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RAPID COMMUNICATION

Role of Additional Mutations outside the YMDD Motif of Hepatitis B Virus Polymerase in L(−)SddC (3TC) Resistance

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ABSTRACT. L(−)SddC (3TC) has been shown to be the most promising nucleoside analogue used for the treatment of hepatitis B virus (HBV) infection. Unfortunately, it has been reported that about 12% of HBV-infected patients experience a recurrence of HBV after a period of treatment with 3TC. Point mutations were detected in the HBV polymerase of those viruses from 3TC-resistant patients. A common mutation occurred at methionine in the YMDD motif. In this report, we present mutants that were generated from the HBV genome (adr subtype) by site-directed mutagenesis based on clinical reports from other investigators. With the transient transfection system, it was found that by changing methionine to valine or isoleucine at the YMDD motif, the viral DNA replication would be more than 100-fold less efficient than that of the wild-type virus. Some additional mutations outside the YMDD motif could enhance the replication of the virus containing a YMDD mutation. Various levels of resistance to 3TC were observed in HBV mutants containing point mutations both inside and outside the YMDD motif. These results suggest that the mutations outside the YMDD motif compensate the YMDD mutation to some extent for the viral replication and may also contribute to clinical viral resistance to 3TC. BIOCHEM PHARMACOL **55**;10:1567–1572, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. hepatitis B virus (HBV); L(−)SddC (3TC); drug resistance; YMDD motif; site-directed mutagenesis; mutations outside YMDD motif

HBV†, a causative agent of acute and chronic hepatitis, affects 300–350 million people around the world. Chronic infection has been shown to be closely related to the development of hepatocellular carcinoma [1, 2]. A selective anti-HBV compound would be useful, not only for the treatment of HBV infection but also for delaying the onset or decreasing the incidence of hepatocellular carcinoma. 3TC is the most promising compound discovered thus far by investigators in our laboratory and others [3–5]. During the past year, several groups reported that some patients initially responsive to 3TC had a rebound of HBV following long-term treatment with 3TC. Point mutations were detected in the HBV genome, and all of these cases displayed a change of methionine to valine or isoleucine in the conserved YMDD motif (encoding for tyrosine, methionine, aspartate, aspartate) of HBV polymerase [6–9]. This motif which is also present in HIV reverse transcriptase [10], is involved in nucleotide binding. The mutation of methionine to either isoleucine or valine at the YMDD motif of HIV reverse transcriptase could render HIV resistant to 3TC [11–15]. Site-directed mutagenesis of methionine to valine at the YMDD motif in duck HBV polymerase also confers resistance to 3TC *in vitro* [16]. Other point mutations beyond this site also have been detected. Whether the YMDD mutation alone was responsible for clinical resistance of HBV to 3TC treatment, and what role other mutations play in the drug resistance, are unclear. In this paper, mutants were constructed by introducing a mutation(s) into the human HBV genomic clone (adr subtype) pHBV-adr. The drug sensitivity to 3TC of the wild-type virus and the mutants was examined in transient transfected HepG2 cells. It was found that a YMDD mutation alone may be essential but not sufficient to establish 3TC resistance. Other mutations outside the YMDD motif could increase the viral progeny DNA replication by compensating for the impact of a YMDD mutation and thus play an important role in the development of clinical resistance.

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†*Abbreviations:* HBV, hepatitis B virus; 3TC, L(−)SddC; SEAP, secreted alkaline phosphatase; PCV, penciclovir; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; and HBcAg, HBV core antigen.

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MATERIALS AND METHODS

HBV Genome Construction and Site-Directed Mutagenesis

The HBV (subtype adr) genomic clone pHBV-adr was a gift from Professor Yuan Wang, Shanghai Institute of Biochemistry, Academia Sinica. Because the HBV replication is via

reverse transcription of a 3.5-kb pregenomic RNA, the entire transcription unit, starting from enhancer II upstream of the core promoter, was cloned into the pBS(+) vector, which was 1.1-fold the length of the HBV genome. It contained a 3′-terminal redundant region necessary for the transcription of all viral RNAs. The linearized genome could express HBeAg, HBcAg, and HBsAg, and could replicate virions efficiently when transfected into HepG2 cells. The expression was controlled by HBV autologous promoters and enhancers other than any foreign regulation elements [17, 18]. It was used as a wild-type construction in the transfection system and as a template in the site-directed mutagenesis. Point mutations in the HBV polymerase were generated by the polymerase chain reaction quick change method (Stratagene). Sequences of the *P* gene have been verified by DNA sequencing. Mutagenic oligonucleotides used in this study are shown below:

HBV nucleotide sequence	5′ CTTTCAGTTAT**A**TGGATGATGTGGT 3′
	nt730 nt754
forward primer M550Vf	5′ CTTTCAGTTAT**G**TGGATGATGTGGT 3′
reverse primer M550Vr	3′ GAAAGTCAATA**C**ACCTACTACACCA 5′
HBV nucleotide sequence	5′ CTTTCAGTTATAT**G**GATGATGTGGT 3′
	nt730 nt754
forward primer M550If	5′ CTTTCAGTTATAT**T**GATGATGTGGT 3′
reverse primer M550Ir	3′ GAAAGTCAATATA**A**CTACTACACCA 5′
HBV nucleotide sequence	5′ CTCAGTCCGTTTCTC**C**TGGCTCAGTTTAC 3′
	nt654 nt682
forward primer L526Mf	5′ CTCAGTCCGTTTCTC**A**TGGCTCAGTTTAC 3′
reverse primer L526Mr	3′ GAGTCAGGCAAAGAG**T**ACCGAGTCAAATG 5′
HBV nucleotide sequence	5′ TCCCCCACTGTTTGG**C**TTTCAGTTATATGGATGATG 3′
	nt715 nt750
forward primer A546Vf	5′ TCCCCCACTGTTTGG**T**TTTCAGTTATATGGATGATG 3′
reverse primer A546Vr	3′ CCGAAAGGGGGTGACAAACC**A**AAAGTCAATATACCTA 5′
HBV nucleotide sequence	5′ TCCCCCACTGTTTGG**C**TTTCAGTTATATGGATGATG 3′
	nt715 nt750
forward primer A546VM550If	5′ TCCCCCACTGTTTGG**T**TTTCAGTTATATTGATGATG 3′
reverse primer A546VM550Ir	3′ CGAAAGGGGGTGACAAACC**A**AAAGTCAATATA**A**CTA 5′
HBV nucleotide sequence	5′ AGTTATATGGATGAT**G**T**G**GTATTGGGGGCCAAGTCTG 3′
	nt735 nt771
forward primer V553If	5′ AGTTATATGGATGAT**A**T**C**GTATTGGGGGCCAAGTCTG 3′
reverse primer V553Ir	3′ GAAAGTCAATATACCTACTA**T**A**G**CATAACCCCCGGTTC 5′
HBV nucleotide sequence	5′ AGTTATAT**G**GATGAT**G**T**G**GTATTGGGGGCCAAGTCTG 3′
	nt735 nt771
forward primer M550IV553If	5′ AGTTATAT**T**GATGAT**A**T**C**GTATTGGGGGCCAAGTCTG 3′
reverse primer M550IV553Ir	3′ AAAGTCAATATA**A**CTACTA**T**A**G**CATAACCCCCGGTTCAG 5′

Cell Culture and Transient Transfection

Human hepatoma cells HepG2 (from ATCC) were maintained in Minimum Essential Medium with 10% fetal bovine serum. Cells (5×10^5) were seeded onto 100-mm culture dishes. Twenty micrograms of plasmid containing HBV genome was transfected by calcium precipitation [19]. Two micrograms of pSEAP was co-transfected with the HBV construction as an internal control. SEAP activity was detected to normalize against the variation of the transfection efficiency among different samples [20].

Viral Antigen Expression

The culture medium of transfected HepG2 cells was harvested every day for the total 9-day observation. HBsAg and HBeAg in 200 μL of medium or its diluted solution were measured by an immunoassay system (Abbott Laboratories). Absorbance at 492 nm, representing the quantity of antigens in 200 μL of medium, was plotted against days after transfection.

HBV Viral DNA Extraction

EXTRACELLULAR. The culture medium was harvested and the virus was precipitated by PEG 8000 (10%) in 0.5 M of NaCl at 4° overnight. After centrifuging for 30 min at 16,000 *g*, the virus was resuspended in 50 mM of Tris-HCl (pH 8.0), 10 mM of $MgCl_2$. The exogenous plasmid DNA was degraded by DNase I (100 μg/mL) at 37° for 30 min, followed by protein digestion with proteinase K (100 μg/mL) in 15 mM of EDTA, 100 mM of NaCl, and 0.5% SDS for 3 hr at 50°. After phenol extraction, the secreted viral DNA was recovered by ethanol precipitation.

INTRACELLULAR. Cells were harvested and lysed in 1% NP-40, 50 mM of Tris-HCl, and 1 mM of EDTA at 37° for 10 min. The digestion of exogenous DNA and virus capsule protein was the same as above. The viral DNA was detected by Southern hybridization, and the quantities were normalized against the activity of the co-transfected reporter gene SEAP.

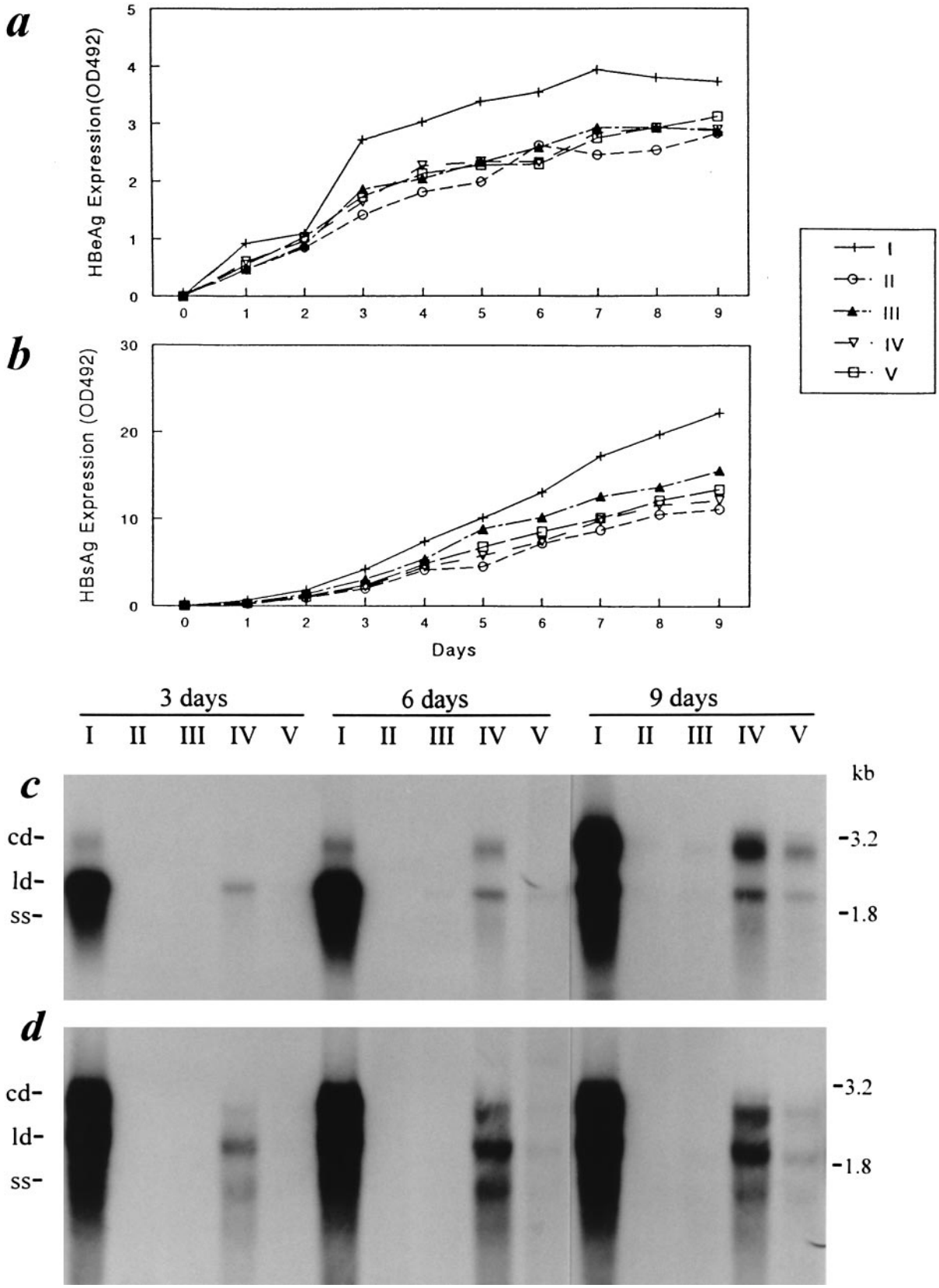

FIG. 1. Time course of HBV antigen expression and progeny DNA replication. HepG2 cells were transient transfected with wild-type and mutated HBV genome. Culture medium and cells were harvested at certain days, as indicated, and detected by ELISA (Abbott) or Southern hybridization. (*a*) HBV e antigen expression. (*b*) HBV surface antigen expression. (*c*) Extracellular HBV progeny DNA. (*d*) Intracellular HBV progeny DNA. Key: (I) pHBV-adr, (II) pM550I, (III) pM550V, (IV) pL526M, and (V) pL526MM550V. Abbreviations: cd, circular partial double-stranded HBV DNA; ld, linear partial double-stranded HBV DNA; and ss, single-stranded HBV DNA. Results of (*a*) and (*b*) are the means of the two separate experiments. Variance from the mean was ≤10% for all values. Panels (*c*) and (*d*) are of an experiment representative of three.

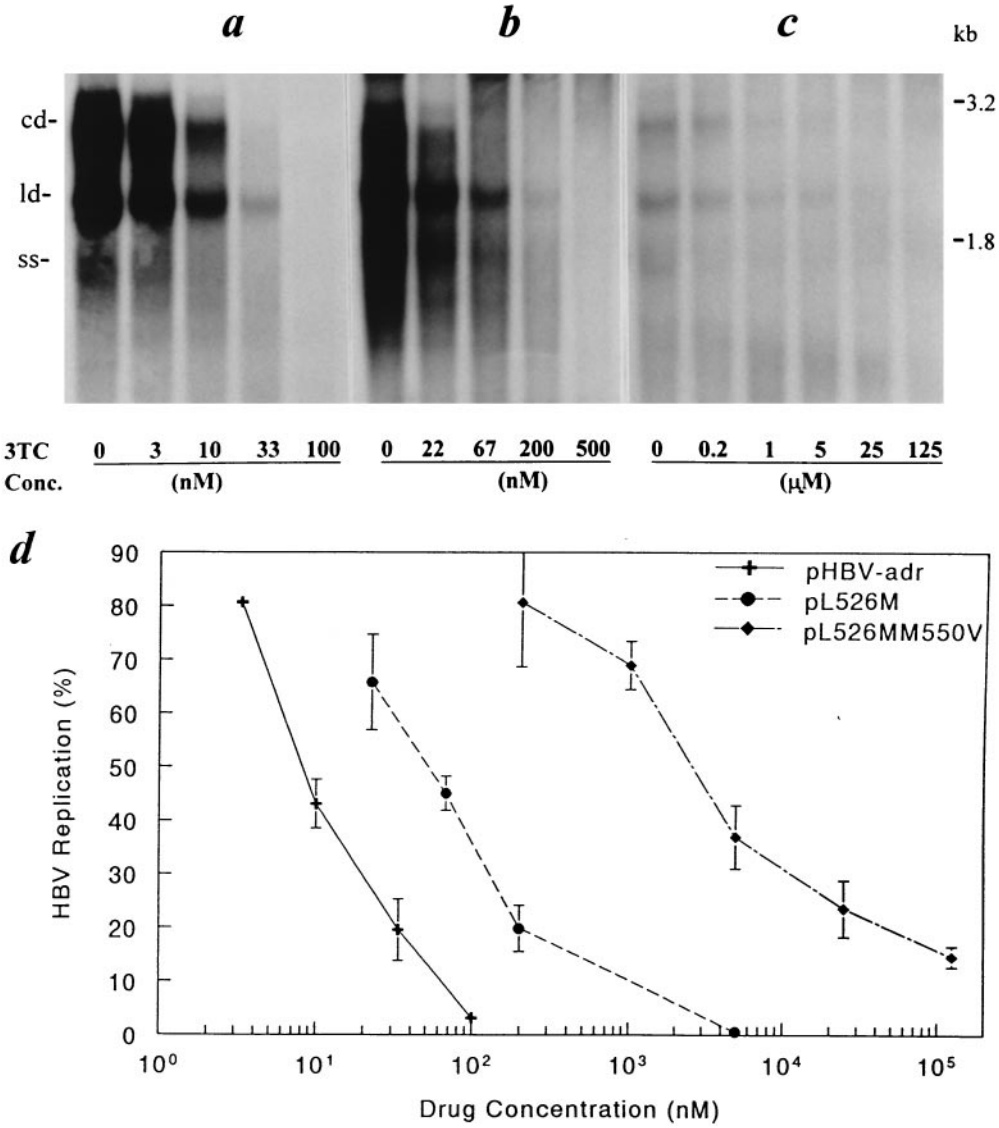

FIG. 2. Drug sensitivity of HBV wild-type and mutated genome to 3TC. HepG2 cells transfected with HBV genome were treated with 3TC for 9 days. Intracellular HBV progeny DNA was extracted and detected by Southern hybridization. (*a*) HBV wild-type genomic clone pHBV-adr. (*b*) HBV mutated genomic clone pL526M. (*c*) HBV mutated genomic clone pL526MM550V. (*d*) Concentration-dependent relationship of 3TC. Values are means ± SD, $N = 3$. Abbreviations: cd, circular partial double-stranded HBV DNA; ld, linear partial double-stranded HBV DNA; and ss, single-stranded HBV DNA.

Assay of the Antiviral Effects of 3TC

Transient transfected HepG2 cells were treated with the appropriate concentration of 3TC when the calcium-phosphate precipitated medium was replaced. Every 3 days, a half volume of fresh medium containing 3TC at a certain concentration was added. After 9 days, the cultures were harvested, and the HBV progeny DNA products were detected by Southern hybridization, as described above.

RESULTS AND DISCUSSION

To examine the impact of the YMDD mutation on the sensitivity of human HBV to 3TC, mutants pM550V and pM550I were generated from the human HBV genomic clone (adr subtype) pHBV-adr by mutating methionine to valine or isoleucine at the YMDD motif. Transient transfection was employed to introduce the wild-type virus and the mutants into HepG2 cells. In this construct, the expression of the viral genome is controlled by its own promoters and enhancers. It was found that the progeny DNA replication efficiency of the two mutants was 0.8% of the wild-type genome, although there was no significant difference in the expression of HBeAg and HBsAg among the wild-type virus and the mutants (Fig. 1). Similar results were also observed by another group [21]. Thus, this mutation alone may not be sufficient to explain the reappearance of HBV in patients undergoing long-term treatment with 3TC.

It was shown that there were other mutations in the HBV DNA polymerase in addition to the methionine

TABLE 1. Variation in HBV progeny DNA replication and drug sensitivity to 3TC

	HBV polymerase amino acid residues				Viral	3TC		HBV surface antigen amino acid residues				
Plasmid	526	546	550	553	yield (%)	IC_{50} (nM)	Fold	192	195	196	198	199
pHBV-adr	L	A	M	V	100	8	1	L	I	W	M	W
	Mutation(s)							Resulting amino acid changes				
Group 1*												
pM550V			V		0.8 ± 0.1	ND	ND		M			
pL526M	M				14.3 ± 1.9	50	6					
pL526MM550V	M		V		3.9 ± 0.2	2.5×10^3	312		M			
Group 2†												
pM550I			I		0.8 ± 0.1	ND	ND			L		
pV553I				I	96.9 ± 4.3	20	3				I	S
pM550IV553			I	I	3.2 ± 1.6	5×10^4	6.25×10^3			L	I	S
Group 3‡												
pM550I			I		0.8 ± 0.1	ND	ND			L		
pA546V		V			91.9 ± 5.4	10	1	F				
pA546VM550I		V	I		2.2 ± 0.5	2×10^3	250	F		L		

The mutation sites in HBV DNA polymerase were based on the HBV virion sequence from patients who had lost sensitivity to 3TC. Viral yield values are means ± SD, $N = 3$. The IC_{50} values were generated from the analysis of concentration-dependent curves representing the means of triplicate independent experiments. ND, not detected.

*Patient No. 1 in Ref. 6.

†Patient in Ref. 7.

‡Patient No. 3 in Ref. 8.

mutation at the YMDD motif of HBV virions from patients no longer responding to 3TC treatment. We suspected that the mutation(s) outside the YMDD motif might compensate for the effect of the methionine mutation at the YMDD motif with regard to the propagation of HBV virions in patients. Because L526M mutation, in addition to M550V mutation, was reported in the virions from a 3TC-resistant patient [6], mutants pL526M and pL526MM550V were constructed, in which pL526M contained only one mutation of leucine to methionine 23 amino acids upstream from the YMDD motif, and pL526MM550V contained both the YMDD mutation and the upstream mutation. No significant difference was observed in the time courses of the expression of HBeAg and HBsAg (Fig. 1, *a* and *b*). When the HBV progeny DNA replication was examined, it was found that the single mutation outside the YMDD motif decreased the HBV replication, too. The virion yield of pL526M was only 14% of the wild-type pHBV-adr. However, pL526MM550V had a five times higher virion yield than pM550V (Fig. 1, *c* and *d*). This suggested that this mutation, outside the YMDD motif, compensated for the impact of YMDD mutation on viral replication.

The pHBV-adr, pL526M, and pL526MM550V were selected as models to examine the 3TC sensitivity of the HBV genome with mutations introduced into the HBV DNA polymerase sequence. The HepG2 cells transfected with HBV genomes were treated with different concentrations of 3TC. The intracellular HBV progeny DNA was extracted and detected by Southern hybridization as shown in Fig. 2 (*a*, *b*, and *c*). Based on the concentration–response relationship of the wild-type virus and its mutants (Fig. 2*d*), the difference in sensitivity to 3TC was obvious. The concentration required to inhibit 50% of viral DNA from the wild-type pHBV-adr was 8 nM, and those of the mutants pL526M and pL526MM550V were 50 nM and 2.5×10^3 nM, respectively. The HBV genome with the codon 526 mutation, which was also detected in HBV patients resistant to PCV treatment [22], was 6-fold resistant to 3TC when compared with the wild-type virus. When the genome was mutated at both codon 526 and 550, it was 300-fold more resistant to 3TC. This result suggested that both mutations observed in the viral genome may be required for the clinical resistance of HBV infection to 3TC treatment.

Since mutations at sites other than L526 were reported in the HBV virions of other 3TC-resistant HBV-infected patients, which also have the M550 mutation [7, 8], the impact of these mutations on the M550 mutation in terms of viral DNA replication and 3TC sensitivity was assessed. Table 1 summarizes the characteristics of the mutants. Due to the gene open reading frame overlap of the *P* gene (encoding HBV polymerase) and the *S* gene (encoding HBsAg), the mutations also changed the amino acids of HBsAg. The impact of mutations of HBsAg on antigenicity and viral envelope assembly was unclear. The expression of HBeAg and HBsAg in the mutants was at the same level as the wild-type virus, but the viral yield of pM550I and pM550V was lower than 1% of the wild-type. The impact of the mutation of methionine to isoleucine or valine in YMDD resulted in a low yield of the progeny DNA. The viral yield from the mutants pV553I and pA546V was the same as that of the wild-type virus. The mutants pM550IV553I and pA546VM550I, both with the YMDD mutation and other mutations outside the YMDD motif, had a higher viral DNA replication than pM550I, which

had only the YMDD mutation. Thus, the mutation of V553I and A546V could help the DNA replication of the M550I mutant. pM550IV553I and pA546VM550I shared the same YMDD mutation. The difference between the two clones is a downstream 553 mutation in one and an upstream 546 mutation in the other. There was a 25-fold difference of 3TC resistance between the two mutants.

In summary, mutation of methionine to valine or isoleucine at the YMDD motif of HBV DNA polymerase may be essential but is not sufficient to explain the rebound of HBV in HBV-infected patients who were initially responsive to 3TC treatment. Other mutations, such as L526M, V553I, or A546V, in HBV polymerase could also be important in contributing to the clinical resistance to 3TC in these drug-treated patients. It should be noted that the mutation of L526M occurred in the virus from patients refractory to PCV treatment [22]. Thus, patients refractory to PCV treatment may have a higher probability of developing resistance to 3TC treatment. This should be investigated. Our study employed the adr-strain of HBV, which is the most common strain of HBV in Asia. Whether the methionine to valine or isoleucine mutation at the YMDD motif in other strains of HBV, such as adw, would have the same impact on HBV DNA replication should also be investigated, since there is a difference of amino acid sequence in HBV DNA polymerase among different strains of HBV.

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