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## Naturally occurring hepatitis B virus reverse transcriptase mutations related to potential antiviral drug resistance and liver disease progression

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### Abstract

The annual number of deaths caused by hepatitis B virus (HBV)-related disease, including cirrhosis and hepatocellular carcinoma (HCC), is estimated as 887000. The reported prevalence of HBV reverse transcriptase (RT) mutation prior to treatment is varied and the impact of preexisting mutations on the treatment of naïve patients remains controversial, and primarily depends on geographic factors, HBV genotypes, HBeAg serostatus, HBV viral loads, disease progression, intergenotypic recombination and co-infection with HIV. Different sensitivity of detection methodology used could also affect their prevalence results. Several genotype-dependent HBV RT positions that can affect the emergence of drug resistance have also been reported. Eight mutations in RT (rtL80I, rtD134N, rtN139K/T/H, rtY141F, rtM204I/V, rtF221Y, rtI224V, and rtM309K) are significantly associated with HCC progression. HBeAg-negative status, low viral load, and genotype C infection are significantly related to a higher frequency and prevalence of preexisting RT mutations. Preexisting mutations are most frequently found in the A-B interdomain of RT which overlaps with the HBeAg "a" determinant region, mutations of which can lead to simultaneous viral immune escape. In conclusion, the presence of baseline RT mutations can affect drug treatment outcomes and disease progression in HBV-infected populations via modulation of viral fitness and host-immune responses.

**Key words:** Polymerase; Hepatocellular carcinoma; Reverse transcriptase; Preexisting mutations; Hepatitis B virus

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**Core tip:** The prevalence of preexisting reverse transcriptase (RT) mutations in treatment-naïve patients largely depends on geographic factors, HBV genotypes, HBeAg serostatus, hepatitis B virus (HBV) viral loads, disease progression, intergenotypic recombination, co-infection with HIV and the method used for detecting the mutation. Genotype-dependent polymorphic amino acid substitutions in RT may affect the emergence of drug resistance, and genotype C exhibits relatively elevated spontaneous RT mutation rates. HBeAg-negative status and low viral loads are significantly associated with a higher frequency and prevalence of HBV preexisting RT mutations. Preexisting mutations are most frequently found in the A-B interdomain of RT, mutations of which can lead to simultaneous viral immune escape.

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## INTRODUCTION

Although an effective and safe vaccine against hepatitis B virus (HBV) has been available since 1982<sup>[1]</sup>, approximately 257 million people are chronic carriers of the virus. The annual number of deaths caused by HBV related diseases, including cirrhosis and hepatocellular carcinoma (HCC), was estimated as 887000 in 2015 (WHO, 2017)<sup>[2]</sup>.

Reverse transcriptase (RT) conducts the major enzymatic activity required for viral replication. Nucleos(t)ide analogs (NAs) such as lamivudine<sup>[3]</sup>, adefovir dipivoxil<sup>[4]</sup>, entecavir<sup>[5]</sup>, telbivudine<sup>[6]</sup>, and tenofovir<sup>[7]</sup>, for treatment of HBV infection, mainly target RT and function as reverse transcriptase inhibitors by mimicking natural nucleosides and integrating within the DNA molecules to interfere with viral replication<sup>[8,9]</sup>. However, due to the lack of proof reading ability of RT, the error rate for viral genome replication is as high as  $10^{-7}$  per nucleotide, which is 10-fold greater than that of other DNA viruses<sup>[10]</sup>, resulting in the emergence of antiviral-drug resistance mutations<sup>[11-15]</sup>. These NA-resistant (NAr) mutants are the greatest challenge for treatment of HBV because they change the conformational structure of RT and lower the effectiveness of NAs by impeding their binding<sup>[16]</sup>. In addition, RT partially overlaps with HBV surface antigen (HBsAg) and RT mutation may simultaneously generate HBsAg mutations, which can alter the antigenicity, immune recognition, replication capacity, and virulence of HBV<sup>[17-19]</sup>.

The reported prevalence of preexisting HBV polymerase RT mutations is varied and the impact of

preexisting RT mutations on treatment-naïve patients remains controversial. In addition, the relationship between preexisting RT mutations and advanced liver diseases, such as cirrhosis and HCC, has not been fully investigated<sup>[20]</sup>. Therefore, this review focuses primarily on factors affecting the prevalence and types of preexisting RT mutations in treatment-naïve patients and the relationship between these mutations and disease progression.

## DISTRIBUTION OF PREEXISTING HBV NAR MUTATIONS IN SAMPLES FROM TREATMENT-NAÏVE PATIENTS

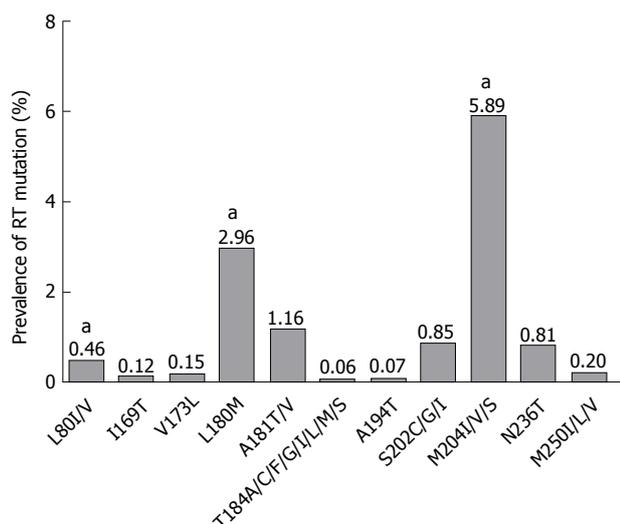
Liu *et al.*<sup>[21]</sup> identified pre-existing HBV RT mutations in 42 potential NAr RT positions from 192 treatment-naïve Chinese patients and arranged them into following four mutation categories: primary drug resistance (Category 1); secondary/compensatory mutation (Category 2); putative NAr (Category 3); and pretreatment (Category 4) (Table 1). To understand the global prevalence of these 42 naturally occurring NAr resistance mutations of RT, we reviewed a total of 50 previous studies<sup>[12,20-68]</sup> and collated their results (Figure 1). These include 32 articles published from institutions based in Asia (12 published from China, four from Iran, four from Turkey, four from India, three from Japan, two from Taiwan, and one each from Korea, Jordan, and Indonesia), 11 articles published from institutions based in Europe (six from Italy, two from Germany, and one each from Austria, Ireland, and Spain), four articles published from institutions based in North America (three from United States and one from Canada), two articles published from institutions based in South America (both from Brazil), and one article published from an institution in South Africa (Supplementary Table 1). Among the 50 studies, 36<sup>[12,20,21,32-58,60-65]</sup> used direct PCR sequencing methods, 11<sup>[22-28,59,66-68]</sup> used the INNO-LIPA line assay, and 3<sup>[29-31]</sup> detected RT mutations by ultra-deep pyrosequencing (UDPS). Seventeen articles<sup>[21,26,27,30,31,34,35,37-39,42,43,46-48,53,58]</sup> included treatment-naïve patients infected with genotypes B and C, one study<sup>[32]</sup> with genotypes A and D, eleven studies<sup>[22,28,29,36,50,51,55,56,60,63,68]</sup> with genotype D, one study<sup>[33]</sup> with genotype C, and fifteen studies<sup>[20,23-25,40,41,44,45,52,54,57,61,64,65,67]</sup> with more than three genotypes (*e.g.*, A, C, and D or A, B, C, and D). In five studies, genotypes of patients were not mentioned. Our literature-based study demonstrated that preexisting RT mutations were also found in treatment-naïve patients at 40 of 42 precisely identified NAr RT positions, the two exceptions were rF242A, a pretreatment mutation and rF166L, a lamivudine (LMV)-associated putative mutation. The distribution and overall incidence of RT mutations is presented in Figures 1 and 2.

Primary drug resistance mutations are amino acid changes that cause direct NA resistance by decreasing viral susceptibility to NAs<sup>[69-71]</sup>. Mutated RT positions known to induce primary drug resistance are rt169,

**Table 1** Distribution of preexisting RT mutation in 42 potential NA<sup>r</sup> regions in treatment naïve patients

Mutation	RT mutation type	Change in HBsAg <sup>a</sup>	Drug resistance	Genotype	Location	Ref.
Primary <sup>1</sup>	I169T		EIV	B, C	China	[20,53]
	A181T/V	sW172 stop	LMV, Ldt, ADV, TNF	A, B, C, D	Canada, Italy, China, United States	[24,25,30,39,45,51,53]
	T184A/C/F/G/I/L/M/S		EIV, LMV	A, B, C, D	Canada, China, South Korea	[24,27,33]
	A194T	no change in HBsAg	ADV, TNF	B, C, D	Indonesia, Italy	[38,51]
	S202C/G/I		EIV, LMV	A, B, C, D	Canada, China, Spain, Italy	[24,27,30,57,59]
	M204I/V/S	V-sI195M, I- sW196S/L/Stop	LMV, Ldt, ADV, TNE, ETV	A, B, C, D	Canada, Italy, South Korea, China, Indonesia, United States, Turkey	[24,25,27,30,33,36,38, 39,47,52,53]
	N236T		ADV, TNF	B, C	China	[27,30,47]
	M250I/L/V		EIV	A, B, C, D	Canada, Italy, China, United States	[24,25,27,45]
	L80I/V	no change in HBsAg	LMV	A, B, C, D	Canada, Italy, South Korea, Indonesia, China	[24,25,33,38,47,53]
	V173L	sE164D	LMV	A, B, C, D	China, Canada, Italy	[20,24,25,30,39,47]
Secondary <sup>1</sup>	L180M	no change in HBsAg	LMV, ETV, Ldt	A, B, C, D	Canada, South Korea, China, Italy, Indonesia	[24,25,27,30,33,38, 39,47,53]
	S53N		LMV	B, C	China, South Korea	[21,33,58]
	T54N	sP46T	ADV	A, B, C, D	Italy	[83]
	L82M		LMV	C	South Korea	[33]
	V84M, S85A		ADV	B, C	South Korea, China	[33,53,127]
	I91L	no change in HBsAg	LMV	B, C, D	China, Indonesia, India	[21,38,54,58]
	Y126C/R/H	C-sI118A	ADV	A, B, C, D	South Korea, China, India	[33,54,58]
	T128I, T128N	N-sG145R, I-sP120S	LMV	B, C, D	Indonesia, China, India	[38,53,54,58]
	N139D/E/Q			A	India	[54]
	W153Q/R/E	Q- sP120T/ sG145R, E-sD144E/G145R	LMV	B, C, D	South Korea, Indonesia, China, India	[33,38,54,58]
Putative <sup>2</sup>	F166L	sF158Y	LMV			
	V191I	sW182 stop	LMV, ADV	B, C, D	South Korea, China, Italy	[33,39,51,58]
	A200V	sL192F	LMV	B, C	South Korea, China	[27,33,42]
	V207I	sW199 stop, sM198I	LMV	A, B, C, D	Germany, China, Italy	[27,32,39,83]
	S213T		LMV, ETV	A, B, C, D	China, India	[39,40,42,58]
	V214A	T-sS204R	ADV	B, C, D	South Korea, Italy, China,	[30,33,53,83]
	Q215E/H/P/S		LMV, ADV	A, B, C, D	China, Italy, India, Turkey	[30,39,51,53,55]
	L217R, E218D, F221Y	D-sS210I/T	ADV	A, B, C, D	South Korea, China	[21,33,42,58]
	L229G/V/W		LMV	B, C	South Korea, China	[33,42,58]
	L229F	F-sC221L, V-sF220L	LMV	B, C, D	China, India	[54,58,128]
Pretreatment <sup>2</sup>	I233V		ADV	A, C, D	Indonesia, Italy, India, China, South Korea, Germany	[25,38,40,58,78,79]
	P237H, N238D/S/T, Y245H	N/A	ADV	B, C	South Korea, China	[21,33,39,58]
	S/C256G		LMV, ETV	B, C	China, Indonesia	[21,38,58]
	T38A, T38K	K-sQ30K		B, C	South Korea, China	[33,58]
	Y124H/D/N			B, C,	China, South Korea	[21,33,58]
	D134E/N	sI126S/N		B, C, D	China, South Korea, Indonesia	[21,33,38,58]
	N139K/H	K-sT131N, T-sI131P, H-sG139N		A, B, C	South Korea, India, Indonesia, China	[33,38,54,58]
	I224V	No change in HBsAg		B, C,	China, South Korea	[21,33,39,58]
	R242A					

<sup>1</sup>Well known NA resistance mutations (primary and secondary) with phenotypic data; <sup>2</sup>Putative and pretreatment mutations relevant to NA resistance but not experimentally confirmed; <sup>3</sup>Changes in HBsAg, reported in Sheldon *et al* [47], Liu *et al* [21], Locarnini *et al* [73], and Yang *et al* [71]. RH139 is shared in both Categories 3 (N139D/E/Q) and 4 (N139K/H). Overall, 42 positions in the RT region were studied. ADV: Adefovir dipivoxil; ETV: Entecavir; Ldt: Telbivudine; LMV: Lamivudine; TNF: Tenofovir.



**Figure 1** Pooled incidence and distribution of preexisting primary and secondary reverse transcriptase mutations compiled using data from 50 previous studies. The distribution and overall incidence of RT region is presented; numbers indicate the pooled incidence rate of the RT mutation in a total of 8,435 treatment-naïve patients. <sup>a</sup>Pre-existing RT mutation associated with the progression of HCC in treatment-naïve patients.

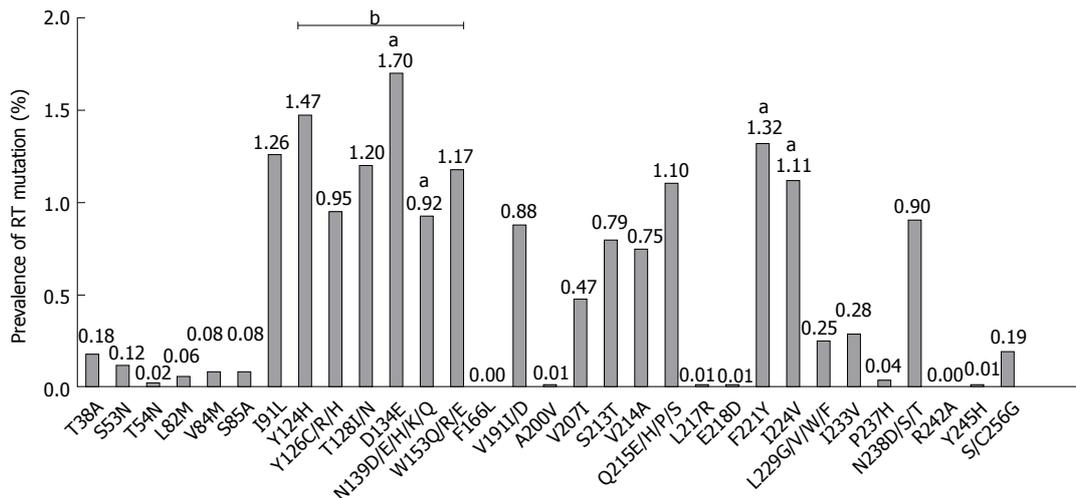
rt181, rt184, rt194, rt202, rt204, rt236, and rt250. The mutations rtA181T/V, rtM204I, and rtM204V also cause the simultaneous HBsAg mutations, sW172 stop, sW196S/L/Stop, and sI195M, respectively<sup>[17,72]</sup> (Table 1). Our literature based pooled incidence data showed that of primary drug resistance mutations, M204I/V is the most frequently encountered in treatment-naïve patients<sup>[14,28,33]</sup> (5.89%), which was far more than the pooled mutation rate of rtA181T/V, rtS202C/G/I and rtN236T (incidence: 1.16%, 0.85% and 0.81%, respectively). Mutation of rtI169T (0.12%), rtT184G (0.06%), rtA194T (0.07%), and rtM250V/L (0.20%) had a very low pooled incidence (Figure 1). A systematic review by Zhang *et al.*<sup>[73]</sup> revealed that the global incidence of rtM204I/V/S is 4.85%. Several other studies<sup>[24,28,34-37]</sup> have also reported the frequent incidence of rtM204I/V/S in treatment-naïve patients. For examples, Kobayashi *et al.*<sup>[34]</sup>, Lee *et al.*<sup>[35]</sup>, Tuncbilek *et al.*<sup>[36]</sup>, Fung *et al.*<sup>[24]</sup>, and Huang *et al.*<sup>[37]</sup> reported rtM204I/V/S mutation frequencies in Japanese, Taiwanese, Turkish, Canadian, and Chinese treatment-naïve patients reached of 27.8%, 57%, 7.8%, 12%, and 26.9%, respectively.

Secondary, or compensatory, mutations refer to amino acid substitutions that compensate for replication defects caused by primary drug resistance mutations and may reduce drug susceptibility by restoring viral replication fitness<sup>[38,69,74]</sup>. The mutations rtL80I/V, rtV173L, rtL180M are known for secondary resistance mutations. Our literature based incidence data showed that rtL180M had the highest natural incidence (2.96%), which was higher than the pooled mutation rate of rtL80I/V and V173L (incidence: 0.46% and 0.15%, respectively) (Figure 1). Similarly, Zhang *et al.*<sup>[73]</sup> reported that the overall frequency of rtL180M mutation

is 2.67%. Other studies, including Fung *et al.*<sup>[24]</sup>, Yamani *et al.*<sup>[38]</sup>, and Mirandola *et al.*<sup>[25]</sup> reported that the prevalence rates of rtL180M were 10.0%, 2.08%, and 1.18% in Chinese, Indonesian, and Italian HBV carriers, respectively. The rtL80I/V mutation also occurs frequently in treatment-naïve patients. Yamani *et al.*<sup>[38]</sup>, Kim *et al.*<sup>[33]</sup>, and Mirandola *et al.*<sup>[25]</sup> found pre-existing rtL80I/V mutation frequencies of 1.07%, 3.82%, and 0.78%, respectively. Notably, Kim *et al.*<sup>[33]</sup> reported that rtL80I/V was the most frequently encountered preexisting mutation of secondary drug resistance mutations in South Korea (3.8%, 5/131 patients), even higher than rtL180M frequency (2.3%, 3/131 patients). Another compensatory RT mutation, rtV173L, was also detected in several studies of treatment-naïve patients, where Zheng *et al.*<sup>[20]</sup>, Wang *et al.*<sup>[39]</sup>, and Mirandola *et al.*<sup>[25]</sup> reported that it occurred in 0.6%, 0.56%, and 0.39% of their patients, respectively.

RT mutations which have been identified as associated with drug resistance, but have not been confirmed experimentally *in vitro*, are defined as putative NA resistant mutations<sup>[75-77]</sup>. A total of 26 types of RT mutations, including rtS53N, rtT54N, rtL82M, rtV84M, rtS85A, rtI91L, rtY126C, rtT128I/N, rtN139D, rtW153Q, rtF166L, rtV191I, rtA200V, rtV207I, rtS213T, rtV214A, rtQ215P/S, rtL217R, rtE218D, rtF221Y, rtL229G/V/W, rtI233V, rtP237H, rtN238D/S/T, rtY245H, and rtS/C256G, are considered putative drug resistance mutations (Category 3) (Table 1)<sup>[21]</sup>. Other amino acid substitutions, which were detected before treatment, but for which the association between their occurrence and drug resistance has not been evaluated, are defined as pretreatment mutations, these include rtT38A, rtY124H, rtD134E/N, rtN139K/H, rtI224V, and rtR242A (Category 4) (Table 1)<sup>[21,38]</sup>.

Recently, it has been proven through *in vitro* and *in vivo* experiments that several putative or pretreatment mutations, including rtL229F, rtS13T, and rtI233V, can also contribute to the development of drug resistance<sup>[40,78,79]</sup>. In addition, several studies have reported that treatment-naïve patients with only putative RT mutations, and without primary or secondary changes, developed drug resistance since treatment initiation<sup>[41]</sup>. Our literature based pooled incidence data showed that several putative or pretreatment mutations, including rtI91L, mutations in 6 positions of A-B interdomain (rtY124H, rtY126C/R/H, rtT128I/N, rtD134E/N, rtN139D/E/H/K/Q and rtW153Q/R/E), rtF221Y and rtI224V, were encountered with high frequency from the treatment naïve patients<sup>[20,21,33,38,39,42]</sup>. Of these, a RT mutation in the A-B interdomain, rtD134E/N, which also cause a simultaneous sI126N/S mutation of the HBsAg "a" determinant, was found to have the highest frequency in treatment-naïve patients (1.70%) (Figure 2). Of note, the following four putative or pretreatment mutations found in treatment naïve patients, rtD134E/N, rtN139D/E/H/K/Q, rtF221Y, and rtI224V, are also reported as associated with progression of severe liver diseases, such as HCC and cirrhosis (described below).



**Figure 2** Pooled incidence and distribution of preexisting putative and pretreatment reverse transcriptase mutations compiled using data from 50 previous studies. The distribution and overall incidence of RT region is presented; numbers indicate the pooled incidence rate of the RT mutation in a total of 8435 treatment-naïve patients. <sup>a</sup>Pre-existing RT mutation associated with the progression of HCC in treatment-naïve patients; <sup>b</sup>A-B interdomain region.

Moreover, some of these mutations also overlapped with genotype-dependent polymorphic sites, as described in the next section.

## DISTRIBUTION OF GENOTYPE-DEPENDENT AMINO ACID POLYMORPHIC SITES IN TREATMENT-NAÏVE PATIENTS

To date, a total of 10 HBV genotypes (A-J) and several sub-genotypes have been identified; genotypes are separated from each other by sequence differences of more than 8% by phylogenetic analysis, based on whole genome sequences<sup>[80,81]</sup>. HBV genotypes, including genotypes A-J and the various sub-genotypes, are associated with several distinct traits, including geographical distribution, host ethnicity, and pathogenicity<sup>[82]</sup>. Since specific mutational patterns of mutation can be restricted by structural/functional constraints to particular genotypes, HBV genotype can influence the evolution frequency, or types, of mutations associated with NAr in treatment-naïve patients, as described by Liu *et al.*<sup>[21]</sup>. Moreover, HBV genotypes can affect LAM-resistant mutations in the YMDD motif of viral RT in patients with chronic infections after long term drug treatment<sup>[42,43,83]</sup>. Two recent studies<sup>[83,84]</sup> reported that genotype A favors the rtM204V mutation, while HBV-B, C, and D select for rtM204I at higher rates, compared with rtM204V. Moreover, Liu *et al.*<sup>[21]</sup> identified eight genotype-dependent AA polymorphic positions, (*i.e.*, rt53, rt91, rt124, rt134, rt221, rt224, rt238, and rt256) useful as signature for B- and C-genotypes with mutations at the 42 positions associated with NAr, and which are important for the distinction between mutations and mere polymorphisms during genotypic mutation analysis of samples from infected patients. These specific, genotype-dependent AA polymorphic positions in RT functional domains, (*i.e.*, rt91 in domain

A, rt238 in domain D and rt256 in domain E) affect drug treatment outcomes<sup>[21,85]</sup>. Liu *et al.*<sup>[21]</sup> also showed that rtL91 and rtI91 were generally favored by genotypes B and C, respectively, and that rtS256 was prevalent in both genotypes, with rtC256 more common in genotype C than in genotype B. rtL91 and rtC256 were also more closely correlated with failure of extended LMV therapy, compared with other polymorphisms (rtI91 and rtS256) leading to the suggestion that they may represent potential pretreatment markers<sup>[86]</sup>.

Mirandola *et al.*<sup>[83]</sup> further extended the eight genotype dependent polymorphic-sites suggested by Liu *et al.*<sup>[21]</sup> into a total of 27 polymorphic-sites potentially affecting treatment, *via* sequence analysis of 200 treatment-naïve chronically infected patients from north-east Italy, infected with the six HBV genotypes; A, B, C, D, E, and F. In this study, substitutions at residues rt53, rt54, and rt91 of the 27 genotype-dependent polymorphic sites were the most frequent single AA substitutions, and HBV-DNA levels of patients with these mutations were significantly lower than those of patients with no mutations, suggesting that these changes contributed to reducing viral fitness during infections. The authors also found that patients with multiple mutations were mainly infected with genotype D, rather than other genotypes (A, B, C, E, or F), strongly supporting previous results indicating that HBV-D may have the highest genetic variability among all HBV-genotypes<sup>[87]</sup>.

Yamani *et al.*<sup>[38]</sup> demonstrated that the distribution of primary and secondary drug resistance mutations was not significantly different between genotypes B and C; however, significant differences were identified in some genotype-dependent polymorphic sites. For example, rtL91I and rtY221F were more common in genotype C, compared to genotype B ( $P < 0.001$ ) (Table 2). Moreover, some genotype dependent mutations, such as rtM129L, rtD134N, rtM145L, and rtE263H/D/Q,

**Table 2** Genotype-dependent amino acid polymorphic sites and reverse transcriptase mutations in treatment-naïve patients

RT position	Drug resistance	Mutations in RT region of four genotypes <sup>1</sup>				Polymorphism	Ref.
		A	B	C	D		
38		T (4.4) <sup>4</sup>			T (14.0) <sup>4</sup>	A/T/T/A	[83]
53	LMV		D/T (1.8) <sup>4</sup>			I/N/S/N	[21]
54	ADV	N (2.2) <sup>2</sup>				T/T/T/H	[83]
84				I (0.5) <sup>2</sup>		V	[127]
				I (1.5) <sup>2</sup>			[21]
85				T (0.6) <sup>2</sup>		S	[127]
91	LMV		I (20.0) <sup>4</sup>	L (23.5) <sup>2</sup>	I (16.7) <sup>4</sup>	I/L/I/L	[83]
					I (14.3) <sup>2</sup>		[44]
103		I (100) <sup>3</sup>			I (1.67) <sup>3</sup>	V	[40]
122		H (47.0) <sup>2</sup>			H (6.66) <sup>2</sup>	F	[40]
					L/V/I(25.0) <sup>2</sup>		[44]
124		H (2.2) <sup>3</sup>	H (20.0) <sup>3</sup>	H (11.8) <sup>3</sup>		N/N/Y/H	[83]
			H (3.6) <sup>3</sup>	H (6.6) <sup>3</sup>			[21]
			D (5.5) <sup>3</sup>				
126		H (6.7) <sup>4</sup>			R (23.7) <sup>4</sup>	Y/H/H/H	[83]
					R (17.9) <sup>4</sup>		[44]
					Y (1.4) <sup>4</sup> Q (0.5) <sup>4</sup>		[83]
128	LMV	N (2.2) <sup>2</sup>			N (1.4) <sup>2</sup> I (1.4) <sup>2</sup>	T	[83]
			I (1.9) <sup>2</sup>				[38]
129				L (60.0) <sup>2</sup>	L (21.4) <sup>2</sup>	M	[44]
			L (1.9) <sup>2</sup>	L (26.2) <sup>2</sup>			[38]
		L (100.0) <sup>2</sup>		L (9.0) <sup>2</sup>	L (3.3) <sup>2</sup>		[40]
134				N (40.5) <sup>2</sup>		D/N/D/D	[38,54]
				E (23.1) <sup>3</sup>	E (5.0) <sup>3</sup>		[21,54]
			I/S (1.8) <sup>3</sup>	E (5.8) <sup>3</sup>			[21]
139	LMV		K (3.7) <sup>2</sup>		K (11.9) <sup>2</sup>	Q/N/N/N	[38]
					K (2.3) <sup>3</sup>		[83]
				K (1.5) <sup>3</sup>			[21]
145			L (3.7) <sup>2</sup>	L (40.5) <sup>2</sup>		M	[83]
153	LMV	Q (100) <sup>2</sup>				W/R/R/R	[40]
191	LMV			V (8.3) <sup>2</sup>		V/I/I/V	[39]
		I (4.6) <sup>2</sup>		F (7.7) <sup>2</sup>			[54]
200	LMV	V (2.2) <sup>2</sup>				A	[83]
207	LMV		M (6.0) <sup>2</sup> L (2.3) <sup>2</sup>			V	[26]
				I (5.9) <sup>2</sup>			[83]
				I/L/G (2.1) <sup>2</sup>			[39]
214	LMV/ADV			A (5.9) <sup>2</sup>	A (2.3) <sup>2</sup>	V	[83]
		A (0.5) <sup>2</sup> I (0.5) <sup>2</sup>		I (0.6) <sup>2</sup>	A (0.8) <sup>2</sup> E (0.7) <sup>2</sup>		[127]
215	ADV			E (7.7) <sup>2</sup>	H (5.0) <sup>2</sup> S (5.0) <sup>2</sup>	Q	[54]
			H (0.9) <sup>2</sup>		H (3.0) <sup>2</sup> S (4.3) <sup>2</sup>		[127]
					P (2.8) <sup>2</sup> S (4.2) <sup>2</sup>		[83]
217		L (6.7) <sup>4</sup>			R (0.9) <sup>2</sup>	R/L/L/L	[83]
221	ADV			F (40.5) <sup>2</sup>		Y/Y//F/F	[38]
					Y (5.1) <sup>2</sup>		[83]
226			H/T (33.3) <sup>2</sup>	H/T (2.4) <sup>2</sup>		N	[38]
237					T (6.4) <sup>2</sup>	P	[127]
238	LMV, ETV	H(1.0) <sup>2</sup> W(1.0) <sup>2</sup>	Q (3.9) <sup>2</sup>	T (8.7) <sup>2</sup>	H (2.3) <sup>2</sup>	N/H/N/N	[127]
			Q (1.82) <sup>2</sup>	S (2.19) <sup>2</sup> T (0.73) <sup>2</sup>			[21]
				T (3.87) <sup>2</sup>			[39]
		D (2.2) <sup>2</sup> T (2.2) <sup>2</sup>			D (1.4) <sup>2</sup>		[83]
245					H (1.4) <sup>2</sup>	Y	[83]
248	ADV		H (7.4) <sup>3</sup>	H (4.8) <sup>3</sup>		N	[38]
256	LMV			G (40.0) <sup>2</sup>	G (10.7) <sup>2</sup>	S/S/S/C	[44]
			G (20.0) <sup>2</sup>		G (3.7) <sup>2</sup>		[83]
			G (5.45) <sup>2</sup>				[21]
					G (3.7) <sup>2</sup>		[26]

A total of 29 reported genotype-dependent amino acid polymorphic sites in the RT region in treatment-naïve patients are shown. The first column contains the RT positions and the second column details the relationship between mutations and drug resistance. Column three to six indicate the prevalence of each mutation as percentages, according genotype. Consensus amino acids are presented in column seven. <sup>1</sup>Incidence (%) of mutations in the RT region; <sup>2</sup>Putative mutation; <sup>3</sup>Pretreatment mutation; <sup>4</sup>Novel mutation. ADV: Adefovir dipivoxil; ETV: Entecavir; Ldt: Telbivudine; LMV: Lamivudine; TNF: Tenofovir.

were more frequent in in genotype C than genotype B viruses ( $P < 0.001$ ). Notably, rtN226H/T was the

only pretreatment mutation, which is more common in genotype B than genotype C ( $P < 0.001$ ). Singla *et al*<sup>[44]</sup>

**Table 3** Positive relationships between HBeAg negative serostatus and preexisting reverse transcriptase mutation frequency in the treatment-naïve patients

HBeAg-positive		HBeAg-negative		HBV genotype (%)	Location	Ref.
Mutations <sup>1</sup>	HBV-DNA <sup>2</sup>	Mutations <sup>1</sup>	HBV-DNA <sup>2</sup>			
3/14 (21.4)	7.8	11/14 (78.6)	5.7	B, C, E	California	[26]
6/24 (25.0)	5.5	18/24 (75.0)	3.9	B, C, B-C	China	[27]
0/4 (0.0)	7.2	4/4 (100.0)	4.7	A, B, C, D, F	California	[45]
3/6 (50.0)	8.0	3/6 (50.0)	3.2	D	Turkey	[36]
8/12 (67.0)	7.9	4/12 (33.0)	6.9	NA	Taiwan, China	[35]
27/43 (62.8)	5.7	16/43 (37.2)	4.7	B, C	China	[46]
8/13 (61.5)	6.3	5/13 (38.5)	5.4	B, C	China	[47]
0/5 (0.0)	NA	5/5 (100.0)	NA	NA	Japan	[34]
0/4 (0.0)	NA	4/4 (100.0)	NA	NA	Japan	[88]

<sup>1</sup>Number of patients with RT mutation (%); <sup>2</sup>HBV-DNA level (log<sub>10</sub> IU/mL).

also showed that rtL91I and rtM129L are more common in samples from genotype C, than genotype D, infected patients. Overall, these findings indicate that distribution of genotype dependent polymorphic sites in treatment-naïve patients could affect drug treatment outcomes via modulation of viral fitness or replication. The distribution of the 29 genotype-dependent polymorphic-sites in the HBV RT region among treatment naïve patients identified in other reports is summarized in Table 2.

## GENOTYPE DISTRIBUTION OF PRIMARY RT MUTATIONS IN TREATMENT-NAÏVE PATIENTS

Mirandola *et al.*<sup>[25]</sup> identified the different genotype different distributions of antiviral drug resistant RT mutations using INNO LiPA line probe analysis of samples from treatment-naïve patients; RT mutations were detected in 13 (5%) of 255 HBV infected patients. Of these, 10 patients had mutations associated with primary resistance or reduced sensitivity, including three cases with a YMDD mutation (rtM204V), three with the mutation, rtM250L/V, which is associated with ETV resistance, and four with the mutation rtI233V, which is associated with reduced sensitivity to ADV. Notably all the three patients with the rtM204V mutation also had coexisting L180M compensatory mutations, and all were infected with HBV-C genotype viruses, suggesting that naturally occurring LMV-resistant HBV may be more frequent in patients infected with genotype C virus. This hypothesis is strongly supported by the recent report of Kim *et al.*<sup>[33]</sup> of the high frequency of the YMDD mutation, (rtM204V/I) (6.87%, 9/131 patients), in Korean treatment-naïve patients with HBV genotype C2 infections. Wang *et al.*<sup>[39]</sup> also reported that RT mutations were only found in genotype C treatment-naïve patients; however, no primary or secondary RT mutations were found in genotype B patients. In addition, a systemic meta-analysis review by Zhang *et al.*<sup>[73]</sup> showed that rtM204V/I had the highest incidence of 4.89% (95%CI: 4.13%-5.65%) among primary and secondary RT mutations. These authors also found, *via*

the subgroup analysis by genotype, that HBV genotype C had a tendency of toward a higher spontaneous YMDD mutation frequency (19.32%) than genotype B (15.01%) or D (14.79%). The increased spontaneous mutations in the viral genome of HBV genotype C could translate to a higher risk of primary NA resistance in HBV endemic areas, where genotype C infections are prevalent, including China and South Korea.

## CLINICAL FACTORS (HBEAG SEROSTATUS AND HBV VIRAL LOADS) AFFECTING INCIDENCE OF PREEXISTING RT MUTATIONS IN TREATMENT-NATIVE PATIENTS

The majority of studies have consistently reported a significant association between the prevalence of preexisting RT mutations and lower HBV DNA loads, or HBeAg-negative status, in treatment-naïve patients<sup>[26,27,34-36,45-47,88]</sup> (Table 3). Vutien *et al.*<sup>[26]</sup> reported that treatment-naïve patients with HBeAg-negative status had higher RT mutation frequencies (78.57%), compared with HBeAg-positive patients (21.42%). These authors also showed that HBeAg-negative patients had significantly lower HBV DNA viral loads compared with HBeAg-positive patients (5.65 log<sub>10</sub> IU/mL vs 7.82 log<sub>10</sub> IU/mL, respectively). Zhao *et al.*<sup>[27]</sup> also reported similar results showing that 75% of patients with RT mutations were HBeAg-negative and had lower HBV DNA levels (3.92 log<sub>10</sub> IU/mL) whereas 25% of patients with RT mutations were HBeAg-positive with higher HBV DNA loads (5.54 log<sub>10</sub> IU/mL). Similarly, Zhu *et al.*<sup>[89]</sup> found that Chinese patients with chronic HBV carrying preexisting RT mutations had significantly decreased serum baseline HBV DNA loads ( $P = 0.0363$ ) and blood platelet counts ( $P = 0.0181$ ) compared with those without RT mutations.

Several other studies<sup>[34,45,88]</sup> also found RT mutations only in HBeAg-negative patients, and the patients were also more likely to have decreased HBV DNA levels compared with those who were HBeAg-positive<sup>[45]</sup>.

Kobayashi *et al.*<sup>[34]</sup> reported that all asymptomatic HBV carriers with YMDD mutation were HBeAg-negative and eAb-positive, suggesting that sustained host immune pressure may be a major force driving potential NAr mutations. Zhang *et al.*<sup>[73]</sup> also reported a systemic meta-analysis finding that patients with chronic hepatitis B (CHB) and genotype C infections, who were male and HBeAg-negative tended to have higher spontaneous mutation rates in subgroup analysis. Xu *et al.*<sup>[46]</sup> reported no significant correlation between pre-existing mutations and the majority of clinical factors including gender, age, HBV genotype, ALT, HBeAg, and HBV DNA loads, in a Chinese population; however, subgroup analysis indicated that pre-existing mutations were strongly associated with lower HBV DNA levels in HBeAg sero-negative, but not HBeAg sero-positive, patients (HBeAg+ vs HBeAg-: 5.74 log<sub>10</sub> IU/mL vs 4.72 log<sub>10</sub> IU/mL,  $P = 0.0112$ ). These findings suggest that preexisting RT mutations might lead to lower HBV viral loads in treatment-naïve patients with HBeAg-negative serostatus. Several other studies have reported similar positive associations between the frequency of pre-existing RT mutations and decreased HBV viral loads<sup>[33,42,83]</sup>.

Taken together, there appears to be a clear causal link between preexisting RT mutations and HBeAg-negative status, decreased HBV DNA load, or liver disease progression. This may be because mutations in the RT active domain, could impair enzyme activity, particularly at the HBeAg negative immune clearance stage, thus decreasing the efficacy of virus replication and, resulting in liver disease progression and poor treatment outcomes<sup>[17,42,90,91]</sup>.

## GENOTYPE DISTRIBUTION AND GEOGRAPHICAL FACTOR AFFECTING THE INCIDENCE OF PREEXISTING RT MUTATIONS

Reports of the incidence of preexisting RT mutations in treatment-naïve patients are highly variable, ranging from 0% to 57%<sup>[25,26,28,32-35,48,92,93]</sup>. This huge discrepancy among studies may be due to differences in factors such as the geographical or ethnic backgrounds of studied patients, sample size, and viral genotype<sup>[27]</sup>. A number of studies have reported prevalence rate of preexisting RT mutations (primary and secondary RT mutations) of more than 5% in treatment-naïve patients (Table 4). Fung *et al.*<sup>[24]</sup> found a higher rate of baseline RT mutations (12% M204I/V, 10% L180M) by using the INNO-LIPA v.3 assay. In this study, many patients, most of whom were infected with genotype D, carried rtL180M, rtM204V/I, and rtL80V/I mutations. In addition, Nishijima *et al.*<sup>[94]</sup> identified a high mutation rate (35.7%) in 14 treatment-naïve patients in Japan, using UDPS. Also, a recent study using direct sequencing<sup>[33]</sup> of samples from

131 treatment-naïve patients infected with genotype C2 reported an overall rate of 12.98% for primary (rtT184A/C/F and rtM204I/V) or compensatory (rtL80I and rtL180M) mutations. According to a systemic meta-analysis review conducted by Zhang *et al.*<sup>[73]</sup>, the overall prevalence of spontaneous mutations among treatment-naïve patients worldwide was 5.73%. The highest pooled prevalence (8.00%) was identified in samples from China, followed by Japan, Turkey, Korea, South America, and Europe at 6.62%, 6.43%, 5.72%, 3.89%, and 2.53%, respectively. Another study of 325 genotype D infected treatment-naïve patients using direct PCR sequencing<sup>[50]</sup> reported overall incidence of 15.69% for primary and secondary drug resistance mutations, including L80V/I, L180M, M204I/V, and S213T/N.

In contrast, several studies have reported prevalence rates of less than 5 % for pre-existing RT mutations (primary and secondary RT mutations) in treatment-naïve patients (Table 4). For example, using direct sequencing of samples from treatment-naïve patients from the United States, Nguyen *et al.*<sup>[45]</sup> demonstrated that only four (0.9%) of 472 patients were infected with viruses with primary and secondary mutations (rtA181A/S, rtA194S, and rtM250I). Similarly, Zollner *et al.*<sup>[32]</sup> screened a total of 96 patients infected with HBV genotypes A and D (52.08% and 47.92%, respectively) using a direct sequencing assay, but found no primary or secondary resistance mutations. Another study by Salpini *et al.*<sup>[51]</sup> using the direct sequencing method reported that, of 140 treatment-naïve patients infected with genotype D, only 1.4% had primary drug resistance mutations, while 2.1% carried secondary mutations.

Overall, preexisting RT mutation prevalence clearly reflects the geographical distribution of HBV infection. For example, China is an area with high levels of endemic area of HBV infection (8%, according to a national survey in 2006) and also has higher prevalence of pre-existing RT mutations<sup>[73]</sup>. Meanwhile, in Europe, which has low levels of endemic HBV infection (approximately 2%), there is a low incidence of spontaneous mutations (2.53%)<sup>[73]</sup>. Since the HBV geographic distribution has also a close relationship with the genotype distribution, the majority of countries in Asia with prevalent genotype B and C infections have high rates of spontaneous RT mutation ( $\geq 5\%$ )<sup>[27,33-35,39,47,73]</sup>, whereas countries in Europe, where genotype A and D infections are dominant, tended to have low incidences ( $\leq 5\%$ )<sup>[32,49,51]</sup>.

## HBV INTERGENOTYPIC RECOMBINATION AND COINFECTION WITH HIV AFFECTING THE INCIDENCE OF PREEXISTING RT MUTATIONS

HBV intergenotypic recombination between different

Table 4 Variation in the prevalence of preexisting reverse transcriptase mutations according to mutation detection methods, genotype, and geographic distribution

Prevalence	Location	No. of cases	Genotype	HBV DNA loads (log <sub>10</sub> IU/mL)	RT mutations prevalence	Mutation detecting methods	Ref.	
HBV DNA RT mutations ≥ 5%	Italy	255	A, C, D	5.0	5.0% mutations overall	INNO-Liipa HBV DR v.3	[25]	
	China	269	B, C, B-C	4.9	8.9% mutations overall	INNO-Liipa HBV DR v.3	[27]	
	Canada	209	A, B, C, D	7.0	12% M204I/V, 10% L180M, 9% L80V/I, 3% V173L	INNO-Liipa HBV DR v.3	[24]	
	Turkey	71	NA	NA	18.3% YMDD mutations	INNO-Liipa HBV DR v.1	[28]	
	South Korea	131	C2	6.5	12.98% mutations overall	Direct Sequencing	[33]	
	Turkey	77	D	7.3	7.8% YMDD mutations	Direct Sequencing	[36]	
	China	213	B, C	6.2	6.1% mutations overall	Direct Sequencing	[47]	
	China	104	B, C, B-C	4.5	26.9% YMDD mutations	Direct Sequencing	[93]	
	Japan	18	NA	NA	27.8% YMDD mutations	Direct Sequencing	[34]	
	Iran	325	D	NA	15.69% mutations overall	Direct Sequencing	[50]	
	Taiwan, China	28	NA	7.5	57% YMDD mutations	Direct Sequencing	[35]	
	China	357	B, C	6.3	16.8% mutations overall	Direct Sequencing	[39]	
	Meta-analysis (China)	8156	B, C, D	NA	8.00% mutations overall	Record screening	[73]	
	HBV DNA RT mutations < 5%	Japan	14	B, C	4.9	35.7% YMDD mutations	Ultra-deep sequencing	[94]
		Iran	147	D	NA	None	Direct sequencing	[56]
China		328	B, C	6.9	3.6% mutations overall	Direct sequencing	[53]	
Japan		20	NA	NA	None	Direct sequencing	[48]	
California		472	A, B, C, D, F	5.3	< 1% mutations overall	Direct sequencing	[45]	
Italy		100	NA	NA	None	Direct sequencing	[49]	
Italy		140	D	4.0	3.5% mutations overall	Direct sequencing	[51]	
Germany		96	A, D	NA	None	Direct sequencing	[32]	
Brazil		189	A, C, D, F	3.2	overall 6.0% in Northeast/ 0% in North	Direct sequencing	[41]	
California		198	B, C	4.2	1% mutations in polymerase	INNO-Liipa HBV DR v.3	[26]	

genotypes is regarded as an important strategy for HBV genetic diversity and may impose challenges on vaccine designation and antiviral therapy strategies<sup>[95,96]</sup>. In particular, the high prevalence of vertical infections in HBV endemic areas, such as Asia or Africa, could lead to a life-long chronic infection<sup>[97]</sup>, resultantly leading to a high probability of co-infection and a high risk for virus recombination<sup>[96,98-101]</sup>. Previous studies on HBV recombination have identified different types of intergenotypic recombinants in HBV RT, most of which have recombination in RT/S overlapping region<sup>[96,98-103]</sup>. Of note, a recent study conducted by Liu *et al.*<sup>[100]</sup> demonstrated that, through full-length HBV RT sequences analysis from 201 Chinese chronic hepatitis B (CHB) patients, 38.10% (24/63) infected with genotype B had recombination with genotype C in the 3'-terminal RT sequences. These authors also showed that these intergenotypic recombinants were associated with enhanced viral DNA load and higher RT point mutation rates, compared with their parental genotype B or C, highlighting the importance of monitoring intergenotypic RT recombinants in HBV endemic areas to ensure optimal management.

Approximately, 10% of HIV-infected persons worldwide are chronically infected with HBV, and co-infection of two viruses is most frequently identified (up to 25%) in sub-Saharan Africa and Asia<sup>[104]</sup>. HBV and HIV co-infection is a major cause of morbidity and mortality because it could contribute to an increased risk of liver cirrhosis and HCC<sup>[105]</sup>. In general, previous studies showed a predominance of HBV genotype A in HIV infected individuals, compared with other genotypes<sup>[106,107]</sup>. In particular, Makondo *et al.*<sup>[108]</sup> reported that the ratio of genotype A to non-A (97% to 3%) was higher in the HBV/HIV co-infected Southern Africa patients compared with mono-infected individuals. These authors also showed that 10 percent, 3 out of 29 patients prior to the initiation of antiretroviral therapy (ART), had drug resistance mutations rtV173L, rtL180M+rtM204V, and rtV214A. In South Africa, rtM204I has been mainly detected in treatment-naïve HBV/HIV co-infected individuals<sup>[12]</sup> with rtM204V in treated HBV mono-infected participants<sup>[109]</sup>, suggesting HIV co-infection could affect HBV preexisting RT mutation pattern. A study of South African patients conducted by Selabe *et al.*<sup>[12]</sup> demonstrated that HBV lamivudine - resistant strains were detected in three out of 15 treatment-naïve mono - infected chronic hepatitis B patients,

**Table 5 Relationship of preexisting reverse transcriptase mutations with disease severity**

Type of mutation in RT	Chang in HBsAg	Genotype	Location	Disease progression	P value	Ref.
rtL80I <sup>2</sup>	NC	C	South Korea	HCC	0.036	[33]
rtD134N <sup>4</sup>	sI126S/N	B, C	China	HCC	0.007	[114]
rtN139K/T/H <sup>4</sup>	sT131N/P	C	South Korea	HCC	0.008	[33]
rtY141F <sup>5</sup>	sM307T	Ce	Taiwan	HCC	0.029	[37]
rtM204I/V <sup>1</sup>	sW196L/S/W	C	South Korea	HCC	0.021	[33]
rtF221Y <sup>3</sup>	NA	B,C,D	China	HCC, poor survival rate	0.028/0.004	[20,115]
rtI224V <sup>4</sup>	NC	C	China	HCC	0.005	[116]
rtM309K <sup>5</sup>	NA	C	China	HCC	0.007	[116]

HBV polymerase RT mutation; <sup>1</sup>Primary; <sup>2</sup>Secondary; <sup>3</sup>Putative; <sup>4</sup>Pretreatment; <sup>5</sup>Novel RT mutation. HCC: Hepatocellular carcinoma; NA: Not available; NC: Not changed; RT: Reverse transcriptase.

whereas detected in 10 out of 20 treatment-naïve HBV/HIV-coinfected patients. In contrast, a multinational study of HIV/HBV-coinfected individuals carried out by Thio *et al.*<sup>[110]</sup> demonstrated that no subject had preexisting RT mutations in the majority population of the quasispecies, suggesting no need for HBV drug-resistance testing prior to starting anti-HBV therapy in HIV-HBV co-infected individuals. It is also supported by a recent study of Ghana patients conducted by Archampong *et al.*<sup>[111]</sup>. Taken together, geographical factors and HBV genotypes could have effects on the preexisting HBV RT mutation in treatment-naïve HBV/HIV-coinfected patients.

rtA181T mutation (1.0% ratio). Similarly, Aberle *et al.*<sup>[66]</sup> also compared the detection efficacies for preexisting RT mutations between the INNO-LiPA assay and direct sequencing. The former identified additional mutations in 8 (14%) of 56 patient samples, which could not be detected using the latter method, indicating the superiority of the former over the latter for RT mutation detection. Overall, these data demonstrate that the method used for detecting the mutations can affect the prevalence estimates of preexisting RT mutations in treatment-naïve patients, which may cause discrepancies among the results of different studies.

### DIFFERENT SENSITIVITY OF DETECTION METHODOLOGY USED CAN AFFECT THE REPORTED PREVALENCE OF PREEXISTING RT MUTATIONS: LIMITATION OF THE STUDIES IN PREEXISTING RT MUTATIONS

The detection methods used can also have a profound effect on the reported incidence results of preexisting RT mutations. The majority of studies have used direct sequencing methods, which can lead to the underestimation of preexisting RT mutations, due to the relative low sensitivity of these assays. Wang *et al.*<sup>[39]</sup> reported that the sensitivity of direct sequencing-based protocols declined when circulating viral subspecies (AA substitutions) levels were at ratio below 20%-25%. Similarly, there were several studies have reported discordance in the incidence of pre-existing RT mutations detected by direct sequencing and other screening methods, such as the INNO-LiPA assay, or UDPS. For example, Margeridon-Thermet *et al.*<sup>[52]</sup> reported that direct sequencing found an average of 5.9 mutations per sample, while UDPS identified an additional 4.6 mutations per sample, which could not be detected by direct sequencing. In that study, two of 17 treatment-naïve patients had mutations which were detected only by UDPS, but not by direct sequencing; one rtM204I mutation with (1.3% mutant ratio) and the other an

### PREEXISTING RT MUTATIONS ARE RELATED TO THE PROGRESSION OF LIVER DISEASES

Although the clear association between preexisting RT mutations and advanced liver disease has not been fully investigated, several types of HBV mutations in RT have previously been reported as related to the progression of liver diseases, such as cirrhosis and HCC (Table 5). Kim *et al.*<sup>[33]</sup> compared types and frequencies of pre-existing RT mutations between CHB and HCC treatment-naïve patients. These authors found a significantly higher rate of RT mutations in HCC patients than in those with chronic hepatitis (3.17% vs 2.09%,  $P = 0.003$ ) and also identified a total of three NAr mutations (rtL80I, rtN139K/T/H, and rtM204I/V) significantly associated with HCC progression. RT mutations rtN139K/T/H and rtM204I/V also cause simultaneous mutations in the overlapped HBsAg coding sequence (sT131N/P and sI195M, or sW196S/L/Stop)<sup>[17,21,72]</sup>. Of these, the YMDD-motif mutation (rtM204I/V) was found in 9 patients of 131 patients (8 HCC and 1 CHB) with the other two types of mutation, rt204I and rt204V, in 8 and 1 patients, respectively. The other HCC-related mutation (rtL80I) was first identified as a compensatory mutation associated with LMV resistance<sup>[69,112]</sup>. Its relationship with clinical deterioration is also corroborated by other reports that it was associated with increased viral loads, accompanied by an elevation in serum aminotransferase activity, and exacerbation of liver disease in every

**Table 6** Distribution of preexisting reverse transcriptase mutations among reverse transcriptase domains

Domains <sup>2</sup>	Mutation frequency (%) <sup>1</sup>		P value <sup>5</sup>	Ref.
	A-B interdomain <sup>3</sup>	Non-A-B interdomains <sup>4</sup>		
1.45	3.51	2.58	< 0.001	[38]
1.37	4.4	3.77	< 0.001	[20]
1.07	7.5	3.16	0.008	[33]
0.43	3.82	0.52	0.0014	[21]

<sup>1</sup>Mutation frequency was calculated as the number of mutations found in a specific RT domain divided by the total number of sites in the domain; <sup>2</sup>Domain including RT mutation sites: rt38, rt84, rt207, rt233, rt238, and rt256; <sup>3</sup>A-B interdomain including RT mutation sites: rt53, rt191, rt213, rt218, rt229, and rt242; <sup>4</sup>Non-A-B interdomains including RT mutation sites: rt124, rt126, rt128, rt134, rt139, and rt153; <sup>5</sup>P-values of comparisons of mutation frequencies between A-B interdomain and other functional domains.

case<sup>[113]</sup>. Interestingly, Kim *et al.*<sup>[33]</sup> also showed that rtL80I was combined with the rtM204I/V mutation in five of nine rtM204I/V cases, and that patients with L80I had increased HBV replication compared with those without this mutation, suggesting that, together with rtM204I/V, it may contribute to HCC generation in treatment-naïve patients by compensating for the defective replication of caused by rtM204I/V.

In another study, Yin *et al.*<sup>[114]</sup> analyzed the association of the mutations of HBV polymerase with postoperative survival in 92 patients with HBV-related HCC using direct sequencing. They discovered three nucleotide sites, one (31st nucleotide) in a spacer region and two [529<sup>th</sup> ( $P = 0.007$ ) and 1078<sup>th</sup> ( $P = 0.038$ )] in the RT region, which could be considered independent predictors of postoperative survival in HBV-related HCC. Of the two sites in RT related to HCC outcomes, rtD134N (mutation G529A) was associated with lamivudine resistance, further supporting previous findings of potential correlation between resistance to the anti-HBV nucleoside analog, lamivudine, and HCC prognosis<sup>[114]</sup>. Since rtD134N also causes an amino acid change in HBsAg (sI126N/V), it can induce changes in the antigenic properties of HBsAg. Further functional studies are necessary to determine whether the rtD134N mutation can induce HCC via modulation of RT activity or through its effects on HBV replication.

Huang *et al.*<sup>[37]</sup> found seven viral single nucleotide polymorphisms (SNPs) in HBV polymerase, which enhance viral replication and liver disease progression in HBeAg negative subjects. Of these SNPs, rtY141F (Y487F), which is located in the RT region of HBV polymerase was associated with increased viral load and HCC ( $P = 0.0291$ ). Moreover, rtY141F, a genotype C-related SNP, also led to a simultaneous amino acid change in the overlapping 'a' determinant region of HBsAg (sM307T). In addition, Li *et al.*<sup>[115]</sup> and Zheng *et al.*<sup>[20]</sup> reported that the rtF221Y mutation was strongly related to HCC prognosis after liver resection (hazard ratio, 2.345;  $P = 0.001$ ). Moreover, the

rtF221Y mutation was also associated with poor overall survival (hazard ratio, 2.557;  $P = 0.004$ ), suggesting that it is a potential independent risk factor and viral marker for HCC. Those results were consistent with the report of Li *et al.*<sup>[115]</sup>, which identified the rtF221Y mutant as an independent risk factor for recurrence of HCC and poor overall survival ( $P = 0.001$  and  $P = 0.004$ , respectively). Wu *et al.*<sup>[116]</sup> also investigated preexisting RT mutations potentially related to HCC in Chinese patients and identified rtI224V and rtM309K as significant risk factors for HCC ( $P = 0.005$  and  $P = 0.007$ , respectively).

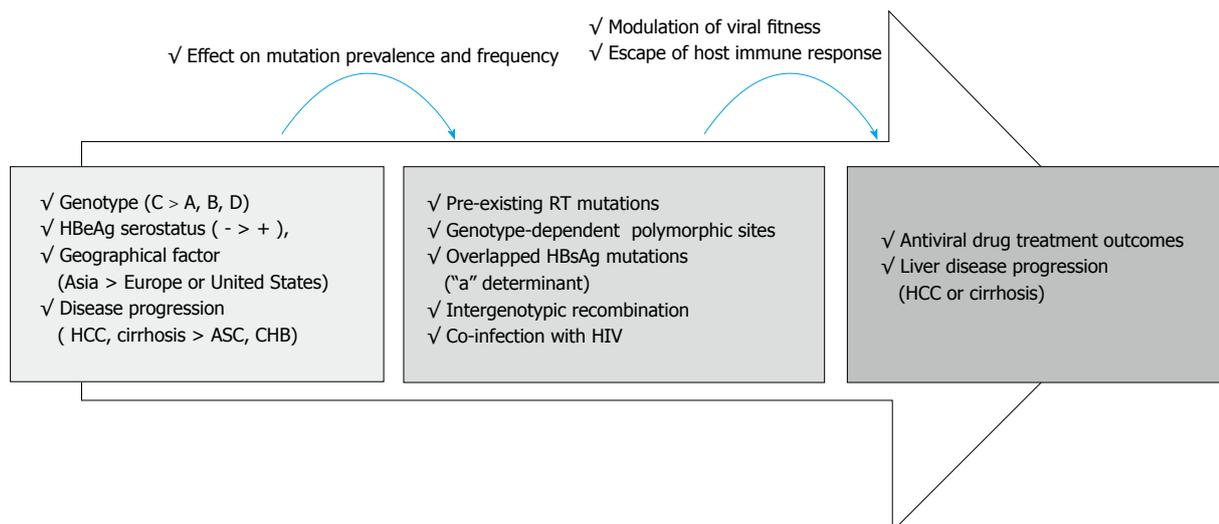
In addition, the number of RT mutations is associated with the liver disease progression. Zhu *et al.*<sup>[89]</sup> revealed that patients with multiple RT mutant sites showed a significantly higher rate of liver fibrosis ( $P = 0.0128$ ), suggesting a link between viral mutation and clinical progression of chronic hepatitis, and also highlighting that the natural accumulation of RT mutations is a process involved in viral survival during chronic liver fibrosis.

Overall, eight mutations in the RT region, namely rtL80I, rtD134N, rtN139K/T/H, rtY141F, rtM204I/V, rtF221Y, rtI224V, and rtM309K, are significantly related to liver disease progression. The majority of HCC-related RT mutations were reported from studies of treatment-naïve patients infected with genotype C HBV. This supports previous reports that HBV genotype C is more likely to lead to severe and aggressive liver disease than other HBV genotypes<sup>[112,117-122]</sup>. Of note, association of the following three mutations, rtM204V, rtL80I, and rtD134N, with disease progression provides a likely explanation for the positive relationship between lamivudine resistance and liver disease progression.

## DISTRIBUTION AND FREQUENCY OF PREEXISTING RT MUTATIONS IN DIFFERENT RT REGIONS

HBV RT consists of seven functional domains (G, F, A, B, C, D, and E) and five inter-domains (F-A, A-B, B-C, C-D, and D-E) which link the functional domains<sup>[18,31,123]</sup>. Previous studies<sup>[20,21,33,38]</sup> reported a higher frequency of preexisting RT mutations in the A-B inter-domain, compared with other regions.

Liu *et al.*<sup>[21]</sup> revealed that all six sites in the A-B interdomain, rt124, rt126, rt128, rt134, rt139, and rt153, exhibit mutations (6/6, prevalence 100%), indicating high genetic variability of this region compared with other sites within RT domains (sites with mutations: 6/22, 27.27%;  $P = 0.0014$ ). In this study, the mutation frequency of the A-B interdomain (44/1152, 3.82%) was also significantly higher than those in other RT domains (Table 6). This result is in line with that reported by Zheng *et al.*<sup>[20]</sup>, who demonstrated that A-B interdomain exhibits higher mutation frequencies (4.3%, 5.3%, 3.6%) than those of other RT domains (1.4%, 1.4%, 1.3%) in Chinese



**Figure 3** Schematic representation indicating the role of preexisting hepatitis B virus reverse transcriptase mutations in liver disease progression and treatment outcomes. HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; ASC: Asymptomatic carriers; CHB: Chronic hepatitis B; HIV: Human immunodeficiency virus.

treatment-naïve patients with CHB, cirrhosis, and HCC. Specifically, they found that there was a clear tendency toward frequent mutations of the A-B interdomain in patients with cirrhosis suggesting a relationship between mutations in the A-B inter-domain and the development of this condition.

Similarly, Yamani *et al.*<sup>[38]</sup> also reported that the A-B interdomain had the highest mutation prevalence and frequency ( $3.51\% \pm 2.53\%$ ) compared with functional domains and non-A-B interdomains ( $1.45\% \pm 1.05\%$  and  $2.58\% \pm 0.51\%$ , respectively) in Indonesian treatment-naïve patients (Table 6). Moreover, they found that genotype C had substantially higher mutations rates in the A-B interdomain than genotype B ( $P < 0.001$ ). Kim *et al.*<sup>[33]</sup> also revealed that mutations within the A-B interdomain were most frequent in treatment-naïve Korean patients infected with genotype C2, compared with other domains, with 46 of 79 patients (58.22%) with preexisting RT mutations having changes in the A-B inter-domain. In this study, rtD134E/N/C was the most frequently encountered hot spot site among the six A-B inter-domain sites and was mutated in 12/79 patients (15.2%). The authors also showed that the mutation frequency of A-B interdomain (59/786, 7.50%) was higher than that of non A-B interdomain (3.16%) (Table 6). Our pooled incidence also supported the previous notion of higher frequency of persisting RT mutations in A-B interdomain compared with other region in RT (Figure 2).

RT and HBsAg mutations can occur simultaneously, due to the overlap of RT region and HBsAg gene sequences<sup>[19,124]</sup>. Liu *et al.*<sup>[21]</sup> reported that 14 of 18 mutated positions in RT overlapped with HBsAg, and that RT mutations at 12 out of 14 RT positions (except those at rt124 and rt126) also led to simultaneous HBsAg mutations of 19 types in 16.67% (32/192) of isolates (Figure 1). Notably, these authors also

found that RT mutations in the A-B interdomain could lead to simultaneous AA substitutions sI126A/N/S/, sG130N, sT131N/P, and sG145R of the overlapped 'a' determinant of HBsAg, including the most frequently described immune-escape mutation sG145R (1/192, 0.52%)<sup>[125,126]</sup>. Similarly, Kim *et al.*<sup>[33]</sup> demonstrated that RT mutations at 10 of 42 NAr positions could lead to 15 types of simultaneous overlapped HBsAg mutations in 32.06% (42/131) patients. Of interest, they also found that the RT mutations at 3 NAr positions (rt134, rt139, and rt153) located in the overlapped HBsAg "a" determinant region from 22 treatment-naïve patients also had simultaneous "a" determinant mutations in two positions, S126 and S131, in 15 patients (15/22, 68.2%) (12 patients with mutations at rt134, leading to 10 changes of AA S126, and 8 patients with mutations at rt139, leading to 5 alterations of AA S131).

Overall, preexisting RT mutations are distributed in a non-random manner, and most frequently found in the A-B interdomain, overlapped with the HBsAg "a" determinant region, than in other domains. Moreover, the A-B interdomain also contains the most abundant mutations, indicating that these positions might be preexisting mutation hotspots in treatment-naïve patients. Of six positions' mutation in the A-B interdomain, three RT mutations, rtD134E/N, rtN139D/E/H/K/Q, and rtW153E/Q/R, that overlap with HBsAg "a" determinant region are hotspots found most frequently in treatment-naïve patients, which could contribute to HBV viral persistence via generation of immune escape "a" determinant mutants proteins. In general, A-B interdomain mutations are prevalent in patients with genotype C2 infections and could contribute to HBV-associated disease, such as HCC and cirrhosis.

## CONCLUSION

Preexisting HBV RT mutations in treatment-naïve

patients are related to potential drug resistance and progression of liver disease, such as HCC or cirrhosis. In addition, genotype-dependent polymorphic amino acid substitution in RT can also affect the emergence of drug resistance and treatment outcomes. The reported prevalence of spontaneous RT mutations in treatment-naïve patients is varied, and largely depends on geographic factors, HBV genotypes, HBeAg serostatus, HBV viral loads, disease progression, intergenotypic recombination, and co-infection with HIV. Different sensitivity of detection methodology used could also affect their prevalence results. The INNO-LiPA assay and UDPS method detect higher prevalence rates of preexisting RT mutations compared with direct PCR sequencing in treatment-naïve patients. Genotype C infection, HBeAg-negative status, and low viral loads are significantly associated with higher frequencies and prevalence rate of pre-existing HBV RT mutations. Higher frequencies of preexisting RT mutations were also generally associated with liver disease progression, including of HCC and cirrhosis. Eight mutations in RT region, rT180I, rT134N, rT139K/T/H, rT141F, rT204I/V, rT221Y, rT224V, and rT309K were significantly associated with progression of HCC in treatment-naïve patients. Of RT domains, preexisting RT mutations occur most frequently in the A-B interdomain which overlaps with the HBsAg "a" determinant region, in which mutations can lead to simultaneous viral immune escape (Figure 3). In conclusion, the presence of baseline preexisting RT mutations can affect drug treatment outcomes and disease progression in populations by modulation of viral fitness and host-immune responses.

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