Original article

Profile of HBV antiviral resistance mutations with distinct evolutionary pathways against nucleoside/nucleotide analogue treatment among Chinese chronic hepatitis B patients

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Background: Antiviral drug-resistant HBV mutants under a variety of treatment protocols are complex and only partly understood. Here, a population-based cross-sectional study was performed to analyse the profile of resistance mutations in distinct evolutionary pathways refractory to different nucleoside/nucleotide analogues (NAs).

Methods: Serum samples of 199 chronic hepatitis B patients undergoing NA treatment from five hospitals in four northern cities of China were obtained between January 2007 and July 2009. The genotypic resistance of HBV in these samples was characterized. The full-length HBV reverse transcriptase region was amplified, sequenced and analysed with particular focus on the following NA-resistant changes: rtL80, rtI169, rtV173, rtL180, rtA181, rtT184, rtA194, rtS202, rtM204, rtN236 and rtM250.

Results: Among 199 HBV isolates, 30 (15.08%) and 169 (84.92%) were genotypes B and C, respectively, and 65 (32.66%) harboured NA-resistant mutations. The prevalence of mutations at rtM204 was 34.33% in 134 patients who had received or who had been exposed to lamivudine-based therapy. Five cases of rtN236 mutations were detected exclusively among 75 patients receiving adefovir-dipivoxil-based therapies. A total of 19 cases of multidrug resistance rtA181 mutations were observed in those with lamivudine-, adefovir-dipivoxil- or telbivudine-based treatment (186 cases), but not in those undergoing entecavir treatment (13 cases). Mutations were not found at rtI169, rtT184, rtA194 or rtS202. rtM204 mutations (27 rtM204I, 15 rtM204V and 5 rtM204I/V cases) were detected at the highest frequency among 65 mutants (72.30% [47/65]) and found to display 16 combination mutation patterns, in which rtM204I and rtM204V were significantly associated with rtL80I/V and rtL180M, respectively ($P<0.01$).

Conclusions: One-third of the studied population harboured NA-resistant HBV with complicated mutation patterns. Monitoring HBV genotypic resistance mutation markers and patterns is therefore shown to be beneficial for optimizing antiviral therapies and for avoiding clinical deterioration.

Introduction

Nucleoside/nucleotide analogues (NAs) are relevant medications in the current clinical treatment of chronic hepatitis B (CHB). Lamivudine (3TC), adefovir dipivoxil (ADV), entecavir (ETV) and telbivudine (LdT) are NAs currently used in China, whereas tenofovir disoproxil fumarate (TDF) has only recently been approved as a novel anti-HBV NA in Europe and in the US [1–8]. These five NAs are known for their ability to inhibit the activity of HBV reverse transcriptase (RT) [1–8]. However, a long duration of NA treatment is known to be associated with an increased risk of development of NA resistance (NAr), which is now recognized as the

single most important factor in NA drug failure [3,9]. It is well-known that reduced susceptibility and NAr are commonly conferred by genotypic mutations at distinct amino acid (AA) positions in the HBV RT region. Although an increasing number of NAr-associated mutation positions have been reported [10–13], 11 residues within the HBV RT region alone have been considered as the classical antiviral resistance mutation positions. This has been confirmed on the basis of *in vitro* phenotypic analyses and in a clinical setting [3,4].

Of the 11 residues, 8 (rtI169, rtA181, rtT184, rtA194, rtS202, rtM204, rtN236 and rtM250) in the RT region are associated with primary drug resistance and are responsible for reduced treatment susceptibility resulting from NA monotherapy or therapy with a group of multiple antiviral agents [3,9,12,14,15]. Conversely, secondary and/or compensatory mutations at three other residues (rtL80, rtV173 and rtL180) result in restoration of functional defects in RT activity caused by primary drug resistance [3,12,16]. Although the current emerging patterns of antiviral drug genotypic resistance in the HBV polymerase are as complex as the NAs are diverse, they can be categorized (on the basis of the NA treatment used) into five specific evolutionary pathways. These pathways include the L-nucleoside pathway (rtM204I [or V or I/V]) against 3TC and LdT as well as 3TC-experienced ETV treatment; the acyclic phosphonate pathway (rtN236T) refractory to ADV; the shared pathway (rtA181T [or V or T/V]) of both L-nucleoside and acyclic phosphonate against 3TV and ADV; the treatment-naive ETV resistance pathway (rtL180M+rtM204V with one of either rtT184, S202 or M250 residue changes); and multidrug resistance pathways (rtA181T+rtI233V+rtN236T +rtM250L) [3,9,12].

With increasingly wide use of NAs against HBV, more attention is being paid to the roles of these known genotypic markers of NAr within the HBV RT region. Many studies have shown the importance of monitoring these markers for surveillance and prediction of the development of antiviral resistance during NA treatment of patients infected with HBV genotypes A and D [17–19]. However, the profile of classical antiviral resistance mutations belonging to different evolutionary pathways and their effects on treatment outcome of various options of antiviral therapies have not been extensively investigated in Chinese patients infected with HBV genotypes B or C. There is a strong need for the availability of data pertaining to genotypic resistance in the NA-treated population to guide and optimize rescue therapies, to avoid the accumulation of new drug resistance mutations and to decrease the probability of cross-resistance to other anti-HBV drugs [9,20]; therefore, this study aimed to characterize the molecular features of AA substitutions at the aforementioned 11 classical NAr mutation positions belonging to different NAr pathways within HBV RT sequences. This was achieved by examining 199 NA-treated CHB patients attending five clinics (Peking University Third Hospital, Beijing, China; Beijing Haidian Hospital, Beijing, China; Qinhuangdao Third Hospital, Qinhuangdao, China; Shandong Jining Infectious Disease Hospital, Jining, China; and The First Affiliated Hospital of Xinjiang Medical University, Urumqi, China) in four northern cities of China between January 2007 and July 2009.

Methods

Patients

Our laboratory (Reference Laboratory for Viral Hepatitis of Peking University, Beijing, China), has been conducting HBV genotypic resistance analyses upon the request of clinics since 2007. The work was approved by the Ethics Committee of Peking University Health Science Center (Beijing, China) in accordance with the Declaration of Helsinki. Data obtained from 199 CHB patients from five clinics in four northern cities of China between January 2007 and July 2009 were analysed in this study. The studied population included 41 (20.60%) females and 158 (79.40%) males with a median age of 39 years (range 17–81). All patients were receiving NAs in single, sequential (switch-to) or combined (add-on) treatment when the samples were obtained. The inclusion criteria were a hepatitis B surface antigen (HBsAg)-positive status and an HBV-DNA-positive status for >6 months. The exclusion criteria included HCV or HIV coinfection, autoimmune liver disease and alcohol or drug abuse. The clinical diagnosis of CHB was made according to the 2003 European Association for the Study of the Liver guidelines [21].

Laboratory tests

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured using a Hitachi Automatic Clinical Analyzer 7170 (Hitachi High-Technologies, Tokyo, Japan) with 40 IU/l as the upper limit of normal. Serum HBsAg, hepatitis B e antigen (HBeAg), HBsAg antibody, HBeAg antibody and antibody against hepatitis B core antigen were detected using electrochemiluminescence immunoassays (Roche Diagnostics, Shanghai, China). HBV DNA was quantified by fluorescence quantitative PCR (Daan Gene, Guangzhou, China) with the lowest detection limit of 1,000 copies/ml.

HBV DNA extraction, amplification and sequencing

HBV DNA was extracted from 200 µl serum samples using a QIAamp DNA Blood kit (Qiagen GmbH,

Hilden, Germany) according to the manufacturer's instructions. Nested-PCR, which was established and optimized on the basis of our previously reported semi-nested PCR method [12], was used to amplify the entire RT region of HBV. A total of 8 µl of DNA extract was added in the first 25 µl reaction using primers P5 (sense 5′-GTGGCTCCAGTTCMGGAACAGT-3′) and P2 (antisense 5′-CTAGGAGTTCCGCAGTATGGAT-3′). PCR conditions were 94°C for 2 min, 30 cycles of 94°C for 30 s, 63°C for 30 s and 72°C for 75 s followed by 72°C for 7 min. The first round of amplified product was diluted 10–50× with sterilized water. A 5 µl aliquot of diluted product was then added into the second 50 µl reaction using primers P3 (sense 5′-CTCCAGTTCCGGAACAGT-3′) and P4 (antisense 5′-GCAGAGGAGCCACAAAGG-3′). The PCR conditions in the second-round reaction were similar to those in the first-round reaction with the exception of the annealing temperature, which was decreased to 55°C. The concentrations of dNTP (200 µM; TaKaRa Biotechnology, Dalian, China), primers (0.4 µM each; Sangon Bioengineering, Beijing, China) and Ex Taq DNA polymerase (50 U/ml; TaKaRa Biotechnology) were the same for both PCR rounds. A PCR fragment with approximately 1,195 base pairs in length was visualized on 1% agarose gel, then purified and sequenced commercially (Sangon Bioengineering). The sequencing primers used were P3 and P4.

Bioinformatics

Nucleotide and deduced amino acid sequences were analysed using DNAStar version 5.0 (DNAStar, Madison, WI, USA) software and HIV-grade bioinformatics tools [22]. HBV genotyping was determined using the NCBI Viral Genotyping Tool and further confirmed by phylogenetic analysis. In total, 11 previously reported NAr mutation positions (including rtL80, rtI169, rtV173, rtL180, rtA181, rtT184, rtA194, rtS202, rtM204, rtN236 and rtM250) in the RT region, belonging to different NAr pathways, were analysed according to bidirectional sequencing results. The identity of coexisting bases at one site found in direct sequencing was determined when each base occupied ≥20% [23]. The mixture of wild-type and mutant residues at single positions was considered as presence of the mutant(s) at that position [20]. Coexistent mutant AAs will be shown separated by a forward slash in the names of mutations, for example, rtM204I/V meaning that both isoleucine (I) and valine (V) emerged at the rtM204 residue.

Statistical analyses

Statistical analyses were performed using SPSS software version 11.0 (SPSS Inc., Chicago, IL, USA). Quantitative values expressed as mean ±SD were compared using the Student's *t*-test. Qualitative values were compared using the χ^2 test. All *P*-values were two-tailed. A *P*-value of <0.05 was considered as statistically significant.

Results

Patient characteristics

Demographic, biochemical and virological characteristics of the 199 NA-treated CHB patients are shown in Table 1. Of them, 15.08% (30/199) and 84.92% (169/199) were infected with HBV genotypes B and C, respectively. This genotype dispersal conformed to the HBV genotype distribution profile in northern China [5]. The HBeAg positivity rate was 69.35% (138/199; Table 1). No significant differences were observed in the male-to-female ratio, HBV DNA, and ALT and AST levels between the patients with HBeAg-positive and HBeAg-negative status; however, HBeAg-negative patients were significantly older than HBeAg-positive patients (*P*<0.01; J-XY *et al*., data not shown).

Analyses of NAr mutations in different treatment regimes

Among the 199 HBV isolates, 32.66% (65/199) harboured NAr mutations, in which 8 (12.31%) and 57 (87.69%) mutants belonged to HBV genotypes B and C, respectively. The NA therapies for the studied population involved seven treatment regimes: monotherapy (3TC, ADV, ETV or LdT), 3TC plus ADV add-on treatment, 3TC switch-to ADV or ETV sequential therapies. Notably, 67.34% (134/199) of the patient population had been receiving or had experienced 3TC treatment at the sampling time points.

Tables 2 and 3 show the results of NAr mutation analyses according to different treatment regimes, in which the mean duration of each therapy was >6 months. NAr mutations were detected at positions rtL80, rtV173, rtL180, rtA181, rtM204, rtN236 and rtM250, but not at positions rtI169, rtT184, rtA194 or rtS202. Mutations at position rtM204, which was considered an important primary NAr mutation position, were observed with prevalence rates ranging

Table 1. Characteristics of the studied population

Characteristic	Value (*n*=199)
Median age, years (IQR)	39 (17–81)
Male/female gender, *n* (% males)	158/41 (79.40)
Mean HBV DNA, $\log_{10}$ copies/ml (±SD)	5.69 (1.31)
Positive/negative HBeAg status, *n* (% positive)	138/61 (69.35)
Genotype B/C, *n* (% genotype C)	30/169 (84.92)
Mean ALT level, IU/l (±SD)	129.59 (201.71)
Mean AST level, IU/l (±SD)	89.22 (142.11)

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBeAg, hepatitis B e antigen; IQR, interquartile range.

Table 2. Prevalence of nucleoside/nucleotide analogue resistance mutations in different treatment regimes

Treatment	Cases, *n*	Isolates with mutations, *n* (%)	Median duration of therapy, months (±SD)
3TC	102	41 (40.20)	20.44 (13.41)[a]
ADV	50	3 (6.00)	11.99 (10.09)
ETV	6	0 (0.00)	12.80 (12.07)
LdT	9	2 (22.22)	6.50 (5.20)
3TC plus ADV	17	10 (58.82)	16.31 (8.66)
3TC switch to ADV	8	5 (62.50)	30.43 (13.69)[b]/25.25 (15.30)[c]
3TC switch to ETV	7	4 (57.14)	36.20 (30.40)[b]/8.14 (5.08)[d]
Total	199	65 (32.66)	NA

[a]A total of 71 patients had a clear duration of therapy among 102 patients treated with lamivudine (3TC) alone. [b]Duration of therapy with 3TC alone. [c]Duration of therapy after switching to adefovir dipivoxil (ADV). [d]Duration of therapy after switching to entecavir (ETV). LdT, telbivudine; NA, not available.

Table 3. Analyses of nucleoside/nucleotide analogue resistance mutations in different treatment regimes

Treatment	Cases, *n*	Mutation patterns	Isolates with mutations, *n*	Prevalence, %
3TC	102	rtM204V/I	35	34.31
		rtL80V/I	20	19.61
		rtL180M	18	17.65
		rtA181T/V	7	6.86
		rtM250L	1	0.98
ADV	50	rtA181T/V	4	8.00
		rtN236T	2	4.00
ETV	6	None	0	0.00
LdT	9	rtM204I	1	11.11
		rtA181T	1	11.11
3TC plus	17	rtM204V/I	6	35.29
ADV		rtL80I	2	11.76
		rtL180M	5	29.41
		rtA181T/V	3	17.65
		rtV173L	2	11.76
		rtN236T	2	11.76
3TC switch	8	rtA181T/V	4	50.00
to ADV		rtM204V/I	2	25.00
		rtL80I	1	12.50
		rtL180M	1	12.50
		rtN236T	1	12.50
		rtM250L	1	12.50
3TC switch	7	rtM204V/I	3	42. 86
to ETV		rtL80I	1	14.29
		rtL180M	4	57.14

ADV, adefovir dipivoxil; ETV, entecavir; LdT, telbivudine; 3TC, lamivudine.

from 25.00% to 42.86% in patients receiving 3TC-based monotherapy, ADV add-on or ADV switch-to, or ETV switch-to therapy. rtN236 mutations (5 cases) were detected exclusively among 75 patients receiving ADV-based therapies. Interestingly, rtA181 mutations (19 cases) were detected in those with 3TC-, ADV- or LdT-based treatment, but not in those undergoing ETV treatment (13 cases).

Analyses of NAr mutation patterns in rtM204 mutation-containing HBV mutants

As shown in Tables 2 and 3, the mutations at rtM204 were detected in 47 isolates among 65 identified mutants with a frequency of 72.31% (47/65), of which rtM204I, rtM204V and rtM204I/V accounted for 57.45% (27/47), 31.91% (15/47) and 10.64% (5/47), respectively.

An in-depth data mining on rtM204V/I-containing mutants was performed and the results are shown in Table 4. In general, 18 types of mutation patterns were observed, including mutations at rtM204 alone and combination mutations at rtM204 and other positions, of which 16 combination mutation patterns were observed.

Interestingly, regarding the selection of combined RT mutation positions rtM204I and rtM204V, the mutants displayed an AA substitution preference. Compared with the rtM204V mutants, rtM204I mutants were preferentially accompanied by rtL80I/V mutations with a statistically significant difference (rtM204I+rtL80I/V [66.67%, 14/21] and rtM204V+rtL80I/V [21.43%, 3/14]; χ^2=6.882, P=0.009). By contrast, rtM204V mutants were found to be coexistent with rtL180M mutations in all isolates containing rtM204V combination mutation patterns (rtM204V+rtL180M [100%, 14/14] and rtM204I+rtL180M [38.10%, 8/21]; χ^2=13.788, P<0.0001; Figure 1).

Discussion

In this study, classical antiviral resistance mutations at 11 residues within the HBV RT region, which belong to diverse evolutionary pathways, were analysed using nested-PCR, direct sequencing and bioinformatic approaches among 199 Chinese patients with CHB [6,7,23]. These patients had undergone a total of seven antiviral treatment protocols employing anti-HBV NAs (3TC, ADV, ETV and LdT) in single, sequential (switch-to) or combined (add-on) treatment in the past 2 years.

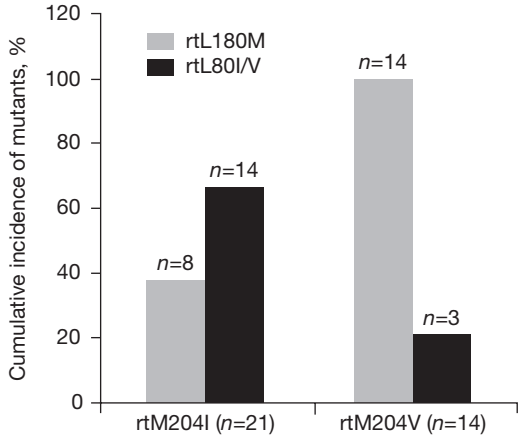
As a result, 32.66% (65/199) of the studied patients were found to harbour antiviral-resistant HBV strains that were involved in three known primary NAr pathways, including the L-nucleoside pathway (rtM204I [or V or I/V]), the acyclic phosphonate pathway (rtN236T) and the shared pathway against both 3TC and ADV (rtA181T [or V or T/V]). Although TDF refractory rtA194T mutations, the substitutions at rtI169, rtT184 and rtS202 residues responsible for primary ETV resistance in the presence of rtL180M+rtM204V mutations, as well as multiple mutations resulting in multidrug resistance pathways were not detected [3,9,24,25], the existence of rtM204I (or V or I/V), rtA181T (or V or T/V) and rtN236T was an important basis from which the NAr mutants could evolve. Importantly, NAr mutations at the rtM204 residue would be able to reduce the susceptibility to ETV and increase the likelihood of selection of additional mutations, which would greatly decrease the sensitivity to other antiviral agents [26,27]. Considering this potential issue, monitoring HBV genotypic resistance provides valuable information for antiviral treatment of CHB patients [7,23].

In this study, up to 72.31% (47/65) of patients had NAr that was associated with the L-nucleoside pathway (rtM204I [or V or I/V]), which could potentially lead to resistance to 3TC, LdT and ETV treatment in 3TC-experienced patients. The highest prevalence rate of genotypic resistance to the L-nucleoside pathway was in agreement with the largest percentage (67.34%, 134/199) of 3TC-experienced patients in our cohort, and further illustrates the characteristics of 3TC, which is the first drug that has a relatively low genetic barrier to resistance with the longest application of CHB treatment in China [3,7]. This suggests that the presence of rtM204I (or V or I/V) mutation carriers might constitute an unavoidable challenge for the effective control of antiviral-resistant HBV in the future because it is difficult to clear such resistant viral strains [13,28]. Meanwhile, this also presents potential risks of transmission to other individuals, as it has been demonstrated that 3TC-resistant HBV strains possessing rtM204V+L180M might spread and could pose a problem with therapeutic management, especially in HIV-coinfected patients [13,28]. Furthermore, the NAr mutations at rtM204 selected by 3TC were cross-resistant to other L-nucleosides and might affect the efficacy of LdT and ETV rescue treatment because they both share the same L-nucleoside pathway [9,26,27]. Although preliminary data using ADV belonging to a different NA group (acyclic phosphonates) from 3TC on 3TC refractory patients seems promising, a shared pathway (rtA181T [or V or T/V]) of both L-nucleosides and acyclic phosphonates could be selected, which would affect ADV sensitivity [9,18]. This was a phenomenon that

Table 4. Mutation patterns detected in 47 isolates with mutations at the rtM204 codon

Types of mutation patterns	Isolates, *n* (%)
Single mutation at rtM204	
rtM204I	6 (12.77)
rtM204V	1 (2.13)
Total	7 (14.89)
Combination mutation patterns including rtM204I	
rtM204I+rtL80I	9 (19.15)
rtM204I+rtL80I/V	1 (2.13)
rtM204I+rtL180M	6 (12.77)
rtM204I+rtL80I+rtL180M	2 (4.26)
rtM204I+rtL80I+rtV173M+rtM250L	1 (2.13)
rtM204I+rtV173L	1 (2.13)
rtM204I+rtL80I+rtA181T	1 (2.13)
Total	21 (44.68)
Combination mutation patterns including rtM204V	
rtM204V+rtL180M	9 (19.15)
rtM204V+rtV173L+rtL180M	2 (4.26)
rtM204V+rtL80I+rtL180M	1 (2.13)
rtM204V+rtL80V+rtL180M	2 (4.26)
Total	14 (29.79)
Coexistence of rtM204I and rtM204V	
rtM204I/V+rtL80I	1 (2.13)
rtM204I/V+rtL180M	1 (2.13)
rtM204I/V+rtL80I+rtL180M+rtA181V	1 (2.13)
rtM204I/V+rtL80I+rtL180M	1 (2.13)
rtM204I/V+rtL80I+rtV173L+rtL180M	1 (2.13)
Total	5 (10.64)

Figure 1. Associations between rtM204I and rtL80L/V as well as those between rtM204V and rtL180M



rtM204I mutants were accompanied by rtL80I/V mutations in 14 of 21 (66.66%) patients, whereas the rtM204V mutants were found coexistent with rtL180M mutations in all 14 (100%) patients. Therefore, rtM204I and rtM204V mutants were significantly associated with rtL80I/V and rtL180M, respectively ($P<0.01$).

was also observed in the studied population (Table 3); therefore, the risk of the propagation of such resistant viral strains with NAr mutations at rtM204 or rtA181 residues is of therapeutic concern and represents a crucial public health threat [3,28].

A total of 18 mutation patterns have been identified among NAr mutants at the rtM204 residue, including a single mutation at rtM204 and multiple mutations at 2–4 residues in addition to rtM204. Although the colocalization of multiple drug resistance substitutions on the same HBV genome still needs to be further confirmed by clonal analyses [18,29], the population-based sequencing approach used in this study did reflect the dominant RT sequence features, and the following findings are worthy of careful attention.

In agreement with previous reports [20,30,31], in this study, the occurrence rate of rtM204I (57.45%, 27/47) was significantly higher than that of rtM204V (31.91%, 15/47; $P<0.0001$). Of the isolates harbouring mutations at the rtL80 residue, rtL80I was predominant (100%, 24/24) and only one rtL80V mutation (4.17%, 1/24) was found to coexist with rtL80I. Although Warner *et al*. [31] have also described the coexistence of rtL80I and rtL80V as a rare event, the difference was that the rtL80I/V coexistent mutants were obtained from a 3TC-treated patient in this study and from 3TC-naive patients in their report. These data suggest that certain mutational patterns might be restricted by structural and/or functional constraints of particular AA positions in the HBV RT region; therefore, different genomic variability acquired in NA-treated patients might further influence the evolution of drug resistance mutants as well as the curative effects and treatment outcome of antiviral therapy [12,31]. This is demonstrated by the findings of Baldick *et al*. [24] who found that only viruses with ETV-resistant changes at either rtT184, rtS202 or rtM250 residues in a 3TC-resistant HBV background of rtM204V and rtL180M, but not that of rtM204I, displayed significant reductions in ETV susceptibility. In addition, the emergence of rtT184, rtS202 or rtM250 mutations preferred HBV strains bearing rtM204V+rtL180M rather than rtM204I mutations. Another convincing example was the observation that only rtM204I was found to be associated with clinical resistance to LdT, which was not active against HBV strains bearing rtM204V+rtL180M or rtM204I with or without rtL80I, but remained active against an rtM204V single mutation strain *in vitro* [32]. Therefore, when identified in the studied population, various primary resistance mutation patterns at the rtM204 residue, although sharing the same L-nucleoside pathway, might potentially benefit from different rescue therapies. For example, the only patient with an rtM204V single mutation, selected out by 7 months of 3TC treatment, experienced clinical resistance and might have benefited from a switch-to LdT therapy. In addition, for the 14 patients possessing rtM204V+rtL180M mutants, it would be beneficial to monitor the dynamic changes of genotypic resistance markers at rtT184, rtS202 and rtM250 residues, and to evaluate the risk of ETV resistance if switch-to ETV was to be the rescue option.

Furthermore, consistent with the previous observation in genotype A and D strains [20,31], in the studied population with genotype B or C infections, rtM204I and rtM204V were found clustering with rtL80I (or I/V) and rtL180M, respectively. The *in vivo* data shown in this study were in accordance with the suggestions from the preceding *in vitro* phenotyping that coselection of these two distinct clusters of mutations occurred because rtL80I (or V) in domain A and rtL180M in domain B of the HBV RT region compensated for the reduced replication efficiency responsible for the acquisition of 3TC resistance conferred by catalytic centre domain C mutants rtM204I (or V) [14,20,31]. Although other secondary/compensatory mutations, rtV173L or rtT184A (or S), were also found clustering with primary resistance mutation rtM204V in genotype D, only two rtV173L mutations, both accompanied by rtM204V but none with rtT184A (or S) mutations, were detected in 3TC-experienced patients in this study. This suggests a potential effect of HBV genotype in modulating resistance development under 3TC pressure, whereby the different clusters of 3TC resistance mutations develop [20]; however, more data will be needed to confirm this hypothesis.

In this study, changes at the rtA181 residue were identified in patients receiving initial monotherapy using 3TC, LdT or ADV, and 3TC-experienced patients switching to or adding on ADV treatment. These observations were in line with the speculation that rtA181T (or V) was associated with resistance to 3TC, LdT and ADV as a potential multidrug resistance pathway refractory to both the L-nucleoside analogues and the acyclic phosphonates [9,18]. It has been observed in cell culture that the rtA181T (or V) mutation induces a decreased susceptibility to 3TC and ADV but remains sensitive to ETV [18]. This has been partially confirmed by the findings in this study because rtA181T (or V) was not detected in the ETV-treated group. Because of the availability of new drugs, the recommendation for first-line oral antiviral medications has been changed to TDF or ETV [6]; however, to date, there are still numerous NA-naive CHB patients receiving 3TC as part of their first antiviral therapeutic regimen in China. Once 3TC resistance occurrs, ADV would be the rescue option either by switch-to or as add-on therapy. A recent study by Villet *et al*. [18] demonstrated that *in*

vivo it would be sufficient for an ADV rescue regime to induce viral breakthrough and NA failure in 3TC-experienced patients with rtA181T (or V or T/V) mutations. In addition, the same clinical deterioration will also occur in ADV-experienced patients who have the same mutation if 3TC was to be chosen as the rescue therapy. Interestingly, in this study, rtA181T was observed in one patient undergoing LdT monotherapy for 7 months. This was, to our knowledge, the first report of rtA181T being identified in initial monotherapy with L-nucleosides other than 3TC, possibly because of the relatively short application of LdT in the clinic, which has strengthened the concept of rtA181T (or V or T/V) as a shared resistant pathway for both L-nucleoside analogues (including 3TC and LdT) and acyclic phosphonates (including ADV), and emphasized the importance of research on the association of rtA181T (or V or T/V) with genotypic resistance to LdT in this particular setting.

Interestingly, substitutions at residue rtM250 were detected in samples from two patients who were ETV-naive. One patient had received 3TC monotherapy for 11 months and displayed a mutation pattern of rtM204I+rtL80I+rtV173M+rtM250L (Table 4), which was consistent with results of Tenney *et al*. [33] indicating that HBV isolates with rtM250L could change in an rtM204I 3TC-resistant background and might be selected under 3TC therapy without ETV treatment. A second patient had 48 months of 3TC treatment followed by 24 months of sequential ADV treatment, and was found to be infected with an HBV strain harbouring rtA181T+rtN236T+rtM250L combination mutations, which is the first report of this kind of mutation pattern according to our knowledge. Whether the rtM250L mutation emerged during ongoing ADV or the preceding 3TC treatment period is difficult to trace. Given the importance of rtM250 substitutions in ETV resistance, the clinical significance of this substitution in ETV salvage therapy for these patients needs to be further investigated.

In summary, the profile of classical antiviral resistance mutations and their potential evolutionary pathways against NAs were analysed among NA-treated Chinese patients with chronic infection with HBV genotypes B or C by a population-based sequencing approach. The study revealed that the double-edged antiviral strategy of a first-line treatment followed by rescue therapy using NAs, although providing effective viral suppression, had induced a complex pool of resistant HBV against future antiviral treatment. The results highlighted the importance of monitoring the shared resistant pathway pertaining to the current first-line antiviral agents and surveillance of genotypic resistance markers to guide and optimize the antiviral therapies in order to avoid the outbreak of clinical deterioration.

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Disclosure statement

The authors declare no competing interests.

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