cirrhosis. Patients co-infected with hepatitis C virus, hepatitis D virus, or human immunodeficiency virus etc. were excluded from this study. Blood samples were collected before, early period of NUC treatment and after 6 months of NUC introduction. Serum samples were stored at -80˚C before analysis. NUC treatment included adefovir (ADV) and entecavir (ETV).

The demographic and clinical data, including age, sex, results of standard test for CHB, were collected from medical record after institutional review board approval was obtained. Written informed consent was obtained from each patient before all the procedures in this study were done. This study was reviewed and approved by the Ethics Committee at Kobe University.

Serological and biochemical tests, and quantification of HBV DNA. The HBsAg titer was determined for all samples using the Lumipulse HBsAg-HQ assay on a fully automated chemiluminescent enzyme immunoassay system (Lumipulse; Fujirebio, Tokyo, Japan). The sensitivity of the Lumipulse HBsAg-HQ assay is 0.005 IU/ml and its coefficient of variation is <5.9% for samples with a low HBsAg titer. Alanine aminotransferase (ALT) levels were determined using standard procedures. The HBV DNA titer was measured using the TaqMan PCR assay (Cobas TaqMan; Roche Molecular Systems, Basel, Switzerland) with a lower limit of detection of 2.1 log copies/ml (14,17).

The Cobas TaqMan methodology uses qPCR to measure PCR product accumulation through a dual-labelled fluorogenic TaqMan probe. The fluorescent signal is generated by the 5'-nuclease activity of the DNA polymerase that digests the probe, separating a fluorescent reporter dye from a quencher dye. The probe specific for the detection of HBV is labelled with a different fluorogenic reporter dye from the QS-specific probe, while both contain the same quencher dye. The accumulation of amplification products is detected by continuously monitoring the release of the fluorescent reporter dyes during the annealing/extension step of each cycle. The PCR cycle at which the increase in normalized fluorescence of a sample exceeds background noise is called the critical threshold value (C_t). The C_t is inversely proportional to the initial copy number of the target template (18).

DNA extraction and HBV amplification. HBV DNA was extracted from 200 μl of serum using a QIAamp DNA blood Mini kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. The entire HBV genome sequence was analyzed using overlapping amplified PCR products. The primers (forward and reverse) were as follows: W1, 5'-GAT TCCTGCTCAAGGAACC-3' (nt 529-547) and 5'-GCCTAC AGCCTCCTAGTAC-3' (nt 1770-1788); W2, 5'-ACTGGG AGGAGTTGGGGGAG-3' (nt 1729-1748) and 5'-GCTGTA GCTCTTGTTCCCAAG-3' (nt 2827-2847); and W3, 5'-GGA CTCATAAGGTGGGAAAC-3' (nt 2461-2480) and 5'-GAC TTGGCCCCCAATACCAC-3' (nt 748-767). The PCR reaction comprised denaturing at 95˚C for 5 min, followed by 35 cycles of denaturing at 95˚C for 1 min, annealing at 55˚C for 1 min and elongation at 72˚C for 1.5 min, and a final extension at 72˚C for 10 min. The presence of an amplified product was confirmed by electrophoresis on 2% agarose gels, stained

with ethidium bromide, and visualized on a UV transilluminator. All of the necessary precautions were taken to prevent cross-contamination. A negative control was included in each assay.

Deep sequencing and analysis of amplified products. Ultra-deep sequencing was used to detect variations in the entire genomes of HBV quasispecies. The concentrations of the PCR products were measured using the Qubit high-sensitivity double-stranded DNA assay (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Next, a library of PCR products (<500 bp) of the viral genome (50 or 0.2 ng) was prepared using the Nextera DNA sample prep kit (Illumina, Inc., San Diego, CA, USA) or Nextera XT DNA sample prep kit (Illumina, Inc.), according to the manufacturer's instructions. The PCR products were uniformly sheared to 500-bp fragments using these kits, and the PCR product libraries were mixed with 1% 8 pM PhiX as a control and run on a MiSeq sequencer (Genome Analyzer; Illumina, Inc.) for paired-end 151 bp sequencing. Finally, image-based detection of the fluorescent signals was performed using MiSeq control software (Illumina, Inc.), and the images were used to develop the sequence data in the FASTQ format. Illumina paired-end sequencing was performed to generate overlapping read pairs (ORP) among relatively short-sequencing-fragment libraries combined with relatively long reads. A quality check and data trimming were necessary before assembling the sequences with Genomics Workbench software version 6.0.1 (CLC bio, Aarhus, Denmark). The sequencing results used in this analysis had a read quality of >80% of the consensus sequence estimated from a quality score of 30 (Q30) according to the manufacturer's (Illumina, Inc.) data. ORP and Q30 filtering were used to recover sequencing errors and eliminate any false-positive variants generated by PCR errors during sequencing, to increase the confidence that the sequence reads could be used to detect the viral variants with low abundance. The assembled sequence reads were mapped against the reference HBV genome of genotype C2 (GenBank accession no. AB246344). The viral quasispecies were evaluated in terms of their prevalence in the viral population, which was done using the setting 'read conflict' in Genomics Workbench. The nucleotide(nt) alterations leading to an amino acid (aa) changes were defined as variants. The percentage of the variant population was determined from the proportion of nt substitutions that changed the aa coverage depth (entire sequence reads in each nt position) (14). The viral quasispecies variants detected in >1% of the total viral population were classified into a major population (>10% of the total population) and a minor population (>1% to <10% of the total population). A free DNA translation tool (available at https://www.expasy.org/) was used to compare the variant population before and early period of treatment.

Calculation of antigenicity and secondary structure. To determine whether the aa changes affect the structure of polymerase protein and the antigenicity of HBsAg, hepatitis B core antigen (HBcAg) and HBx, we used the Jameson-Wolf Antigenic Index Prediction algorithm in Lasergene Protean v14.1 software (DNUCTAR Inc., Madison, WI, USA). The Antigenic Index Prediction analysis uses information from the primary aa sequence to predict the secondary structure (Chou-Fasman and Robson-Garner) (19).

Biostatistical analysis. Statistical analyses were performed with SPSS version 24.0 (IBM Corporation, S&I, Tokyo, Japan). Shapiro-Wilk test was used for checking the normal distribution of each pair. Differences between groups were examined using paired samples t-test (nominal variables) and Wilcoxon signed rank test (non-normal) and analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

Results

Clinical characteristics. The subjects' demographic, biochemical and virologic data are shown in Table I. Based on the entire genome, the HBV genotype was C2 in all eleven patients. The HBV DNA titer in samples collected from early period of NUC treatment was significantly lower than those collected before treatment (6.09±0.92 vs. 8.08±1.17 log copies/ml, respectively; P<0.000). The HBsAg titer was also lower during early period of treatment than before treatment (4.02±0.43 vs. 4.21±0.55 log10 IU/ml, respectively), but it did not show statistically significant between before and early period of treatment (P<0.091). The ALT level was significantly decreased during early period of treatment compared with before treatment (93±35 vs. 132±66 IU/l, respectively; P<0.037). A mean of 3,146,683±1,935,574 (range 898,165 to 6,576,948) bp nucleotides before treatment and 3,171,583±2,283,172 (range 1,125,580 to 8,863,180) bp nucleotides during early period of treatment were mapped into the reference consensus, and mean coverage depth of 82,839±48,562 (range 22,023 to 173,168) and 100,545±83,475 (range 31,440 to 311,408) was achieved for each nucleotide site of HBV sequences, respectively. Although we could not calculate statistically due to lack of data, the amount of HBV DNA and HBsAg level have been clearly decreased after six months of NUC treatment (Table I).

Frequency of viral quasispecies variants for each genomic domain. The frequency in major and minor viral quasispecies for each genomic domain was compared between before and early period of NUC treatment (Fig. 1). In the major population, the variation in the polymerase region was significantly greater during early period of NUC treatment than before treatment (3.51±1.47 vs. 3.08±1.28%, respectively; P<0.008; Fig. 1A). Of note, the variation in the RT domain of polymerase gene and the overlapping small S domain of the surface gene were significantly different between before and early period of treatment (3.76±1.25 vs. 4.52±1.37%, respectively, P<0.012; 6.81±3.26 vs. 7.81±3.26%, respectively, P<0.040; Fig. 1C). In the minor populations, although there were slight variations between before and early period of treatment, these were not statistically significant (Fig. 1B and D).

Amino acid changes in viral population ongoing NUC treatment. The aa mutations/substitutions that accounted for >1% of the variations in the total viral population were examined, and the variations were compared between before and early period of treatment. The positions were analyzed if they were detected in ≥6 patients (>50% of subjects). The nt and corresponding aa mutations that differed significantly

Table I. Demographic, biochemical, and virologic characteristics of the 11 patients.

No.	Age/gender	Antiviral treatment	Duration (days)	HBV DNA (logcopy/ml)			HBsAg (log10 IU/ml)			ALT (IU/l)		
				Before	Early	After 6 M	Before	Early	After 6 M	Before	Early	After 6 M
1	57/F	ETV	43	7.4	5.8	3.1	3.34	3.45	3.53	120	63	35
2	30/M	ETV	32	8.8	6.4	3.1	4.65	3.72	3.23	165	90	131
3	39/M	ETV	148	9.3	7.3	6.1	4.31	4.45	4.35	90	132	86
4	66/F	ETV	106	9.1	6.7	5.7	4.68	4.46	4.42	62	49	40
5	28/M	ADF	85	8.7	6.5	2.3	3.76	3.63	3.62	363	82	95
6	48/F	ETV	127	7.0	4.5	4.3	3.37	3.36	3.35	163	17	18
7	75/M	ETV	64	8.9	6.4	4.0	4.03	4.13	3.76	32	43	36
8	70/M	ETV	63	9.3	6.6	ND	4.75	4.55	ND	123	105	ND
9	58/F	ETV	14	5.7	4.8	2.7	3.95	3.91	4.09	39	29	24
10	26/M	ADF	123	7.2	5.1	3.7	4.78	4.13	4.00	192	77	56
11	67/F	ETV	57	7.6	6.9	4.6	4.65	4.42	4.30	99	41	69
Mean				8.08	6.09		4.21	4.02		132	66	
SD				1.17	0.92		0.55	0.43		93	35	
P-value				0.000			0.091			0.037		

B/E, before treatment/early period of treatment; ETV, entecavir; ADF, adefovir; HBsAg, hepatitis B virus surface antigen; ALT, alanine aminotransferase; SD, standard deviation; ND, no data; After 6M, after 6 months of NUC introduction.
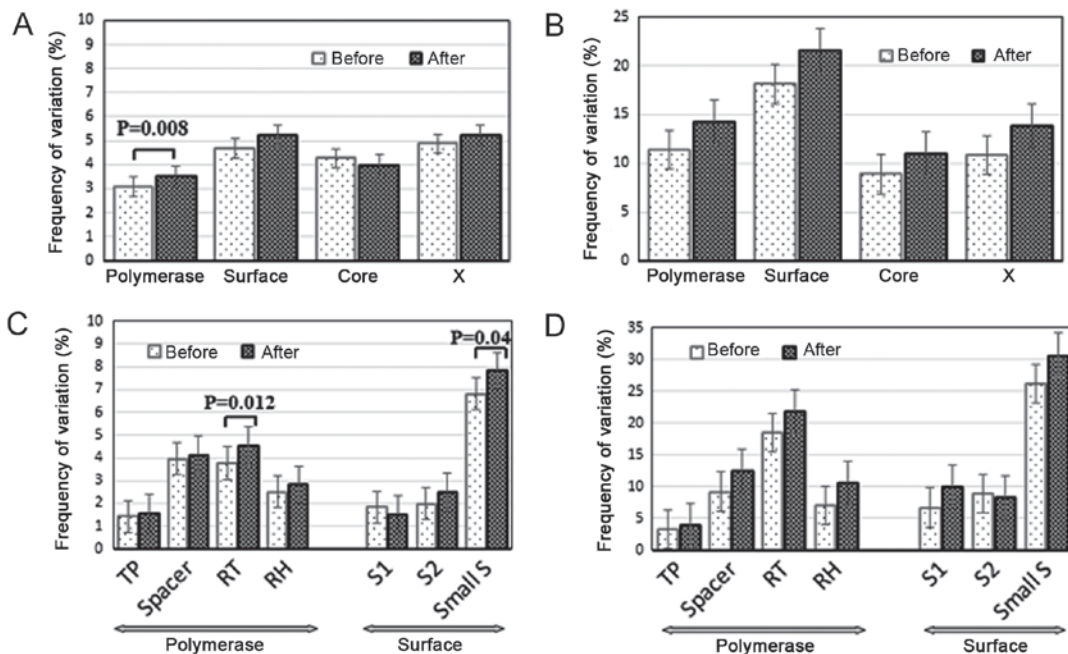

Figure 1. Frequencies of viral variants before and early period of nucleos(t)ide analogues (NUC) treatment in the major (A and C) and minor (B and D) populations. The variation in the polymerase region in the major population was significantly greater in samples collected during early period of NUC treatment than in samples collected before treatment (A). The variation in the RT domain of the polymerase region and the overlapping small S region of the surface gene was significantly different between before and early period of NUC treatment (C). Although there were slight variations in the minor populations between before and early period of treatment, these were not statistically significant (B and D). The value showed as a percentage. (A) frequency of variation in 4 main domains in major population (B) frequency of variation in 4 main domains in minor population (C) frequency of variation in sub-domains in major population (D) frequency of variation in sub-domains in minor population.

are listed in Table II. Numerous mutations were detected in the RT region, which is targeted by NUC. Fig. 2 shows a map of the polymerase domain boundaries. Interestingly, most of these mutations were located in the RT region,

RT1 motif (aa21-aa51) which may affect viral replication. It was suggested that the RT1 motif in the RT region was more sensitive than other subdomains in the early period of treatment. In addition, we found significant variation in the

Table II. Sites and variations of mutations according to genomic region.

Position (nt)	Polymerase (RT)	Surface (small S)	Core	X	Before treatment	Early period of treatment	P-value
		Region (aa)			Variations of viral quasispecies (%), (mean ± SD)		
A162G		N177S (N3S)			98.40±1.77	95.84±7.62	0.037
G217T	V376F (V30F)				18.15±5.93	21.02±5.35	0.029
G217C/T		L195F (L21F)			19.06±6.18	22.00±5.55	0.027
G220T	D377Y (D31Y)				13.12±3.73	15.03±3.30	0.044
G220C/T		L196F (L22F)			13.67±3.88	15.77±3.38	0.039
A221C	D377A (D31A)	T197P (T23P)			1.54±0.56	2.01±0.40	0.027
A221G	D377G (D31G)	T197A (T23A)			2.89±1.39	3.71±0.92	0.013
A221T	D377V (D31V)	T197S (T23S)			4.50±2.69	6.11±2.18	0.017
C222T		T197I (T23I)			2.02±1.11	2.70±0.82	0.018
T228C		I199T (I25T)			1.03±0.46	1.44±0.26	0.023
A233C	H381P (H35P)	T201P (T27P)			10.83±2.96	12.53±2.82	0.011
A235C	N382H (N36H)				5.67±1.68	7.09±1.53	0.005
T241C	T384P (T38P)				3.68±1.06	4.32±1.07	0.041
T247G	S386A (S40A)				10.18±2.25	11.65±3.34	0.044
A245C/T247A/G		S205R (S31R)			12.82±3.02	14.77±3.88	0.036
C248G	S386C (S40C)	L206V (L32V)			1.00±0.49	1.60±0.49	0.010
T249C		L206P (L32P)			1.82±0.77	2.43±0.89	0.035
C255T		S208L (S34L)			1.13±0.42	1.55±0.48	0.022
A272C	Q394P (Q48P)	N214H (N40H)			4.20±1.72	5.27±2.28	0.025
A280T	R397W (R51W)				2.42±1.20	3.39±1.72	0.046
G281T	R397M (R51M)	G217W (G43W)			1.61±0.64	1.97±0.81	0.043
A293C	H401P (H55P)	T221P (T46P)			2.30±1.41	3.05±1.74	0.013
A333G		H234R (H60R)			0.92±0.33	1.21±0.27	0.043
A337G	T416A (T70A)				1.02±0.47	1.39±0.27	0.023
G633A		R334K (R160K)			3.76±4.61	5.48±4.55	0.036
A1138C	N683H (N337H)				79.16±40.77	75.86±39.95	0.005
G1613A	R841K				14.8±22.7	19.91±26.60	0.040
T1753C				I127T	11.77±25.07	16.67±26.71	0.036
A1965C			D51A		0.93±0.41	1.33±0.21	0.036

Values show mean mutation frequency and standard deviation (SD): The mean of the ratio of total mutant clones to total aligned coverage at each nucleotide sites were detected from eleven subjects; nt: nucleotide; aa, amino acid; RT, reverse transcription.

small S region which overlaps with RT region that may affect HBsAg synthesis. In terms of the other regions, we found several substitutions in the core region and X region but only cD51A, xI127T showed statistically different during early period of treatment.

Changes in the predicted antigenicity of HBV protein. To determine the changes in antigenicity, we used the Jameson and Wolf antigenic index prediction model (19). We used DNAStar software to compare the antigenicity between before and early period of treatment for the aa substitutions/mutations in which the variant population occurred in >1% of the total population. The aa variations linked to changes in the secondary structure and their predicted antigenicities are summarized in Table III.

Discussion

HBV infection is one of the most significant medical problems worldwide. It can cause a variety of liver diseases, including acute hepatitis, chronic hepatitis, liver cirrhosis, and HCC. Therapies for CHB are likely to delay the progression of the severe liver disease. Patients with CHB are treated with NUC, which are effective inhibitors of HBV replication. However, few patients achieve HBsAg clearance, and most patients require long-term NUC treatment. In this study, adefovir and entecavir were selected by the doctor's decision as both of them were first line drug for CHB.

Adefovir belongs to acyclic phosphate class. The structural similarity of adefovir to its natural substrate dATP, together with its small flexible linker, allows it accessibility of adefovir
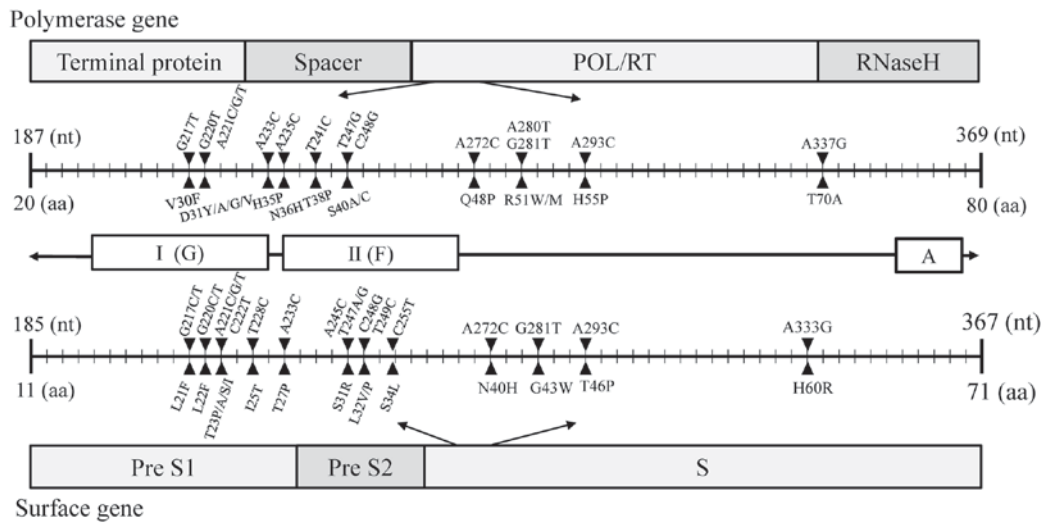
Figure 2. Structures of the polymerase and surface genes. The polymerase gene contains four domains, with seven conserved boxes (A-G) in the RT domain. Boxes A-E are not shown or are incomplete in this figure. The figure also shows part of the small surface gene, which is overlapped by the polymerase gene. Most of mutations which showed significantly different were located in the RT region, RT1 motif (aa21-aa51) which may affect viral replication. From this figure, the RT1 motif in the RT region was more sensitive to NUC treatment than other subdomains in the early-duration of treatment. In addition, the variants detected from S region located in the small S region which overlaps with RT region. NUC, nucleos(t)ide analogues.
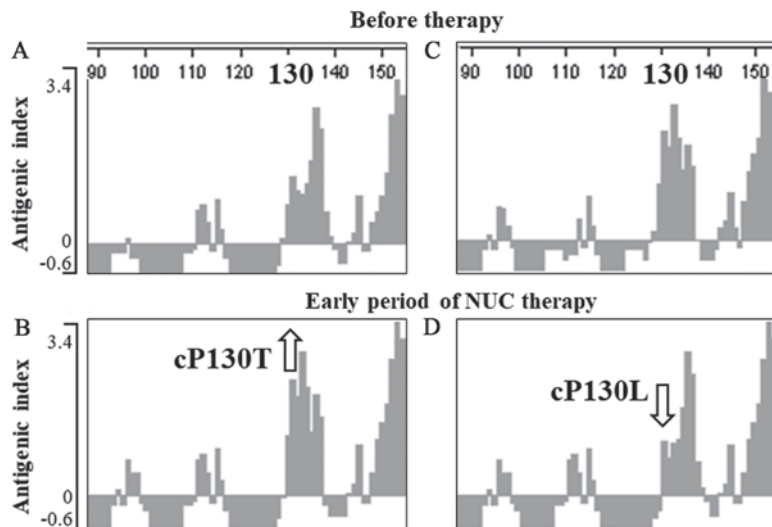

Figure 3. Antigenicity plots based on the amino acid sequences of HBcAg. Two aa variations (cP130T and cP130L) were observed to result in a marked altera-tion on a large area surrounding the amino acid distribution; the P130T pattern caused an increased antigenicity index in their surroundings (A and B), while the P130L pattern caused marked decreased antigenicity after early period NA treatment (C and D). The major changes in each antigenic index are indicated by an arrow. NUC, nucleos(t)ide analogues; HBcAg, hepatitis B virus surface antigen.

to the polymerase (20). The primary mutations associated with adefovir resistance are rtA181T/V, rtI233V, and rtN236T. The secondary mutations include rtV84M, rtS85A, rtV214A, rtQ215S, rtN238T/D (5-7). The rtN236T mutation may disrupt the hydrogen bond between rtN236 and rtS85 and rtN236 and adefovir diphosphate, thereby decreasing the binding affinity for adefovir. Similarly, other mutations (rtP237H, rtN238T/D, rtV84M, and rtS85A) around this region may also disrupt the triphosphate binding site. The mutation rtA181V/T in domain B may bend the α helices through hydrogen bond formation, and drug resistance may thus result from an allosteric change in the catalytic site. Other mutations (rtV214A, rtQ215S) do not directly interact with the nucleotide but may cause allo-steric changes affecting the polymerase function (21-23).

Entecavir belongs to the cyclopentane class and has potent and selective activity against HBV. Two resistance patterns have been recognized: rtM250V + rtI169T + M204V + L180M and rt184G + rtS202I + rtM204V + rtL180M. The require-ment of multiple mutations for entecavir resistance suggests a higher genetic barrier to resistance. The rt250 mutation appears to alter the binding interaction between the primer strand and the template DNA. The rt169 interacts with the dNTP and may work cooperatively with the mutation at rt250. The combination of these mutations may affect elongation of the DNA strand. The rtT184 G and rtS202I mutations interact and affect the hydrophobic core region between the α helix of the DNA template binding region and the β sheet, which encodes the 2 catalytic aspartate residues in the C domain.

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Table III. Mutation patterns in hepatitis B virus proteins.

Author, year	Regions	Mutation nt	Mutation aa	Antigenic index Before treatment	Antigenic index Early period of treatment	Description	(Refs.)
Caligiuri et al, 2016	RT	A841C	N238H↑	3.4	2.76	Drug-resistance mutation	(30)
Salpini et al, 2017	HBsAg	T784G	S210R↓	-0.4	-0.2	HCC development	(37)
Kim et al, 2004; Saha et al, 2014; Carman et al, 2014	HBcAg	C2288A	P130T↑	1.4	2.24	MHC II	(40,51,57)
Kim et al, 2016; Kim et al, 2007; Kim et al 2008	HBx	G1386A	V5M↑	-0.1	-0.3	HBeAg-negative disease	(45,58,59)
Kim et al, 2016; Xie et al, 2014; Li et al, 2015		A1479C	T36P↑	-0.25	-0.25	B cell epitope	(45,60,61)
Wu et al, 2010	RT	C294A	H55Q ↑	0.16	0.31	Drug-resistance mutation	(46)
Fu et al, 2016		T1078G	S317A↑	0.6	-0.3	Drug-resistance mutation	(47)
Thedja et al, 2015	HBsAg	G285A	G44E↑	0.15	1.9	Non-synonymous mutation	(48)
Lee et al, 2013; Mayaphi et al, 2013; Saha et al, 2014; Liu et al, 1995; Akarca et al, 1995; Kim et al, 2012	Pre-core	G1896A	W28*↓	0	-0.4	HCC development	(49-54)
Lee et al, 2013; Saha et al, 2014; Yeh et al, 2015; Inoue et al, 2011	HBcAg	A2189C	I97L↓	0.3	0.45	MHC II	(49,51,55,56)
		C2289T	P130L↑	2.24	1.05	-	-

↑: Increased prevalence of mutation ongoing NUC treatment. ↓: Decreased prevalence of mutation ongoing NUC treatment. -: no previous report nt, nucleotide; aa, amino acid; RT, reverse transcription; HBsAg, hepatitis B virus surface antigen; HBcAg, hepatitis B virus core antigen; HBeAg, hepatitis B e antigen; HBx, hepatitis B virus protein X; HCC, hepatocellular carcinoma; MHC, major histocompatibility complex.

The substitutions at residues rt184 and rt202 are predicted to magnify the impact of the lamivudine-resistant mutation in the YMDD loop, affecting substrate discrimination upon nucleotide loading and addition to the primer terminus. In the absence of the lamivudine-resistant mutations, the rtT184 G and rtS202I mutations do not affect the susceptibility of HBV to entecavir (23).

Although these two therapy show the different of mutagenicity, the potency of ADV treatment on HBsAg reduction is not significantly different with that of ETV treatment (24).

The present study revealed significant reductions in the HBV DNA titer and ALT level during NUC treatment (Table I). However, long-term NUC therapy could lead to the emergence of drug resistance and multidrug resistance mutations in the HBV genome. From previous studies (5-10), the well-known resistance mutations in RT region and deletion mutations were not found in this study. The present study should provide further insight into the molecular mechanism of drug resistance in early duration of treatment.

It is perhaps unsurprising that the difference in variation between before and early duration of treatment was greatest in the RT region, a target of NUC therapy. But it is still interesting that the variation in the major population increased during early period of treatment after analyzed the subdomain of RT region. The polymerase gene contains four domains (TP, Spacer, RT, and RH domains). RT plays an important role in viral replication. Because RT lacks a proofreading ability, the error rate and high viral replication rate (10^{11} virions per day) contribute to the high mutation rate (10^{-5} per nt per generation) (25,26). The RT region is divided into seven boxes (A-F). The RT1 motif, which contains boxes G and F (aa24-36 and aa37-47, respectively), is required for Hε binding (3,26). It was suggested that mutations in the RT1 motif, such as rtV30/rtD31, may disrupt protein priming and affect viral replication (27-29). In this study, it is suggested that RT1 motif region is more sensitive to NUC treatment and might be a crucial factor for inhibiting the RT activity and suppress HBV replication (Table I) in the early period of NUC treatment.

There are two types of mutations associated with NUC resistance: Primary resistance mutations, which are directly responsible for drug resistance, and secondary mutations, which promote or enhance the replication competence of resistant strains. Several well-known mutations, particularly rtM204I/V (primary resistance mutations) and rtL80V/I (secondary mutations), which occur following long-term treatment and increase viral replication, were not detected in this study. However, we did detect several mutations, notably rtH55Q and rtN238H, which are frequently generated during long-term NUC treatment and are associated with M204I/V (30).

The treatment of CHB with all the by NUCs could result in changes to HBsAg, such as rtA181T/sW172*. *In vitro* analysis of rtA181T/sW172* HBV has shown secretion defect of the viral particles to extracellular, resulting in intracellular retention of surface proteins and lower viral loads detected extracellularly (31-33). As shown in this study, the changes in RT 1 motif of polymerase gene can affect small S gene of surface gene because it overlapped with each other. Therefore, the mutation in small S gene can affect the synthesis and secretion of HBsAg that become undetectable by current commercially available kit, resulting in 'false' decrease of HBsAg. In addition, major HBsAg are encoded by small S gene (the large and middle S proteins are expressed at levels of about 5-15 and 1-2% compared with small S protein) (34) and includes the regions involved in the attachment of the virus to hepatocytes as well as the main epitopes recognized by neutralizing antibodies. The aa changes in the surface gene may lead to the adaptive evolution of the viral gene, resulting in altered antigenicity (35). The polymerase gene completely overlaps the HBV surface gene. It is suggested that changes in the S region where overlapped with polymerase gene (RT1 motif) might decrease the synthesis and secretion of HBsAg. Mutations in the polymerase gene decrease replication activity, but some double mutations in the polymerase gene and the 'a' determinant region actually increase replication activity (36). In our study, we found no double mutations in polymerase and the 'a' determinant region. However, several secondary resistance mutations were detected in the MHR region, notably sG44E. In addition, the variation in S210R which has been suggested that long-term therapy is associated with reduced cell cycle activity and liver carcinogenesis (37) was detected from early period of treatment. It is showed that it might change the secondary structure and then alter the antigenicity of HBsAg in the early period of treatment.

In this study, the proportion of rtH55Q, rtN238H, rtS317A, sG44E showed an increasing trend during early period of treatment as potential drug resistance mutations in the early duration of treatment. It was considered that these drug resistance mutations may enhance viral replication activity and thus affect the clinical outcome with the persist treatment; sS210R with decreased proportion may indirectly contribute to the activity of these mutations. Therefore, it was suggested that these substitutions should be monitored since the early period of treatment.

The double A1762T and G1764A mutations in the basal core promoter region are associated with altered expression of HBeAg and viral replication (38). However, the impact of basal core promoter and precore mutations on viral replication remains controversial (39). In this study, the variations A1762T/G1764A were found in 9/11 patients (81.8%), but there were no significant differences in these variants between before and early period of treatment. The most prevalent mutation in the precore region was G1896A (W28*), which introduces a translational stop codon W28* in the HBeAg sequence, and halts protein synthesis. A recent meta-analysis indicated that the precore mutation G1896A was significantly associated with increased risk of HCC and progression of liver disease, especially in Asians (40). In the early period of treatment, W28* was detected from three subjects. In other words, NUC treatment could protect or delay the progression to severe. The variations I97L and P130T in the core regions were decreased by NUC treatment. These mutations are located in the MHC class II restricted region and are significantly associated with the pathogenesis of HCC owing to the loss of HBcAg assembly (41,42). It was also reported that the mutation P130T is strongly correlated with the mutation I97L (43,44). It was suggested that these mutations may affect the secondary structure of HBcAg and increased its immunogenicity, attenuating liver carcinogenesis. cP130L with increased proportion more than 15% during early period of treatment was detected from this study. It was inferred that cP130L might alter the secondary structure of HBcAg and

lead to reduce the antigenicity of HBcAg according to predicted antigenicity (Fig. 3). Subsequently, HBcAg could not be recognized by immune system resulting in immune escape. Therefore, it was considered that cP130L might be one of immune escape variants resulting in the persistence of HBV. The identification of this hypothesis should be supported by further experiments and more data.

HBx is a multifunctional protein involved in viral replication and affects host gene expression related to HCC (45). HBx also plays important roles in the establishment and maintenance of chronic infection by altering the host's innate immunity. In this study, there were trends towards increased proportion of V5M and T36P following NUC therapy, and these mutations may result in the alteration of the secondary structure of HBx. V5M is common in HCC patients, which suggested that this mutation was a genuine HCC-specific mutation that may play a key role in the progression from chronic hepatitis or liver cirrhosis to HCC (45). By contrast, T36P, which is located in the B cell epitope region, may lead to persistent infection by providing the virus with a mechanism to evade the host's humoral immune response. In this study, increased variations in V5M or T36P were found from three patients. It is unknown whether the risk of liver cancer is increased due to the aa variations. Regular follow-up and monitoring the progression of liver diseases is important to predict the clinical outcome of patients with CHB.

This study has some limitations. Only a small number of serum samples could be analyzed because of the high cost of the technique and complexity of the analysis for the HBV whole genome. In addition, it is still unclear that resistant mutations will generate after long-term NUC treatment among these patients. These limitations will be solved by long-term follow up and further studies examining additional patients.

In summary, although the HBV whole genome can be affected by NUC treatment, RT 1 motif and small S region are more sensitive to the early period of NUC treatment. This study suggested the initial changes of HBV quasispecies might affect the long-term drug sensitivity and resistance to NUC treatment.

The whole regions of HBV were affected by NUC treatment. Several variations were detected in quasispecies at several positions of the HBV genome. RT1 motif region and small S region are susceptible in the early period of NUC treatment resulting in inhibiting RT activity and synthesis and secretion of HBsAg. The initial changes of HBV quasispecies might be related to the long-term drug sensitivity and resistance. In this study, it is suggested that B and C subdomains in RT region should not only attached importance to as drug resistance mutations found, others subdomains such as G and F also should be followed during NUC treatment especially early-period of treatment. These results will be enable us to improve the development of diagnostic tools and establish more efficient therapeutic protocols for CHB.

Acknowledgements

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