Antiviral Research 85 (2010) 512–519

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Characterization of potential antiviral resistance mutations in hepatitis B virus reverse transcriptase sequences in treatment-naïve Chinese patients

Bao-Ming Liu[a], Tong Li[a,*], Jie Xu[b], Xiao-Guang Li[b], Jian-Ping Dong[c], Ping Yan[c], Jing-Xian Yang[a], Ling Yan[a], Zhi-Yong Gao[d], Wen-Peng Li[a], Xie-Wen Sun[e], Yu-Hua Wang[e], Xiu-Juan Jiao[e], Chun-Sheng Hou[f], Hui Zhuang[a,**]

[a] Department of Microbiology, Peking University Health Science Center, Xueyuan Road 38, Haidian District, 100191 Beijing, China
[b] Department of Infectious Diseases, Peking University Third Hospital, Beijing, China
[c] Department of Infectious Diseases, Beijing Haidian Hospital, Beijing, China
[d] Beijing Municipal Center for Disease Prevention and Control, Beijing, China
[e] Department of Infectious Diseases, Qinhuangdao Third Hospital, Qinhuangdao, China
[f] Department of Infectious Diseases, Shandong Jining Infectious Disease Hospital, Jining, China

ARTICLE INFO

Article history:
Received 19 July 2009
Received in revised form 28 October 2009
Accepted 15 December 2009

Keywords:
Hepatitis B virus
Resistance
Mutation
Nucleos(t)ide analogue naïve
Genotype
Polymorphic

ABSTRACT

Full-length hepatitis B virus (HBV) reverse transcriptase (RT) sequences were amplified and sequenced among 192 nucleos(t)ide analogue (NA)-naïve Chinese patients with chronic hepatitis B. Deduced amino acids (AAs) at 42 previously reported potential NA resistance (NAr) mutation positions in RT region were analyzed. Patients were found with either B-genotype (28.65%) or C-genotype (71.35%) infections. Rt53, rt91, rt124, rt134, rt221, rt224, rt238 and rt256 were identified as B- and C-genotype-dependent polymorphic AA positions. AA substitutions at 11 classical NAr mutation positions, i.e. rt80, rt169, rt173, rt180, rt181, rt184, rt194, rt202, rt204, rt236 and rt250, were not detected. However, potential NAr mutations were found in 30.73% (59/192) isolates, which involved 18 positions including rt53, rt207, rt229, rt238 and rt256, etc. The concomitant AA changes of HBsAg occurred in 16.67% (32/192) isolates including sG145R mutation. One-third of mutation positions were located in functional RT domains (e.g. rt207 and rt233), A–B interdomains (overlapping HBsAg 'a' determinant and showing most concomitant immune-associated mutations) and non-A–B interdomains (e.g. rt191 and rt213), respectively. Genotypes B and C each showed several preferred positions to mutate. These results might provide insights into understanding the evolution and selection basis of NAr HBV strains under antiviral therapy.

1. Introduction

Chronic hepatitis B virus (HBV) infection remains a serious public health problem in China (Chinese Medical Association, 2007). Although antiviral therapy using nucleos(t)ide analogues (NAs) is known as an effective control measure (Leung et al., 2001; Marcellin et al., 2003), the obstacles are limited kinds of NAs available and inevitable emergence of antiviral resistance conferred by viral mutations during long-term treatment (Locarnini et al., 2004; Locarnini and Mason, 2006). Thus, understanding of the mechanisms of the evolutionary basis of the drug resistance mutants is of importance for prevention and control of them.

Anti-HBV NAs approved currently in China include lamivudine (LMV), adefovir dipivoxil (ADV), entecavir (ETV) and telbivudine (LdT). They exclusively target the activity of the reverse transcriptase (RT) of HBV polymerase (Locarnini and Mason, 2006; Chinese Medical Association, 2007). In line with this, all reported potential antiviral resistance mutations are localized into the RT region (Locarnini and Mason, 2006; Lok et al., 2007). It is well-known that nucleos(t)ide-selected classical antiviral resistance mutations with in vitro phenotypic confirmation are classified into two categories (Locarnini and Mason, 2006; Lok et al., 2007). The first category contains primary drug resistance mutations resulting in amino acid (AA) substitutions directly reducing susceptibility to monotherapy or even a group of multiple antiviral agents, for example, rtM204I refractory to LMV and LdT (Shaw et al., 2006; Langley et al., 2007; Lok et al., 2007; Locarnini, 2008). The other category

Abbreviations: HBV, hepatitis B virus; NAs, nucleos(t)ide analogues; LMV, lamivudine; ADV, adefovir-dipivoxil; ETV, entecavir; LdT, telbivudine; RT, reverse transcriptase; AA, amino acid; NAr, nucleos(t)ide analogue resistance; CHB, chronic hepatitis B; TNF, tenofovir disoproxil fumarate; HBsAg, hepatitis B surface antigen; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBeAg, hepatitis B e antigen.

* Corresponding author. Tel.: +86 10 82802413; fax: +86 10 82802413.
** Corresponding author. Tel: +86 10 82801617; fax: +86 10 82801617.
E-mail addresses: tongli08@vip.sina.com (T. Li), zhuangbmu@126.com (H. Zhuang).

0166-3542/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2009.12.006

Table 1
Potential NAr mutations at 42 positions of HBV reverse transcriptase analyzed in this study.

Mutation category (no. of positions)	Type	Relationship with therapy	Reference
1. Primary drug resistance mutation (8[a])	I169T	ETV	Lok et al. (2007)
	A181T/V	LMV, LdT, ADV, TNF	Lok et al. (2007) and Locarnini (2008)
	T184A/C/F/G/I/L/M/S	ETV	Langley et al. (2007)
	A194T	ADV, TNF	Locarnini (2008) and Shaw et al. (2006)
	S202C/G/I	ETV	Langley et al. (2007)
	M204I/V/S	LMV, ETV, LdT, TNF	Lok et al. (2007) and Locarnini (2008)
	N236T	ADV, TNF	Locarnini (2008) and Shaw et al. (2006)
	M250I/L/V	ETV	Langley et al. (2007)
2. Secondary/compensatory mutation (3[a])	L80I/V, V173L	LMV	Lok et al. (2007)
	L180M	LMV, ETV, LdT	Lok et al. (2007) and Lai et al. (2005)
3. Putative NAr mutation (26[b])	S53N	LMV	Svicher et al. (2009)
	T54N[a]	ADV	Yang et al. (2002)
	L82M	LMV	Sheldon et al. (2006)
	V84M[a], S85A[a]	ADV	Sheldon et al. (2006)
	I91L	LMV	Ciancio et al. (2004)
	Y126C[a]	ADV	Yang et al. (2002)
	T128I, T128N[a], N139D[b], W153Q[a], F166L[a]	LMV	Sheldon et al. (2006), Sheldon and Soriano (2008), Wakil et al. (2002), Torresi et al. (2002), Melegari et al. (1998)
	V191I[a]	LMV, ADV	Yang et al. (2002) and Sheldon et al. (2006)
	A200V[a], V207I[a]	LMV	Fu and Cheng (1998), Pichoud et al. (1999), Sheldon et al. (2006) and Xiong et al. (2000)
	S213T, V214A	ADV	Shaw et al. (2006) and Sheldon et al. (2007)
	Q215P/S	LMV, ADV	Shaw et al. (2006), Wakil et al. (2002) and Kim et al. (2008)
	L217R[a], E218D[a], F221Y	ADV	Yang et al. (2002), Sheldon et al. (2006) and Nguyen et al. (2008)
	L229G/V/W	LMV	Locarnini (2008) and Wakil et al. (2002)
	I233V[a], P237H, N238D/S/T, Y245H[a]	ADV	Curtis et al. (2007), Locarnini (2008), Schildgen et al. (2006), Shaw et al. (2006), Yang et al. (2002) and Yim et al. (2006)
	S/C256G	LMV, ETV	Ciancio et al. (2004) and Colonno et al. (2006)
4. Pretreatment mutation (6[b])	T38A, Y124H, D134E, N139K/H[b], I224V, R242A	Found before therapy	Kim et al. (2008) and Ogura et al. (1999)

[a] With known phenotypic data. Some in vitro test results are given for reference purpose. T54N, Y126C, E218D and Y245H: no resistance to ADV (Yang et al., 2002); V84M and S85A: decreased sensitivity to ADV (Sheldon et al., 2006); T128N and W153Q: partial restoration of the replicative capacity of LMV-resistant HBV (Torresi et al., 2002; Sheldon et al., 2006); F166L: great impairing replication; V191I: decreased replication (Sheldon et al., 2006), sensitive to ADV (Yang et al., 2002); A200V: no influence on the viral yields, partial restoration of fitness of M204I mutant (Fu and Cheng, 1998). L217R: decreased sensitivity to ADV (Sheldon et al., 2006). For the following mutations the conflicting phenotypic data are existing. V207I: no influence on the viral yields, partial restoration of fitness of M204I mutant (Fu and Cheng, 1998), decreased replication and resistance to penciclovir and sensitive to LMV (Pichoud et al., 1999; Sheldon et al., 2006), moderately decreased sensitivity to LMV and sensitivity to ADV (Xiong et al., 2000); I233V: resistance to ADV and sensitivity to TNF (Schildgen et al., 2006), full susceptibility to ADV (Curtis et al., 2007), no significant drug resistance and no significant reduced replication capacity in the absence of selection pressure but compensation for the replication defects associated with acquisition of multidrug resistance (Locarnini, 2008).

[b] Categories 3 and 4 shared rt139, so altogether 42 positions in the RT region were analyzed.

is referred to as secondary/compensatory mutations contributing to AA substitutions restoring functional defects in RT activity associated with primary drug resistance, for instance, rtV173L combined with rtM204V + rtL180M (Lai et al., 2005; Lok et al., 2007).

Besides, the AA changes at some other positions of RT have been reported in NA resistance (NAr) studies. Firstly, several types of AA changes have been reported to be selected during prolonged NA therapy and potentially associated with NAr or replication compensation, although the functional relevancies have not been clarified thoroughly in vitro or in vivo, for example, rtV191I and rtS213T (Fu and Cheng, 1998; Ogura et al., 1999; Pichoud et al., 1999; Torresi et al., 2002; Wakil et al., 2002; Yang et al., 2002; Ciancio et al., 2004; Colonno et al., 2006; Sheldon et al., 2006, 2007; Yim et al., 2006; Kim et al., 2008; Nguyen et al., 2008; Svicher et al., 2009). This group of AA substitutions was named as "putative NAr mutations" in this study. Secondly, other types of AA substitutions have been reported in NA-naïve patients but their relationships with antiviral resistance development have not been clarified yet. In this study we called them "pretreatment mutations" (Ogura et al., 1999; Kim et al., 2008). Thus, totally we discovered 42 potential NAr AA positions scattered in the full-length RT sequences reported so far and grouped them into 4 categories, i.e. primary drug resistance mutation (Category 1), secondary/compensatory mutation (Category 2), putative NAr mutation (Category 3) and pretreatment mutation (Category 4) (Table 1).

Puzzled by so many reported potential NAr mutations, we wondered how often they presented as naturally occurring polymorphic mutations in NA-naïve chronic hepatitis B (CHB) patients and whether those belonging to Categories 3 and 4 (Table 1) contributed to the antiviral resistance property of HBV. As we know, many of the previous investigations on NA-naïve patients focused on classical primary and secondary mutations and on A- or D-genotype infections, and the results on the presence of drug-resistant mutations as natural genome variability were different among various studies (Kobayashi et al., 2001; Matsuda et al., 2004; Ramezani et al., 2008). Therefore, this study aims to characterize the AA substitutions at the aforementioned 42 potential NAr mutation positions in HBV RT sequences in a cohort of 192 Chinese NA-naïve CHB patients commonly with B- or C-genotype infections.

2. Materials and methods

2.1. Patients

The study was approved by the Ethics Committee of Peking University Health Science Center in accordance with the Helsinki Declaration. Informed consent was obtained from each patient. One hundred and ninety-two Chinese CHB patients from outpatient clinics in 4 hospitals were consecutively enrolled in a period from December 2007 to August 2008 with the inclusion criteria of hepatitis B surface antigen (HBsAg) positive, HBV DNA positive, as well as LMV, ADV, ETV and LdT treatment-naïve. Exclusion criteria included hepatitis C virus or human immunodeficiency virus co-infection, autoimmune liver disease, and alcohol or drug abuse. The clinical diagnosis of CHB was according to EASL 2003 guideline (de Franchis et al., 2003).

2.2. Liver biochemistry, HBV serology, and HBV DNA assays

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using Hitachi Automatic Clinical Analyzer 7170 (Hitachi High-Technologies, Tokyo, Japan) with cut-off value of 40 IU/L. Serum HBsAg, hepatitis B e antigen (HBeAg), anti-HBs, anti-HBe, and anti-HBc were detected using electrochemiluminescence immunoassay (Roche Diagnostics, Shanghai, China). HBV DNA was quantified with fluorescence quantitative PCR (Daan Gene, Guangzhou, China) with the lowest detection limit of 1000 copies/mL.

2.3. HBV DNA extraction, amplification and sequencing

HBV DNA was extracted from 200 μL serum samples using QIAamp DNA Blood Kit (Qiagen, Hilden, Germany) and eluted with 100 μL sterilized water. Semi-nested polymerase chain reaction (snPCR) was used to amplify HBV full-length RT region. Five microliters of DNA extract was added in the first 25 μL reaction using primers P1 (nt 2744–2767, 5′-TTATTTACATACTCTKTGGAAGGC-3′) and P2 (nt 1285–1264, 5′-CTAGGAGTTCCGCAGTATGGAT-3′). The nucleotide sequence numbering was according to the suggested reference strain sequence D00329 (Stuyver et al., 2001). PCR conditions were 94 °C for 2 min; 30 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 2 min; then 72 °C for 10 min. Second round PCR was performed in a 50 μL reaction containing 4 μL first-round PCR product and using primers P5 (nt 63–84, 5′-GTGGCTCCAGTTCMGGAACAGT-3′) and P2. PCR conditions were 94 °C for 2 min; 30 cycles of 94 °C for 1 min, 63 °C for 1 min and 72 °C for 1.25 min; then 72 °C for 10 min. Both PCR rounds shared the same final concentration of $MgCl_2$ (1.5 mM), dNTP (200 μM), primers (0.8 μM each) and *Taq* Plus DNA polymerase (50 U/mL) (Dongsheng Biotech, Guangzhou, China). An approximately 1223 bp PCR fragment was purified and sequenced commercially (Sangon Bioengineering, Beijing, China) using primers P5u (nt 63–84, 5′-GTGGCTCCAGTTCAGGAACAGT-3′) and P2.

2.4. HBV genotyping

HBV genotyping was determined using NCBI Viral Genotyping Tool (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi) and phylogenetic analysis with MEGA 4.0 software.

2.5. Mutation analysis and definitions

Forty-two potential NAr relevant AA positions in RT were summarized in Table 1 and analyzed for AA mutations and concomitant influences on HBsAg. The AA mutations were identified by comparing HBV RT sequences with the genotype-matched consensus sequence generated based on the HBV sequences obtained in this study and the reference sequences in previous investigations (Stuyver et al., 2001; Borroto-Esoda et al., 2007). Conserved positions were defined as those with a single AA identity observed across all isolates, while polymorphic positions were those with multiple AA identities (Borroto-Esoda et al., 2007). A mutation type was referred to the replacement of the consensus AA of the corresponding genotype with a novel one, for instance, rtM204I and rtM204V were described as two mutation types (Lok et al., 2007).

2.6. Data analyses

Nucleotide sequences were analyzed using DNAStar 5.0 and MEGA 4.0 softwares. Statistical differences were evaluated by *t*-test, chi-squared test, one-way analysis of variance and multiple comparisons with SPSS 11.5 software where appropriated. All *P* values were two-tailed. $P < 0.05$ was considered to be statistically significant.

2.7. GenBank accession numbers

See supplementary data.

3. Results

3.1. Patient characteristics

HBV RT sequences were determined in all 192 patients with 28.65% (55/192) B-genotype and 71.35% (137/192) C-genotype infections, respectively ($P < 0.0001$), with a prevalent characteristic representative of HBV genotype distribution in northern China (Chinese Medical Association, 2007). HBeAg positive rate was 71.35% (137/192). The main characteristics of the HBeAg-positive and -negative patients were compared in Table 2, showing that HBeAg-negative patients were significantly older ($P < 0.0001$) and had significantly lower HBV DNA than those of HBeAg-positive ones ($P < 0.0001$). Multiple comparisons according to HBeAg statuses and HBV genotypes are shown in Table 3. Significant differences were found in age and HBV DNA level among four groups with different HBeAg statuses and HBV genotype infections ($P < 0.0001$) (Table 3). Further analyses were performed and the following findings were obtained. In HBeAg-positive group, the patients with C-genotype infections were significantly older ($P = 0.023$) than those

Table 2
Main characteristics of the study population.

Characteristics	HBeAg+ CHB ($N = 137$)	HBeAg− CHB ($N = 55$)	*P*-value
Sex (male/female)	86/51	39/16	0.29
Age (years), median (range)	28.00 (16.00–81.00)	42.00 (18.00–76.00)	<0.0001
ALT (IU/L), median (range)	70.00 (11.00–1 730.00)	67.00 (20.00–1 393.00)	0.50
AST (IU/L), median (range)	55.00 (12.00–1 016.00)	63.00 (16.00–1 205.00)	0.63
HBV DNA (Log_{10}copies/mL), median (range)	7.07 (3.00–9.00)	5.41 (3.08–8.38)	<0.0001
Genotype (B/C)	42/95	13/42	0.33

Table 3
Multiple comparisons of main characteristics of the patients with different HBeAg statuses and HBV genotype infections.

Characteristics	HBeAg/HBVgenotype				*P*-value
	HBeAg+/B (*N* = 42)	HBeAg−/B (*N* = 13)	HBeAg+/C (*N* = 95)	HBeAg−/C (*N* = 42)	
Sex (male/female)	28/14	8/5	58/37	31/11	0.53
Age (years), median (range)	27.00 (16.00–63.00)	33.00 (18.00–74.00)	29.00 (16.00–81.00)	43.00 (19.00–76.00)	<0.0001[a]
ALT (IU/L), median (range)	56.50 (11.00–573.00)	50.00 (20.00–152.00)	73.00 (12.00–1 730.00)	79.00 (21.00–1 393.00)	0.39
AST (IU/L), median (range)	42.00 (13.00–285.00)	46.00 (20.00–79.00)	59.00 (12.00–1 016.00)	72.50 (16.00–1 205.00)	0.18
HBV DNA (log_{10}copies/mL), median (range)	7.20 (3.00–8.49)	5.62 (3.30–8.08)	6.96 (3.15–9.00)	5.04 (3.08–8.38)	<0.0001[b]
HBeAg (+/−)	42/13	95/42	0.33		

[a] Comparison of age: HBeAg+/B vs HBeAg+/C, *P* = 0.023; HBeAg−/B vs HBeAg−/C, *P* = 0.14; HBeAg+/B vs HBeAg−/B, *P* = 0.037; HBeAg+/C vs HBeAg−/C, *P* < 0.0001.
[b] Comparison of serum HBV DNA: HBeAg+/B vs HBeAg+/C, *P* = 0.29; HBeAg−/B vs HBeAg−/C, *P* = 0.92; HBeAg+/B vs HBeAg−/B, *P* < 0.0001; HBeAg+/C vs HBeAg−/C, *P* < 0.0001.

with B-genotype infections, but HBV DNA were at similar levels (*P* = 0.29). In HBeAg-negative group there were no significant differences in age and HBV DNA among patients regardless of infected HBV genotypes. In B-genotype infection group, HBeAg-negative patients were significantly older (*P* = 0.037) and had significantly lower HBV DNA (*P* < 0.0001) than those of HBeAg-positive ones, and so was the C-genotype infection group (*P* < 0.0001, respectively).

3.2. Analyses of potential NAr mutations

Upon analysis of 42 positions, genotype-dependent AA polymorphisms were found at 8 sites (Table 4). The results showed that the presence of asparagine or serine at rt53, leucine or isoleucine at rt91, asparagine or tyrosine at rt124, asparagine or aspartic acid at rt134, tyrosine or phenylalanine at rt221, valine or isoleucine at rt224, and histidine or asparagine at rt238 were significantly correlated with genotype B or C, respectively (*P* < 0.0001, respectively). Serine at rt256 was significantly more associated with genotype B than genotype C (*P* = 0.01), though serine was predominant at rt256 for both genotypes. Therefore, it was notable that the consensus (wild-type) AA residue at each of these sites was genotype B or C dependent and defined by the dominant AA at a particular site, while the other AA residues, which existed at low frequencies in this treatment-naïve population, represented naturally occurring polymorphic mutations. In this study, the AA change as compared to the consensus AA at any of the 42 sites was considered when the statistic analysis of potential NAr mutations was done, since all these 42 sites were reported in NAr studies. However, the readers should notice that many of the potential NAr mutations (Categories 3 and 4 in Table 1) needed further investigation regarding nucleos(t)ide resistance in vitro and in vivo.

On these bases, potential NAr mutations were further assessed. Although none of mutations belonging to Categories 1 and 2 in Table 1 were observed, potential NAr mutations belonging to Categories 3 and 4 were found in 30.73% (59/192) HBV isolates, which harbored 76 mutations in total, indicating some isolates harboring multiple mutations. Moreover, 33 AA mutation types (15 previously reported types and 18 novel types) were detected at 42.86% (18/42) screened AA sites (Fig. 1 and Table 5).

3.3. Mutation distribution and frequency in different RT sections

HBV RT region consists of 6 functional domains (F, A, B, C, D and E) and 5 interdomains (F–A, A–B, B–C, C–D and D–E) connecting domains (Stuyver et al., 2001; Warner et al., 2007). Mutation distribution analyses revealed that all 6 studied sites (6/6, 100%) within A–B interdomain had mutations, which displayed the most genetic variability as compared to the sites within RT domains (sites with mutations: 6/22, 27.27%; *P* = 0.0014) and the sites within non-A–B interdomains (6/14, 42.86%; *P* = 0.017) (Table 6 and Fig. 1). Furthermore, the mutation frequency of A–B interdomain (44/1152, 3.82%) was also significantly higher than those of the RT domains

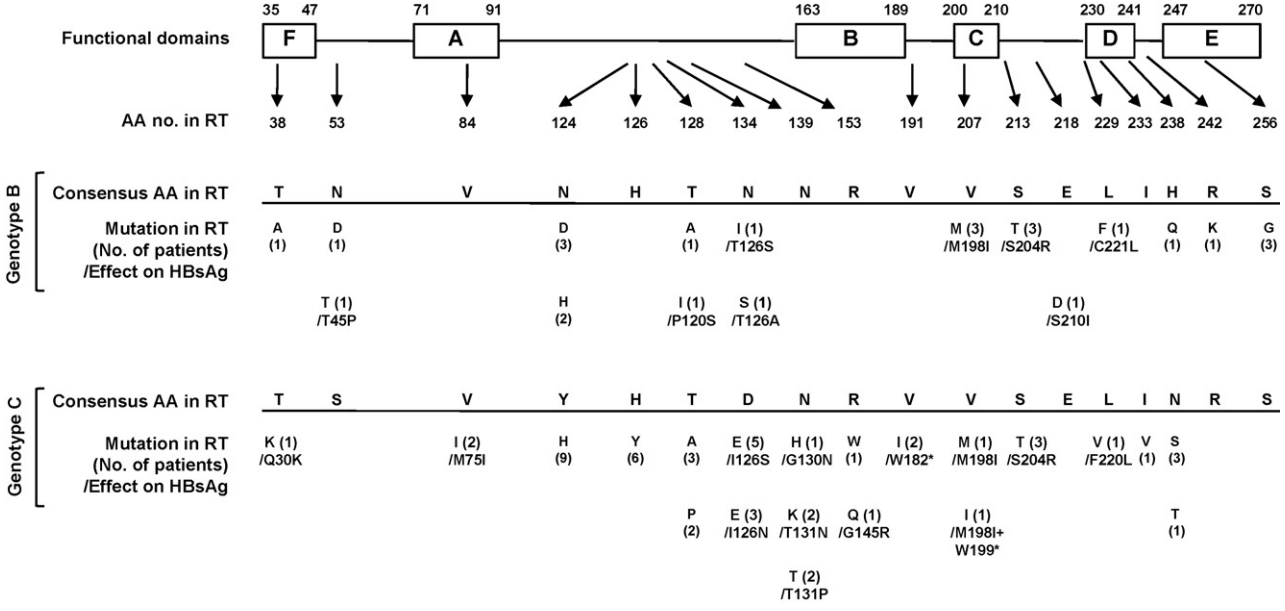

Fig. 1. Mutations identified in RT region and the overlapping HBsAg. Asterisks indicate stop codons.

Table 4
Genotype-dependent AA polymorphic sites found in this investigation.

Genotype	rt53				rt91		rt124				rt134				rt221			rt224		rt238						rt256			
	N	S	D[a]	T[a]	I	L	N	Y	D[a]	H[a]	D	N	E[a]	I[a]	S[a]	F	Y	I	V	N	H	Q[a]	S[a]	T[a]	C	S	G[a]
B (*N* = 55)	53	0	1	1	2	53	50	0	3	2	10	43	0	1	1	0	55	0	55	2	52	1	0	0	1	51	3
C (*N* = 137)	3	134	0	0	134	3	0	128	0	9	118	11	8	0	0	129	8	127	10	131	2	0	3	1	21	116	0
P-value	<0.0001				<0.0001		<0.0001				<0.0001					<0.0001		<0.0001		<0.0001					0.001		

N: asparagine; S: serine; D: aspartic acid; T: threonine; I: isoleucine; L: leucine; Y: tyrosine; H:histidine; E: glutamic acid; F: phenylalanine; V: valine; Q:glutamine; C: cysteine; G: glycine.
[a] Described as naturally occurring polymorphic mutations in this study.

Table 5
Potential NAr mutations identified in HBV RT region.

Mutation category	Mutation types	No. of isolates with mutations
Putative NAr mutation: Category 3 in Table 1	T128I	1
	V191I[a]	2
	V207I[a]	1
	S213T	6
	E218D[a]	1
	L229V	1
	I233V[a]	1
	N238S[b] + N238T[b]	3 + 1
	S256G[b]	3
Total	9 positions, 10 types	20
Pretreatment mutation: Category 4 in Table 1	T38A	1
	Y124H[b]	9
	D134E[b]	8
	N139H + N139K	1 + 2
Total	4 positions, 5 types	21
Novel mutation: new AA mutation type found in this study	T38K	1
	N53D[b] + N53T[b]	1 + 1
	V84I	2
	N124D[b] + N124H[b]	3 + 2
	H126Y	6
	T128A + T128P	4 + 2
	N134I[b] + N134S[b]	1 + 1
	N139T	2
	R153W + R153Q	1 + 1
	V207M	4
	L229F	1
	H238Q[b]	1
	R242K	1
Total	13 positions, 18 types	35

[a] With known phenotypic data (see Table 1 footnote).
[b] Genotype-dependent AA polymorphic positions identified in this study.

(18/4 224, 0.43%, $P < 0.0001$) and non-A–B interdomains (14/2688, 0.52%, $P < 0.0001$) (Table 6). However, the RT domain and non-A–B interdomain did not show any significant differences regarding the percentage of sites with mutations and the mutation frequency (Table 6). In addition, the A–B interdomain displayed the most abundant mutations in terms of mutation types and associated case numbers at rt124, rt128, rt134, rt139 and rt153 (Fig. 2), indicating that these positions might be naturally occurring mutation hotspots in this treatment-naïve population and had potential to evolve toward different directions. In contrast, restricted mutation types and comparably less case numbers were observed in the flanking regions of A–B interdomain, where RT domains and conserved sites were located (Fig. 1 and Table 6).

Additionally, RT and HBsAg mutations can occur concomitantly by the complete overlapping nature of RT region and S gene (Sheldon and Soriano, 2008). In this study 14 out of 18 mutated positions in RT were within the corresponding region of HBsAg. AA mutations at 12 out of 14 RT positions (except mutations at rt124 and rt126) were accompanied by 19 types of AA changes of HBsAg in 16.67% (32/192) isolates (Fig. 1). Remarkably, nucleotide mutations in A–B interdomain could lead to AA substitutions at s126, s130, s131 and s145 in the overlapping 'a' determinant of HBsAg, including the most frequently described immune-escape mutation sG145R (1/192, 0.52%) (Echevarria and Avellón, 2006; Svicher et al., 2009).

3.4. Mutations in B- and C-genotype isolates

The statistic analyses did not show any significant differences between genotypes B and C regarding the number of isolates with mutations (B vs C: 19/55, 34.55% vs 40/137, 29.20%; $P = 0.47$), the

Table 6
Mutation site distributions and mutation frequencies in different sections of HBV RT region.

RT sections	No. of sites studied	Sites with mutations %[a]	Mutation frequency %[b]
Domains[c]	22	27.27 (6/22)	0.43 (18/4224)
Non-A–B interdomains[d]	14	42.86 (6/14)	0.52 (14/2688)
A–B interdomain[e]	6	100 (6/6)	3.82 (44/1152)
Total	42	42.86 (18/42)	0.94 (76/8064)

[a] Comparison of the sites with mutations (%) among different RT sections were as follows: domains vs non-A–B interdomains, $P = 0.34$; domains vs A–B interdomain, $P = 0.0014$; non-A–B interdomains vs A–B interdomain, $P = 0.017$.
[b] The number of mutations detected within a specific RT section divided by the total sites number studied in this section in 192 isolates was defined as the mutation frequency. For instance, the calculation of the mutation frequency of "domains" section was as follows: 18 mutations detected/(22 studied sites × 192 isolates) × 100% = 18/4 224 × 100% = 0.43%. Comparisons of the mutation frequencies among different RT sections were as follows: domains vs non-A–B interdomains, $P = 0.57$; domains vs A–B interdomain, $P < 0.0001$; non-A–B interdomains vs A–B interdomain, $P < 0.0001$.
[c] Mutation sites in this section include rt38, rt84, rt207, rt233, rt238 and rt256.
[d] Mutation sites in this section include rt53, rt191, rt213, rt218, rt229 and rt242.
[e] Mutation sites in this section include rt124, rt126, rt128, rt134, rt139 and rt153.

discovered sites with mutations (B vs C: 12/42, 28.57% vs 14/42, 33.33%; $P = 0.64$) and the mutation frequencies (B vs C: 25/2 310, 1.08% vs 51/5 754, 0.89%; $P = 0.41$). However, the following findings should be mentioned. Firstly, though both genotypes shared 8 common mutation positions, including rt38, rt124, rt128, rt134, rt207, rt213, rt229 and rt238 (Fig. 2), they preferred different mutation types at these sites (except rt213) (Fig. 1). Moreover, genotypes B and C had several unique mutated positions at which the other genotype did not mutate. They were rt53, rt218, rt242 and rt256 for genotype B, and rt84, rt126, rt139, rt153, rt191 and rt233 for genotype C (Fig. 2).

3.5. Characterization of the isolates with NAr mutations

Of 59 isolates with mutations, 47 (79.66%) had single mutation while 12 (20.34%) had multiple mutations, including 9 with double mutations, 2 with triple mutations and 1 with a quintuple mutation (see supplementary data). Comparison of patients with different RT sequence polymorphisms showed that the mean HBV DNA of the patients with multiple mutations ($5.53 \pm 1.22 \log_{10}$ copies/mL) was significantly lower than that of those without mutations ($6.54 \pm 1.31 \log_{10}$ copies/mL, $P = 0.011$) and those with single mutation ($6.37 \pm 1.32 \log_{10}$ copies/mL, $P = 0.049$). No significant differences were observed in sex, age, ALT, AST, HBeAg status or genotypes among the three groups.

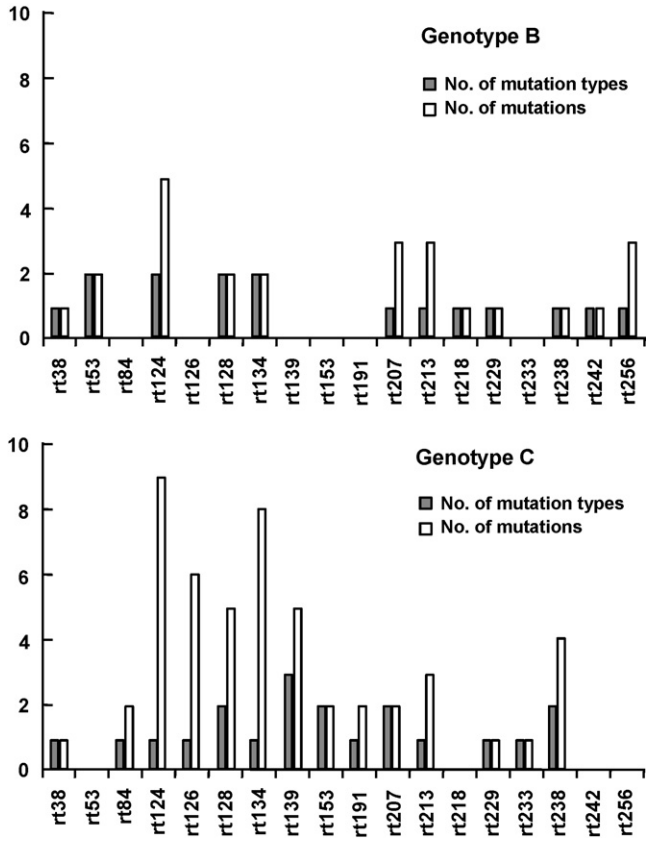

Fig. 2. Potential NAr mutations in B- and C-genotype isolates.

4. Discussion

With the wide use of NAs, potential NAr mutation positions in HBV RT region have been reported increasingly but presented with many puzzles nowadays (Zoulim et al., 2009). Based on publication reviewing, various potential NAr mutation positions in RT region reported so far were grouped into 4 categories in this study (Table 1). Similar to some previous reports (Pollicno et al., 2007; Nguyen et al., 2008), the primary drug resistance mutations and secondary mutations were not found in this study. This might be due to the impossibility of the population-based sequencing approach used for identifying minor quasispecies less than 20% or the property of the studied patient cohort. Anyhow, this result hinted that the naturally occurring NAr strains were not prevalent or predominant in this group of NA-naïve Chinese CHB patients with B- or C-genotype infection.

In this study 8 genotype-dependent AA polymorphic positions, i.e. rt53, rt91, rt124, rt134, rt221, rt224, rt238 and rt256, were identified for B- and C-genotypes. This was particularly important for the definitions of so-called mutations in genotypic mutation analyses. For instance, serine and asparagine at rt53 were described in Table 1 as wild-type and mutant AA according to a previous report on genotype D, respectively (Svicher et al., 2009). However, our results showed that asparagine and serine at rt53 were B- and C-genotype dependent wild-type AAs, respectively. Of interest, we applied the same approach to analyze some RT sequences of D genotype obtained from GenBank and our clinic isolates from Xinjiang, China, and we found that asparagine was actually the consensus AA at rt53 of D-genotype HBV (data not shown). Additional analyses using sequences deposited in GenBank showed that both genotypes A and D also displayed genotype-dependent AA polymorphic property at these 8 sites, i.e. rtI53, rtI91, rtN124, rtD134, rtY221, rtV224, rtN238 and rtS256 for genotype A; rtN53, rtL91, rtH124, rtD134, rtF221, rtV224, rtN238 and rtC256 for genotype D (data not shown). These findings suggest that one has to interpret carefully the genotypic mutation data. In addition, the genotype-dependent polymorphism feature of HBV RT sequences would be an important basis from which the NAr mutants would evolve, though the virtual effects of it on NAr and replication capacity require further study.

An interesting finding was that the positions in RT domains and within non-A–B interdomains especially those close to the

classical NAr mutation positions seemed to have limited mutation frequencies compared with those in A–B interdomain (Table 6). The reason might be that these sections were of importance for RT function and viral replication, thus changes in these sections would not normally confer replication advantages in the absence of antiviral drugs. These in vivo observations were in line with the well-known results obtained by in vitro phenotypic assay, in which the primary drug resistance mutants, for example catalytic center domain C mutant rtM204V, could confer the LMV resistance at the expense of compromised viral replication fitness (Lok et al., 2007). However, by discussing so, we did not mean that the positions with naturally occurring mutations detected in this study were not important at all in terms of antiviral resistance. Our speculation was that those positions located in the functional domains or adjacent to the classical NAr mutation positions, such as rt38, rt84, rt191, rt207, rt233, rt238 and rt256, might be still of significance and needed further attention in the antiviral resistance research and surveillance (Shaw et al., 2006; Sheldon et al., 2006). Some evidence to support our hypothesis appeared in the reports showing rtV207I in domain C conferring a decreased replication capacity (Pichoud et al., 1999), rtI233V near rt236 in domain D compensating for the replication defects in multi-drug resistance mutant (Locarnini, 2008), and rtS256G in domain E associated with ETV treatment (Colonno et al., 2006).

Moreover, the AA sites in A–B interdomain displayed the highest mutation frequency (Table 6). The mutated sites in A–B interdomain were structurally distant from other significant functional domains (Bartholomeusz et al., 2004). Further, due to the overlapping nature of HBV genomes, the most of RT-HBsAg concomitant changes were found concentrated in the A–B interdomain corresponding to the overlapping 'a' determinant with a cluster of documented immune-escape mutations, such as sT126A, sI126N, sI/T126S, sT131N and sG145R (Fig. 1) (Echevarria and Avellón, 2006; Svicher et al., 2009). The results hinted that A–B interdomain seemed less likely to be crucial for RT function and antiviral resistance, rather the mutations within which might be driven mainly by host immune pressures (Ogura et al., 1999; Sheldon and Soriano, 2008).

The potential effects of HBV genotypes on modulating resistance development remains still a matter of controversy (Buti et al., 2002; Ciancio et al., 2004; Svicher et al., 2009). A recent study by Svicher et al. (2009) has demonstrated that genotypes A and D had different preferences for two distinct LMV-resistance mutation clusters, showing possible roles of HBV genotypes in driving RT sequence evolution under LMV treatment. Similarly, the findings in this study also suggested that HBV genotype as a genetic background might contribute to the evolution of drug-resistance mutants. The first observation was the existence of genotype-dependent AA polymorphic positions (Table 4). Particularly, those in the RT functional domains might be more important, for instance, rt91 in domain A, rt238 in domain D and rt256 in domain E. Our study showed that rtL91 and rtI91 were favored by genotypes B and C, respectively, while rtS256 was predominant in both genotypes with rtC256 more prevalent in genotype C than in genotype B. Ciancio et al. (2004) have reported that rtL91and rtC256 were more correlated with extended treatment failure than rtI91and rtS256, which were suggested as potential pretreatment markers to predict the long-term response to LMV therapy. Ciancio et al. further suggested that the presence of rtL91 and rtC256 might represent helpful compensatory changes for restoring replication competence in viruses acquiring the drug-resistance rtM204V/I and/or rtL180M mutations by spatial structural analysis of HBV polymerase (Ciancio et al., 2004). In addition, we found that genotypes B and C each had a few preferred AA positions to change, respectively (Figs. 1 and 2). For instance, rtN53D/T and rtS256G were only detected in genotype B but not in genotype C; while rtL229V near domain D was identified in genotype C alone, which was reported lately clustered with rtM204V mutation (Svicher et al., 2009). These findings could be explained by the implication that certain mutational patterns were restricted by structural/functional constraints to particular genotypes, suggesting that both genotypes had acquired different genomic variabilities in NA-naïve patients and might further influence the evolution of drug-resistance mutants. Surely, a clearer picture regarding the genotype impacts on the development of drug resistant HBV mutants needs further investigation with larger cohorts and in vitro phenotypic testing.

The clinical significance of the preexisting potential NAr mutations in Categories 3 and 4 remains further investigation. Until now, 6 patients (4 without any preexisting potential NAr mutations and 2 with preexisting rtY124H mutations) in this study have started antiviral therapy and will be followed up.

In conclusion, the classical antiviral resistance mutations were not detected while naturally occurring potential NAr mutations were found in NA-naïve Chinese patients with chronic B- or C-genotype HBV infection by population-based sequencing approach. The clinical significance of these mutations needs further investigation. The study revealed that the identification of genotype-dependent AA polymorphic positions, analysis of the localization of a potential NAr position in RT region, and comprehension of the potential roles of HBV genotype as a genetic background might provide rational explanations to elucidate the mechanisms of the evolution and selection of the NAr HBV mutants under antiviral therapy.

Acknowledgements

This study was supported by the financial grants from Chinese National Key Basic Research Project (2005CB523104) and Major Science and Technology Special Project of China Eleventh Five-year Plan (2008ZX10002-004 and 2009ZX10004-314).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2009.12.006.

References

Chinese Medical Association, 2007. Guideline on prevention and treatment of chronic hepatitis B in China. Chin. Med. J. (Engl) 120, 2159–2173.
Bartholomeusz, A., Tehan, B.G., Chalmers, D.K., 2004. Comparisons of the HBV and HIV polymerase, and antiviral resistance mutations. Antivir. Ther. 9, 149–160.
Borroto-Esoda, K., Miller, M.D., Arterburn, S., 2007. Pooled analysis of amino acid changes in the HBV polymerase in patients from four major adefovir dipivoxil clinical trials. J. Hepatol. 47, 492–498.
Buti, M., Cotrina, M., Valdes, A., Jardi, R., Rodriguez-Frias, F., Esteban, R., 2002. Is hepatitis B virus subtype testing useful in predicting virological response and resistance to lamivudine? J. Hepatol. 36, 445–446.
Ciancio, A., Smedile, A., Rizzetto, M., Lagget, M., Gerin, J., Korba, B., 2004. Identification of HBV DNA sequences that are predictive of response to lamivudine therapy. Hepatology 39, 64–73.
Colonno, R.J., Rose, R., Baldick, C.J., Levine, S., Pokornowski, K., Yu, C.F., Walsh, A., Fang, J., Hsu, M., Mazzucco, C., Eggers, B., Zhang, S., Plym, M., Klesczewski, K., Tenney, D.J., 2006. Entecavir resistance is rare in nucleoside naive patients with hepatitis B. Hepatology 44, 1656–1665.
Curtis, M., Zhu, Y., Borroto-Esoda, K., 2007. Hepatitis B virus containing the I233V mutation in the polymerase reverse-transcriptase domain remains sensitive to inhibition by adefovir. J. Infect. Dis. 196, 1483–1486.
de Franchis, R., Hadengue, A., Lau, G., Lavanchy, D., Lok, A., McIntyre, N., Mele, A., Paumgartner, G., Pietrangelo, A., Rodés, J., Rosenberg, W., Valla, D., 2003. EASL International Concensus Conference on Hepatitis B. 13-14 September 2002, Geneva, Switzerland. Consensus statement. J. Hepatol. 39 (Suppl. 1), S3–S25.
Echevarria, J.M., Avellón, A., 2006. Hepatitis B virus genetic diversity. J. Med. Virol. 78 (Suppl. 1), S36–S42.
Fu, L., Cheng, Y.C., 1998. Role of additional mutations outside the YMDD motif of hepatitis B virus polymerase in L(−)SddC (3TC) resistance. Biochem. Pharmacol. 55, 1567–1572.
Kim, D., Ahn, S., Chang, H., Shim, H., Heo, J., Cho, M., Moon, B., Moon, Y., Paik, Y., Lee, K., Han, K., 2008. Hepatitis B virus quasispecies in the

polymerase gene in treatment-naïve chronic hepatitis B patients. J. Hepatol. 48, S211.
Kobayashi, S., Ide, T., Sata, M., 2001. Detection of YMDD motif mutations in some lamivudine-untreated asymptomatic hepatitis B virus carriers. J. Hepatol. 34, 584–586.
Lai, C.L., Leung, N., Teo, E.K., Tong, M., Wong, F., Hann, H.W., Han, S., Poynard, T., Myers, M., Chao, G., Lloyd, D., Brown, N.A., 2005. A 1-year trial of telbivudine, lamivudine, and the combination in patients with hepatitis B e antigen-positive chronic hepatitis B. Gastroenterology 129, 528–536.
Langley, D.R., Walsh, A.W., Baldick, C.J., Eggers, B.J., Rose, R.E., Levine, S.M., Kapur, A.J., Colonno, R.J., Tenney, D.J., 2007. Inhibition of hepatitis B virus polymerase by entecavir. J. Virol. 81, 3992–4001.
Leung, N.W., Lai, C.L., Chang, T.T., Guan, R., Lee, C.M., Ng, K.Y., Lim, S.G., Wu, P.C., Dent, J.C., Edmundson, S., Condreay, L.D., Chien, R.N., 2001. Extended lamivudine treatment in patients with chronic hepatitis B enhances hepatitis B e antigen seroconversion rates: results after 3 years of therapy. Hepatology 33, 1527–1532.
Locarnini, S., 2008. Primary resistance, multidrug resistance, and cross-resistance pathways in HBV as a consequence of treatment failure. Hepatol. Int. 2, 147–151.
Locarnini, S., Hatzakis, A., Heathcote, J., Keeffe, E.B., Liang, T.J., Mutimer, D., Pawlotsky, J.M., Zoulim, F., 2004. Management of antiviral resistance in patients with chronic hepatitis B. Antivir. Ther. 9, 679–693.
Locarnini, S., Mason, W.S., 2006. Cellular and virological mechanisms of HBV drug resistance. J. Hepatol. 44, 422–431.
Lok, A.S., Zoulim, F., Locarnini, S., Bartholomeusz, A., Ghany, M.G., Pawlotsky, J.M., Liaw, Y.F., Mizokami, M., Kuiken, C., 2007. Antiviral drug-resistant HBV: standardization of nomenclature and assays and recommendations for management. Hepatology 46, 254–265.
Marcellin, P., Chang, T.T., Lim, S.G., Tong, M.J., Sievert, W., Shiffman, M.L., Jeffers, L., Goodman, Z., Wulfsohn, M.S., Xiong, S., Fry, J., Brosgart, C.L., 2003. Adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. N Engl. J. Med. 348, 808–816.
Matsuda, M., Suzuki, F., Suzuki, Y., Tsubota, A., Akuta, N., Hosaka, T., Someya, T., Kobayashi, M., Saitoh, S., Arase, Y., Satoh, J., Kobayashi, M., Ikeda, K., Miyakawa, Y., Kumada, H., 2004. YMDD mutants in patients with chronic hepatitis B before treatment are not selected by lamivudine. J. Med. Virol. 74, 361–366.
Melegari, M., Scaglioni, P.P., Wands, J.R., 1998. Hepatitis B virus mutants associated with 3TC and famciclovir administration are replication defective. Hepatology 27, 628–633.
Nguyen, M., Trinh, H., Garcia, R., Nguyen, L., Vutien, P., Ha, N., Nguyen, H., Nguyen, K., Keeffe, E., Ha, N., 2008. Prevalence of HBV DNA polymerase (B-DNA Pol) mutations in 345 patients with treatment-naive chronic hepatitis B (CHB). Gastroenterology 134, A310.
Ogura, Y., Kurosaki, M., Asahina, Y., Enomoto, N., Marumo, F., Sato, C., 1999. Prevalence and significance of naturally occurring mutations in the surface and polymerase genes of hepatitis B virus. J. Infect. Dis. 180, 1444–1451.
Pichoud, C., Seignerès, B., Wang, Z., Trépo, C., Zoulim, F., 1999. Transient selection of a hepatitis B virus polymerase gene mutant associated with a decreased replication capacity and famciclovir resistance. Hepatology 29, 230–237.
Pollicno, T., Isgro, G., DiStefano, R., Ferraro, D., Maimone, S., Brancatelli, S., Di Marco, V., Squadrito, G., Craxi, A., Raimondo, G., 2007. Variability of the HBV Pol gene reverse-transcriptase domain in viral isolates from untreated and lamivudine-resistant chronic hepatitis B patients. Hepatology 46, 660A.
Ramezani, A., Velayati, A.A., Roshan, M.R., Gachkar, L., Banifazl, M., Keyvani, H., Aghakhani, A., 2008. Rate of YMDD motif mutants in lamivudine-untreated Iranian patients with chronic hepatitis B virus infection. Int. J. Infect. Dis. 12, 252–255.
Schildgen, O., Sirma, H., Funk, A., Olotu, C., Wend, U.C., Hartmann, H., Helm, M., Rockstroh, J.K., Willems, W.R., Will, H., Gerlich, W.H., 2006. Variant of hepatitis B virus with primary resistance to adefovir. N. Engl. J. Med. 354, 1807–1812.
Shaw, T., Bartholomeusz, A., Locarnini, S., 2006. HBV drug resistance: mechanisms, detection and interpretation. J. Hepatol. 44, 593–606.
Sheldon, J., Ramos, B., Garcia-Samaniego, J., Rios, P., Bartholomeusz, A., Romero, M., Locarnini, S., Zoulim, F., Soriano, V., 2007. Selection of hepatitis B virus (HBV) vaccine escape mutants in HBV-infected and HBV/HIV-coinfected patients failing antiretroviral drugs with anti-HBV activity. J. Acquir. Immune Defic. Syndr. 46, 279–282.
Sheldon, J., Rodès, B., Zoulim, F., Bartholomeusz, A., Soriano, V., 2006. Mutations affecting the replication capacity of the hepatitis B virus. J. Viral Hepat. 13, 427–434.
Sheldon, J., Soriano, V., 2008. Hepatitis B virus escape mutants induced by antiviral therapy. J. Antimicrob. Chemother. 61, 766–768.
Stuyver, L.J., Locarnini, S.A., Lok, A., Richman, D.D., Carman, W.F., Dienstag, J.L., Schinazi, R.F., 2001. Nomenclature for antiviral-resistant human hepatitis B virus mutations in the polymerase region. Hepatology 33, 751–757.
Svicher, V., Gori, C., Trignetti, M., Visca, M., Micheli, V., Bernassola, M., Salpini, R., Gubertini, G., Longo, R., Niero, F., Ceccherini-Silberstein, F., De Sanctis, G.M., Spanò, A., Cappiello, G., Perno, C.F., 2009. The profile of mutational clusters associated with lamivudine resistance can be constrained by HBV genotypes. J. Hepatol. 50, 461–470.
Torresi, J., Earnest-Silveira, L., Civitico, G., Walters, T.E., Lewin, S.R., Fyfe, J., Locarnini, S.A., Manns, M., Trautwein, C., Bock, T.C., 2002. Restoration of replication phenotype of lamivudine-resistant hepatitis B virus mutants by compensatory changes in the "fingers" subdomain of the viral polymerase selected as a consequence of mutations in the overlapping S gene. Virology 299, 88–99.
Wakil, S.M., Kazim, S.N., Khan, L.A., Raisuddin, S., Parvez, M.K., Guptan, R.C., Thakur, V., Hasnain, S.E., Sarin, S.K., 2002. Prevalence and profile of mutations associated with lamivudine therapy in Indian patients with chronic hepatitis B in the surface and polymerase genes of hepatitis B virus. J. Med. Virol. 68, 311–318.
Warner, N., Locarnini, S., Kuiper, M., Bartholomeusz, A., Ayres, A., Yuen, L., Shaw, T., 2007. The L80I substitution in the reverse transcriptase domain of the hepatitis B virus polymerase is associated with lamivudine resistance and enhanced viral replication in vitro. Antimicrob. Agents Chemother. 51, 2285–2292.
Xiong, X., Yang, H., Westland, C.E., Zou, R., Gibbs, C.S., 2000. In vitro evaluation of hepatitis B virus polymerase mutations associated with famciclovir resistance. Hepatology 31, 219–224.
Yang, H., Westland, C.E., Delaney 4th, W.T., Heathcote, E.J., Ho, V., Fry, J., Brosgart, C., Gibbs, C.S., Miller, M.D., Xiong, S., 2002. Resistance surveillance in chronic hepatitis B patients treated with adefovir dipivoxil for up to 60 weeks. Hepatology 36, 464–473.
Yim, H.J., Hussain, M., Liu, Y., Wong, S.N., Fung, S.K., Lok, A.S., 2006. Evolution of multi-drug resistant hepatitis B virus during sequential therapy. Hepatology 44, 703–712.
Zoulim, F., Durantel, D., Deny, P., 2009. Management and prevention of drug resistance in chronic hepatitis B. Liver Int. 29 (Suppl. 1), 108–115.