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Amino acid similarities and divergences in the small surface proteins of genotype C hepatitis B viruses between nucleos(t)ide analogue-naïve and lamivudine-treated patients with chronic hepatitis B

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ABSTRACT

Entire C-genotype small hepatitis B surface (SHBs) sequences were isolated from 139 nucleos(t)ide analogues (NA)-naïve and 74 lamivudine (LMV)-treated chronic hepatitis B (CHB) patients. The conservation and variability of total 226 amino acids (AAs) within the sequences were determined individually, revealing significant higher mutant isolate rate and mutation frequency in LMV-treated cohort than those in the NA-naïve one ($P = 0.009$ and 0.0001, respectively). Three absolutely conserved fragments (s16–s19, s176–s181 and s185–s188) and seven moderately conserved regions (a few AA sites acquiring increased variability after LMV-treatment) were identified. The significant mutation rate increase after LMV-treatment occurred primarily in major hydrophilic region (except 'a' determinant) and transmembrane domain 3/4, but not in other upstream functional regions of SHBs. With little influence on immune escape-associated mutation frequencies within 'a' determinant, LMV-monotherapy significantly induced classical LMVr-associated mirror changes sE164D/rtV173L, sI195M/rtM204V and sW196L/S/rtM204I, as well as non-classical ones sG44E/rtS53N, sT47K/A/rtH55R/Q and sW182stop/rtV191I outside 'a' determinant. Interestingly, another newly-identified truncation mutation sC69stop/rtS78T decreased from 7.91% (11/139) in NA-naïve cohort to 2.70% (2/74) in LMV-treated one. Altogether, the altered AA conservation and diversity in SHBs sequences after LMV-treatment in genotype-C HBV infection might shed new insights into how LMV-therapy affects the SHBs variant evolution and its antigenicity.

1. Introduction

Hepatitis B virus (HBV) is an enveloped double-stranded DNA virus containing four so largely compact and overlapping open reading frames (ORF) (C, S, P and X) in genome that S ORF is embedded within P ORF (Echevarria and Avellon, 2006). S ORF encodes large (LHBs), middle (MHBs) and small hepatitis B surface proteins (SHBs), while P ORF encodes polymerase harboring error-prone reverse transcriptase (RT) domain required for viral replication (Locarnini and Yuen, 2010). Besides, due to a high rate of viral turnover, there are substantial naturally-occurring variations in SHBs and the overlapping RT in chronic hepatitis B (CHB) patients (Chotiyaputta and Lok, 2009; Liu et al., 2010).

By contrast, iatrogenic mutations within SHBs/RT can be selected under immunological or antiviral drug selection pressure (Chotiyaputta and Lok, 2009). On one hand, vaccine-induced immune-escape mutations in and around 'a' determinant (e.g., sG145R) are responsible for SHBs antigenicity alteration, undermining the efficacy of neutralizing antibodies to SHBs (anti-HBs) (Torresi, 2008). On the other, nucleos(t)ide analogues (NAs)-induced primary resistance mutations within RT (e.g., rtM204I/V) confer resistance to refractory strains, decreasing viral susceptibility to antiviral therapies. Secondary/compensatory mutations (e.g., rtL180M) restore the compromised viral replication fitness caused by the acquired antiviral drug resistance (Lok et al., 2007).

Furthermore, like two sides of one coin, the variations/mutations within SHBs/RT can be coupled due to the shared nucleotide substitution in both codons (e.g., sW196L/S/rtM204I), suggesting a "mirror change" mechanism that the amino acid (AA) change in one viral protein might affect the other (Torresi, 2008; Sheldon and Soriano, 2008). Accordingly, the growing anti-HBV NAs utilization has provoked concerns on the potential effects of NA-induced mutations in RT on SHBs antigenicity (Torresi et al., 2002). It was revealed that a recombinant genotype-A SHBs bearing lamivudine (LMV) resistance (LMVr)-associated mirror changes sI195M/rtM204V + sE164D/rtV173L, could acquire immune evasion

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activity with disrupted 'a' determinants of SHBs (Sloan et al., 2008). Recently in a studied population mainly with genotype-D infection, Pollicino and colleagues reported an increased rate in SHBs-antigenicity-modifying mutations within and around 'a' determinant after LMV-treatment (Pollicino et al., 2009).

However, it is still unknown whether or not LMV-treatment dramatically affects the AA diversity and conservation within or outside 'a' determinant of SHBs in other HBV genotypes infection. To help understand this, a population-based investigation was implemented to characterize and compare full-length genotype-C SHBs sequences isolated from LMV-treated and NA-naïve Chinese CHB patients. Notably, genotype A–D each might have its own preferred wild-type AAs and variation/mutation types at some sites in RT/SHBs (Liu et al., 2010; Mirandola et al., 2012). In order to exclude the influence of HBV genotypes as a variable on clinical data and HBV mutational analysis, this study enrolled genotype-C strains alone which are considered exceedingly prevalent and aggressive in China (Chinese Medical Association, 2007). Moreover, considering the worldwide LMV-experienced patient population, and growing risks of interpersonal transmission and cross-resistance with other L-nucleosides (Lu and Zhuang, 2009; Yeh, 2010; Teo and Locarnini, 2010), LMV, though with a low genetic barrier, was focused on in this research.

2. Materials and methods

2.1. Patients

CHB diagnosis was conducted accordingly (Chinese Medical Association, 2007). The studied SHBs sequences were isolated from 213 genotype-C-infected CHB patients with positive hepatitis B surface antigen (HBsAg) and viral DNA. Among them, 139 never underwent LMV, adefovir-dipivoxil (ADV), entecavir, telbivudine or tenofovir disoproxil fumarate (TNF) treatment, while the remaining 74 had received LMV-monotherapy. Exclusion criteria were hepatitis C or human immunodeficiency virus co-infection, autoimmune liver disease, or alcohol/drug abuse.

2.2. Laboratory tests and HBV sequence analyses

As described elsewhere (Liu et al., 2010; Yang et al., 2010; Li et al., 2012), clinical indexes for CHB were determined, and entire RT sequences covering SHBs were amplified, sequenced and genotyped. Mutation analyses of the nucleotide (nt) and deduced AA sequences were performed accordingly. Notably, the SHBs-serotype-associated polymorphism sK/R122, sP/T/L/I127, sF/Y134, sA/G/V159, sK/R160, sV/A177 and sP/Q178 were considered as wild-type AAs (Echevarria and Avellon, 2006).

Furthermore, we analyzed the distribution of wild-type and mutant AA sites in various SHBs sections. As an integral membrane protein, SHBs consists of four hydrophobic transmembrane domains (TM) (TM1, s8–s22; TM2, s80–s98; TM3/4, s170–s226) (Schadler and Hildt, 2009; Siegler and Bruss, 2013) (Fig. 1A). The N-terminus (s1–s7) before TM1 as well as the major hydrophilic region (MHR, s99–s169) between TM2 and TM3/4 are exposed on virion surface, while the domain s23–s79 between TM1 and TM2 is inside viral particles (Schadler and Hildt, 2009; Siegler and Bruss, 2013) (Fig. 1A). Within MHR, s124–s147 was defined here as 'a' determinant, the major target of anti-HBs (Chotiyaputta and Lok, 2009; Locarnini and Yuen, 2010; Teo and Locarnini, 2010).

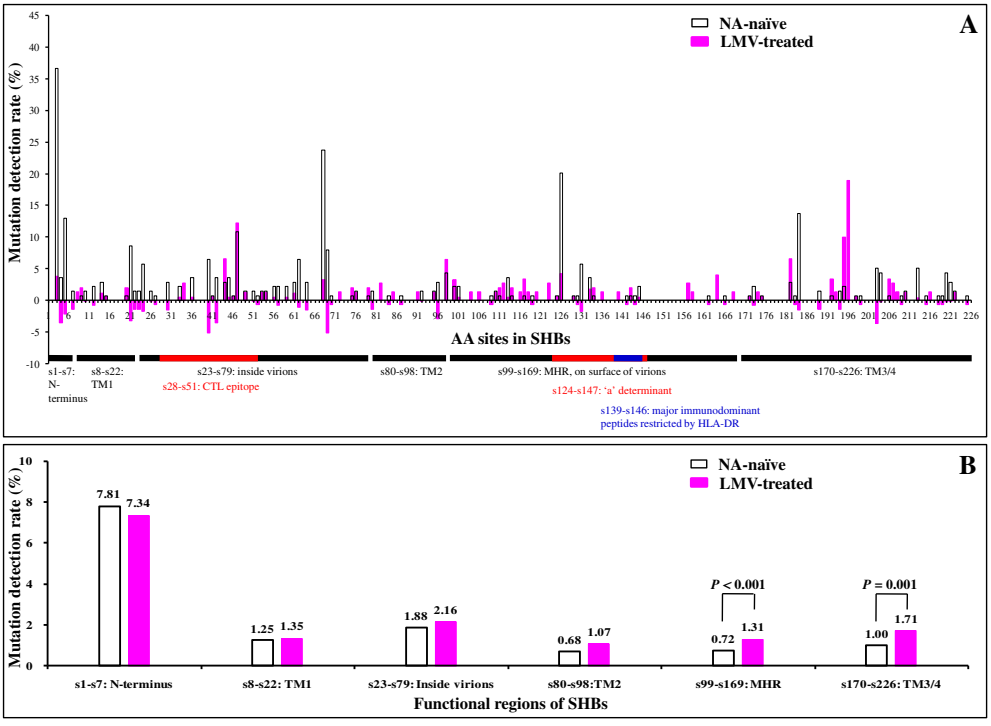

Fig. 1. AA similarity and discrepancy of SHBs variation/mutation profiles between the NA-naïve and LMV-treated CHB patients. *A*, the white bars indicated the absolute mutation rates observed at totally 226 SHBs AA sites among139 NA-naïve patients. The pink ones showed the mutation rate difference of LMV-treated group (74 patients) compared with the rate of the NA-naïve one, the positive value of which (above the X-axis) suggested the former was greater than the latter and vice versa. Notably, SHBs can be divided into four TM regions (TM1, 2 and 3/4) and three non-TM regions [N-terminal s1–s7 and MHR (s99–s169) on the virion surface as well as s23–s79 inside virions] (Echevarria and Avellon, 2006; Schadler and Hildt, 2009). The horizontal black, red and blue lines below the X-axis represented the various functional regions of SHBs, whose biological significance was listed below the lines. *B*, The absolute mutation rates within the seven functional regions in NA-naïve and LMV-treated cohorts were shown by white and pink bars, respectively. The LMV-treated group had significantly higher mutation rates in MHR ($P < 0.001$) and TM3/4 ($P = 0.001$) than NA-naïve one. AA, amino acid; TM, transmembrane region; MHR, major hydrophilic region; CTL, cytotoxic T lymphocytes; HLA-DR, human leukocyte antigen-D related. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. Statistics

Chi-square, Fisher's exact and *t* tests were performed with Graph Pad Prism 5.0 (San Diego, CA) when appropriate, providing two-tailed *P* values.

2.4. Sequence accession numbers

The HBV entire RT sequences isolated in this study were deposited in GenBank with accession numbers of KC191828–KC192040.

3. Results

3.1. Patient characteristics

Table 1 summarized the demographic, biochemical and virological characteristics of NA-naïve and LMV-treated patients, suggesting no significant difference in male-to-female ratio, age, alanine aminotransferase (ALT), aspartate aminotransferase (AST), HBV DNA or hepatitis e antigen (HBeAg) status.

3.2. Overall picture of SHBs variation/mutation and conservation profiles in both cohorts

Mutation rates at individual AA site were determined at aggregately 226 positions of 213 SHBs fragments (Fig. 1A). 88.49% (123/139) of NA-naïve and 98.65% (73/74) of LMV-treated isolates were found with variations/mutations, suggesting a significant increase in mutant SHBs rate after LMV-treatment ($P = 0.009$). There were 419 AA changes at 97 sites among 139 NA-naïve SHBs sequences and 300 substitutions at 94 positions in 74 LMV-treated ones, suggesting significantly higher average mutation frequency in LMV-treated cohort [1.79%, 300/(226 × 74)] than that in NA-naïve one [1.33%, 419/(226 × 139)] ($P = 0.0001$).

Surrounded by SHBs variable regions, ten conserved SHBs areas ($\geqslant$4 AAs long) were identified in both cohorts (Fig. 1A and Table 2), which could be divided into two subgroups: (1) three highly conserved fragments without variations/mutations regardless of treatment; (2) seven moderately conserved regions were intact in NA-naïve cohort, but variable almost within or around 'a' determinant in LMV-treated one. In the latter subgroup, the AAs important for SHBs functions kept constant regardless of treatment (Table 2), e.g., the N-glycosylation site sN146 in s146–s161 (Schadler and Hildt, 2009). This polypeptide fragment was highly conserved in NA-naïve group but affected slightly at its C-terminal fourth and fifth AAs by LMV-treatment. Besides, the second longest conserved fragments s102–s108 (GMLPVCP) and s135–s141 (PSCCCTK) were both in MHR as major B-cell epitopes (Echevarria and Avellon, 2006; Sayiner et al., 2008). These three regions harbored three disulfide-bridge-building cysteine pairs (sC107–sC138, sC137–sC149, and sC139–sC147), which were conserved in all cases (Weber, 2005). This was also true for the remaining cysteine pair (sC121–sC124) within the moderately conserved s120–s124 (Table 2).

3.3. Mutation distribution and frequency in various SHBs sections

AA change distribution within four TM and three non-TM regions of SHBs were compared between NA-naïve and LMV-treated patients (Fig. 1B and Table 3). The N-terminal s1–s7 on virion surface, the hottest mutation spot among all sections, did not display any significant difference in mutation rates between both groups. Neither did TM1/2 nor their linking region s23–s79. By contrast, significant enhancement of AA changes in MHR ($P < 0.001$) and TM3/4 ($P = 0.001$) were observed in LMV-treated group relative to NA-naïve one. Further analysis revealed that there was no significant difference in mutation rate within 'a' determinant between both cohorts, demonstrating that LMV dramatically increased variability in MHR except 'a' determinant which was still mainly surveilled under host immune pressure (Lok et al., 2007; Weber, 2005).

Furthermore, six LMVr-associated SHBs/RT mirror changes exhibited significantly higher mutation rates after LMV-treatment (Table 3), i.e., sG44E/rtS53N and sT47K/A/rtH55R/Q within s23–s79, sE164D/rtV173L within MHR but downstream of 'a' determinant, as well as sW182stop/rtV191I, sI195M/rtM204V and sW196S/L/rtM204I within TM3/4 (Chotiyaputta and Lok, 2009; Svicher et al., 2009; Zhao et al., 2012).

3.4. Further analyses of AA conservation and variability within MHR and TM3/4

To comprehensively understand how LMV-treatment significantly elevate the variability within MHR and TM3/4, the conserved and mutated AA sites were subjected to careful characterization. To begin with, among 71 AA sites of MHR (s99-s169) (Fig. 1A), 50.70% (36/71) were found highly conserved in both cohorts, including eight cysteines, six prolines, four serines and three tryptophans which were harbored by the highly conserved regions identified in Table 2. Meanwhile, 49.30% (35/71) were mutated, with 27 sites having increased variability after LMV-treatment. The overall mutation rate of these 27 sites was significantly higher ($P < 0.0001$) in LMV-treated group [3.30%, 66/(27 × 74)] than in NA-naïve one [1.49%, 56/(27 × 139)], which was attributed not only to enhanced mutation frequency at the 14 shared mutant positions before and after LMV-treatment (e.g., sE164D), but also to the emergence of 13 new mutated sites after LMV-therapy (with significant increase in mutated site number, $P < 0.0001$). As for the distribution of these 27 variable sites along MHR, nine sites in 'a' determinant made less contribution to enhancing overall MHR mutation rate than the other parts of MHR, which could be illustrated by the observation that there was no significant mutation rate increase at sI126T/S, the highly

Table 1
Main characteristics of the study population.

Characteristics	NA-naïve ($N = 139$)	LMV-treated ($N = 74$)	*P*-value[a]
Sex (male/female)	98/41	58/16	0.216
Age (years), median (range)	41.00 (20.00–81.00)	41.00 (13.00–73.00)	0.480
ALT (IU/L), median (range)	45.50 (0.00–1393.00)	73.00 (4.00–1099.00)	0.934
AST (IU/L), median (range)	37.00 (0.00–1205.00)	69.00 (0.00–1207.00)	0.762
HBV DNA ($\log_{10}$ copies/mL), median (range)	6.67 (3.08–8.79)	5.76 (3.26–8.08)	0.065
HBeAg (positive/negative)	97/42	48/18[b]	0.665
LMV exposure (weeks), median (range)	0.00 (0.00–0.00)	56.00 (3.00–364.00)	NA

NA, not available.
[a] *P*-values represented the results of statistical tests between the NA-naïve and LMV-treated groups, respectively. Student's *t* test was performed for age, ALT, AST and HBV DNA, and Fisher's exact test for sex and HBeAg.
[b] HBeAg status of eight patients were not available.

Table 2
Conserved SHBs peptide fragments identified in the studied patients.

Peptide fragments	Predicted location in SHBs[a]	Consensus AA seuqences (N → C)	Mutation rate in NA-naïve group % (*N* = 139)	Mutant sequence and rate (%) in LMV-treated group (*N* = 74)
Highly conserved				
s16–s19	TM1	Q-A-G-F	0.00	0.00
s176–s181	TM3/4	L-V/A-P-F-V-Q	0.00	0.00
s185–s188	TM3/4	G-L-S-P	0.00	0.00
Moderately conserved				
s71–s74	Inside virions	G-Y-R-W	0.00	G-**C**-R-W (1.35%, 1/74)
s88–s91	TM2	L-L-C-L	0.00	L-L-C-**H** (1.35%, 1/74)
s102–s108	MHR, upstream of 'a' determinant	G-M-L-P-V-C-P	0.00	G-M-L-P-**G**-C-P (1.35%, 1/74) G-M-**W**-P-V-C-P (1.35%, 1/74)
s120–s124	MHR, partially in 'a' determinant	P-C-K-T-C	0.00	**T**-C-K-T-C (1.35%, 1/74) P-C-K-**I/A**-C (2.70%, 2/74)
s135–s141	Completely in 'a' determinant of MHR	P-S-C-C-C-T-K	0.00	P-**Y**-C-C-C-T-K (1.35%, 1/74) P-S-C-C-C-**I**-K (1.35%, 1/74)
s146–s161	MHR, partially in 'a' determinant	N-C-T-C-I-P-I-P-S-S-W-A-F-A/V-R/K-F	0.00	N-C-T-C-I-P-I-P-S-S-W-**V**-F-A-R-F (2.70%, 2/74) N-C-T-C-I-P-I-P-S-S-W-A-**S**-A-R-F (1.35%, 1/74)
s167–s171	Spanning MHR and TM3/4	S-V-R-F-S	0.00	S-**I**-R-F-S (1.35%, 1/74)

sA/V159 and sR/K160 were serotype related sites and were considered as wild-type AAs. The AA substitutions were indicated by underlined bold letters.
[a] The location of each peptide fragment was illuminated by Schadler and Hildt (2009).

Table 3
The distribution of AA changes in the various functional regions of SHBs in the NA-naïve and LMV-treated cohorts.

SHBs functional regions	NA-naïve (*N* = 139)		LMV-treated (*N* = 74)		*P*-value for average mutation rates between both cohorts	AA sites with significant difference in mutation detection rates between both cohorts (NA-naïve *vs* LMV-treated)
	No. of mutations	Average mutation rate within this region (%)[a]	No. of mutations	Average mutation rate within this region (%)		
N-terminus (s1–s7)	76	7.81	38	7.34	0.742	Not found
TM1 (s8–s22)	26	1.25	15	1.35	0.803	Not found
Region inside virions (s23–s79)	149	1.88	91	2.16	0.297	sG44E/rtS53N [2.88% (4/139) *vs* 9.46% (7/74), *P* = 0.016] sT47K/A/rtH55R/Q [10.79% (15/139) *vs* 22.97% (17/74), *P* = 0.026]
TM2 (s80–s98)	18	0.68	15	1.07	0.194	Not found
MHR (s99-s169)	71	0.72	69	1.31	< 0.001	sE164D/rtV173L [0.00% (0/139) *vs* 4.05% (3/74), *P* = 0.041]
TM3/4 (s170–s226)	79	1.01	72	1.74	0.001	sW182stop/rtV191I [2.88% (4/139) *vs* 9.46% (7/74), *P* = 0.039] sI195M /rtM204V [2.16% (3/139) *vs* 12.16% (9/74), *P* = 0.004] sW196L/S/rtM204I [0.00% (0/139) *vs* 18.92% (14/74), *P* < 0.001]

[a] Average mutation rate within this region, equals the number of mutations within the functional region divided by the AA number of the functional region and patient number of the cohort. For instance, average mutation rate within N-terminus (s1–s7) among NA-naïve cohort: 7.81% = 76/(7 × 139) × 100%.

active mutation site in this region [20.14% (28/139) in NA-naïve *vs* 24.32% (18/74) in LMV-treated group, *P* = 0.489].

Of the 57 sites within TM3/4 (s170–s226) (Fig. 1A), 45.61% (26/57) were highly conserved regardless of treatment. There were 16 sites having significantly increased overall mutation rate from 1.03% [23/(16 × 139)] in NA-naïve group to 4.48% [53/(16 × 74)] after LMV-treatment (*P* < 0.0001). Mutations at these 16 variable SHBs sites (e.g., sW182stop, sI195M, and sW196S/L) were found with corresponding LMVr mirror changes in RT (e.g., rtV191I and rtM204I/V) from downstream part of B domain to the linking region between C and D domains (Table 3).

3.5. SHBs truncation mutations

We identified four types of SHBs nonsense mutations with their mirror changes in RT (Table 4). sW182stop/rtV191I occurred significantly more frequently in LMV-treated than in NA-naïve group (*P* = 0.039). By contrast, sC69stop rate decreased by 5.21% after LMV-treatment. Interestingly, 76.92% (10/13) of sC69stop and 54.55% (6/11) of sW182stop cases suggested the co-existence of truncated and wild-type AAs at the same site.

4. Discussion

In this investigation, the AA similarity and divergence in SHBs were identified between 74 LMV mono-therapy and 139 NA-naïve genotype-C-infected CHB patients. Consequently, 419 AA changes were observed among 88.49% (123/139) of NA-naïve SHBs sequences and 300 substitutions in 98.65% (73/74) of LMV-treated ones (Fig. 1A), hinting that LMV-treatment could significantly enhance the AA variability in SHBs.

Flanked by variations/mutations in SHBs (Fig. 1A), conserved regions containing three intact fragments were identified regardless of NA-treatment (Table 2). Seven 4–16 AAs long moderately conserved regions were discovered absolutely unchanged in NA-naïve patients but variable at some unimportant sites in LMV-treated cohort (Table 2). These findings suggested that these conserved sites and fragments might be important for stabilizing SHBs structure and ensuring its functions. For instance, the absolutely conserved disul-

Table 4
The rates of truncation mutations in SHBs in the studied patients.

SHBs truncation mutations	Deletion length (AA)	Concomitant mirror change in RT region	Mutation rate in 139 NA-naïve patients (%)	Mutation rate in 74 LMV-treated patients (%)	*P*-value[a] between mutation rates of both cohorts	Mutation rate in total 213 patients (%)
sC69stop	158	rtS78T	7.91	2.70	0.227	6.10
sW172stop	55	rtA181T	0.72	1.35	1.000	0.94
sW182stop	45	rtV191I	2.88	9.46	0.039*	5.16
sW199stop	28	rtV207I	0.72	0.00	1.000	0.45

[a] Fisher's exact or chi-square tests were employed and asterisk symbols indicated a significant difference.

fide bridge-building cysteines were recently found critical for virion secretion (Kwei et al., 2013). Remarkably, the HBV DNA fragment nt589-nt609, covered by the conserved sequence encoding s146-s156, was deemed as a successful RNA interference (RNAi) target for silencing SHBs expression (Panjaworayan and Brown, 2011). Notably, the highly conserved nt203-nt212 encoding s16–s19 identified here, might become a valuable candidate target for development of novel RNAi strategies for both NA-naïve and LMV-experienced HBV strains (Panjaworayan and Brown, 2011; Ivacik et al., 2011).

Further, the overall mutation rates within four TM and three non-TM regions were compared before and after LMV-treatment (Fig. 1B and Table 3), suggesting that significant mutation rate increase after LMV-treatment occurred primarily in MHR (except 'a' determinant) and TM3/4, but not in other upstream functional regions. First of all, although overall MHR mutation rate was significantly higher in LMV-treated cohort than in NA-naïve one (Table 3), no significant difference was found in the mutation rate within 'a' determinant between both cohorts, revealing that immune selection pressure was still the main driving force for the mutations within 'a' determinant during LMV-treatment (Teo and Locarnini, 2010). Moreover, unlike previous findings in genotype-D and -A SHBs variants (Pollicino et al., 2009), we did not observe dramatic changes in immune-escape-associated mutation rates within 'a' determinant in our genotype-C SHBs after LMV-treatment, e.g., sI126T/S, sT131N/P, sM133T, sD144N or sG145R/A/E (data not shown). This might be also explained by plausible existence of genotype-D-specific mutation sites within 'a' determinant (Liu et al., 2010; Mirandola et al., 2012).

By contrast, we found that LMV significantly induced mutations sE164D/rtV173L downstream of 'a' determinant in MHR, as well as sI195M/rtM204V and sW196S/L/rtM204I in TM3/4 in genotype-C infection (Table 3), which were reported to disrupt 'a' determinant conformation and modify SHBs antigenicity (Sloan et al., 2008). Notably, in our LMV-treated cohort, up to 28.38% (21/74) had primary LMVr rtM204I/V mutants but only 5.41% (4/74) carried secondary/compensatory mutation rtV173L (Chotiyaputta and Lok, 2009). The mutation rate gap between rtM204I/V and rtV173L suggested the existence of other secondary/compensatory mutations, e.g., rtL80I and rtL180M (data not shown) preferred by rtM204I and rtM204V, respectively (Yang et al., 2010). One remarkable difference between rtL80I or rtL180M and rtV173L is that the former two mutations do not exert any effect on SHBs codons or expression whereas the latter with its concomitant SHBs mutation sE164D provides rtM204V/sI195M mutant with a reduced epitope-antibody binding avidity in the loops of 'a' determinant as compared with the classical sG145R vaccine-escape mutant (Sloan et al., 2008). Therefore, the combined mutations in same viral genomes (sI195M/rtM204V + sE164D/rtV173L or sW196S/L/rtM204I + sE164D/rtV173L) call for further cautious surveillance and assessing their possible disconcerting consequences for HBV immunization campaign (Torresi, 2008; Locarnini and Yuen, 2010). Besides, non-classical LMVr-associated mutations sG44E/rtS53N and sT47K/A/rtH55R/Q inside virions were found significantly associated with LMV-treatment (Table 3). Remarkably, rtH55R/Q was reported associated with sI195M and sW196S/L(rtM204V/I) specifically in genotype-C HBV (Zhao et al., 2012).

However, the overlapping RT/SHBs theory could not explain the enhanced AA diversity in the moderately conserved region within or around 'a' determinant (Yeh, 2010). These vaccine-escape-like mutants may be attributed to LMV-induced prolonged viral suppression and SHBs seroclearance decreasing or reversing ratio of SHBs to anti-HBs to such an extent that some selection pressure is created (Yeh, 2010). Altogether, our observations revealed that LMV could enhance SHBs genetic diversity outside 'a' determinant by mirror change-dependent mechanism, and did so within it via mirror change-independent approach (Teo and Locarnini, 2010; Yeh, 2010).

The last group of screened mutation types was SHBs truncation mutations (Table 4). sC69stop and sW182stop were found prevalent in our NA-naïve and LMV-treated cohorts, respectively. Similar to our observations, sW182stop/rtV191I were reported not only in NA-naïve blood donors (Meldal et al., 2011) but also as putative NA resistance mutation in various NA treatments including ADV, LMV and TNF (Yang et al., 2002; Wakil et al., 2002; Sheldon et al., 2005; Liu et al., 2010). In our LMV-treated cohort, sW182stop/rtV191I was significantly higher than the ADV-resistance-associated sW172stop/rtA181T [9.46% (7/74) *vs* 1.35% (1/74), $P = 0.029$], suggesting that the former mutation pair was more associated with LMV selection (Chotiyaputta and Lok, 2009). Furthermore, similar to sW172stop/rtA181T with an elevated oncogenicity, sW182stop/rtV191I was reported to contribute to the progression of chronic genotype-C HBV infection towards hepatocellular carcinoma, which partially explained the strong relationship between genotype-C infection and oncogenic progression and also underscored the importance of studying the disease progression of genotype-C-infected, LMV-treated patients carrying sW182stop/rtV191I mutants (Yeh, 2010; Lee et al., 2012).

Additionally, this study identified 13 sC69stop/rtS78T mutants (Table 4), which were rarely reported in previous studies (Locarnini and Yuen, 2010; Yeh, 2010). These 68-AA long mutants were so highly variable that 84.62% (11/13) had distinct variations within the shortened sequences, which might serve as a cytotoxic T lymphocytes epitope pool for mimicking normal SHBs to distract host immune pressure (Fig. 1A). Furthermore, 76.92% (10/13) of these mutations were detected coexistent with wild-type sC69, suggesting the impaired virion secretion by the mutants might be rescued by wild-type strains (Gao et al., 2007; Kwei et al., 2013). Moreover, Table 4 showed that LMV-treatment appeared to decrease sC69stop mutants, whose significance demanded further clarification. To our knowledge, this was the first study to dissect the potential roles of sC69stop in HBV survival and antiviral treatment.

In summary, in our genotype-C-infected patients, SHBs AA conservation at the functionally-important sites and freshly-identified conserved peptide fragments, represented critical SHBs regions not affordable to change regardless of treatment. Although immune selection pressure remains major factor inducing variations within 'a' determinant after LMV-treatment, the AA discrepancy before and after LMV-treatment, reflected the driving force of LMV-treatment on viral diversity within and outside 'a' determinant in differ-

ent pathways. These observations shed new insights into how LMV-treatment alters the evolution and antigenicity of SHBs variants in genotype-C-infected CHB patients. This study also gleaned solid population-based data for future improvement of HBV vaccination programs and LMV-treated patient management strategies, suggestive of important clinical and public health significance. Furthermore, our discoveries advocate vigilant monitoring and phenotypical testing *in vitro* of the novel mutants identified here.

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