Evolution of Multi-Drug Resistant Hepatitis B Virus During Sequential Therapy

Hyung Joon Yim, Munira Hussain, Ying Liu, Stephen N. Wong, Scott K. Fung, and Anna S. F. Lok

Multi-drug resistant hepatitis B virus (HBV) has been reported in hepatitis B patients who received sequential antiviral therapy. *In vitro* studies showed that HBV constructs with mutations resistant to lamivudine and adefovir have marked reduction in sensitivity to combination of lamivudine and adefovir, whereas constructs with mutations resistant to either drug remain sensitive to the other drug. We conducted this study to determine whether mutations conferring resistance to multiple antiviral agents co-locate on the same HBV genome *in vivo* and to describe the evolution of these mutations. Sera from six patients who had been found to have multi-drug resistant HBV mutations to lamivudine + adefovir, lamivudine + hepatitis B immunoglobulin (HBIG), or lamivudine + entecavir on direct sequencing were cloned after nested polymerase chain reaction (PCR). Analysis of 215 clones from 11 samples with multi-drug resistant mutations on direct sequencing showed that 183 (85%) clones had mutations to both therapies on the same genome; 31 clones had lamivudine-resistant mutants only. Clonal analysis of serial samples from three patients showed progressive evolution from all clones with lamivudine-resistant HBV mutations only to mixtures of clones that have multi-drug resistant mutations and clones that have lamivudine-resistant HBV mutations only, and ultimately all clones having multi-drug resistant HBV mutations. In conclusion, mutations conferring resistance to multiple antiviral agents co-locate on the same viral genome, suggesting that combination therapy directed against mutants resistant to each treatment may not be adequate in suppressing multi-drug resistant HBV. *De novo* combination therapy may prevent the emergence of multi-drug resistant mutants. (HEPATOLOGY 2006;44:703-712.)

Treatment of chronic hepatitis B is greatly improved with the availability of nucleoside/tide analogs (NA) such as lamivudine, adefovir, and entecavir.[1-3] However, antiviral resistance has become an increasingly common problem during long-term treatment with NA.[4,5] Viral breakthrough associated with selection of antiviral-resistant hepatitis B virus (HBV) mutants is usually followed by biochemical breakthrough and in some instances hepatitis flares and liver failure.[6-10] Therefore, treatment should be changed when viral breakthrough is detected. However, multi-drug resistant HBV has been reported in patients who received sequential treatment with NA monotherapies.[7,11-14]

Treatment failure caused by drug-resistant HBV is not confined to NA. Mutation of the surface gene (S gene) has been attributed as the cause of recurrent hepatitis B in some patients who received hepatitis B immunoglobulin (HBIG) monotherapy to prevent HBV reinfection after liver transplantation.[15-17] The most common mutation, glycine to arginine change at codon 145 of the small S protein (sG145R), has been shown to have decreased binding to hepatitis B surface antibody.[18-20] More recently, case reports have been published of dual mutations in the polymerase and the S genes in patients who developed recurrent hepatitis B while receiving lamivudine and HBIG prophylaxis.[21-24]

Development of multi-drug resistance may have implications on the efficacy of rescue therapy, as in the case of multi-drug resistant human immunodeficiency virus.[25,26] *In vitro* studies showed that HBV constructs with mutations resistant to lamivudine and adefovir have marked

Abbreviations: NA, nucleoside/tide analogs; HBV, hepatitis B virus; S gene, surface gene; HBIG, hepatitis B immunoglobulin; PCR, polymerase chain reaction.

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Received February 27, 2006; accepted June 1, 2006.

Supported in part by the National Institutes of Health grant U01-DK57577. (A.S.L.). H.J.Y. was supported by the Department of Gastroenterology and Hepatology at Korea University Medical Center, Seoul, Korea.

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Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.21290

Potential conflict of interest: Dr. Lok receives research support and serves on the advisory board of Roche, GlaxoSmithKline, Bristol Myers Squibb, Idenix and Gilead.

reduction in sensitivity to the combination of lamivudine and adefovir, whereas constructs with mutations resistant to either drug remain sensitive to the other drug.[12] These findings suggest that if the antiviral-resistant mutations to lamivudine or adefovir are not present on the same viral genome, combination therapy with these two antiviral agents will still be effective in suppressing mutants that are resistant to one of these drugs. In contrast, if mutations resistant to the two drugs are present on the same viral genome, efficacy of combination therapy will be limited. Therefore, the distinction between these two scenarios is important in determining appropriate rescue therapy and in predicting its likelihood of success. However, no *in vivo* data have been published regarding whether these mutations exist on the same or different viral genomes.

Successive evolution of different patterns of resistant mutations has been reported during long-term lamivudine therapy.[27,28] The initial mutations are usually associated with decreased replication fitness compared with wild-type HBV; however, additional mutations that can restore replication fitness are frequently selected as treatment is continued.[29-31] Successive evolution of mutations may result in a combination of mutations that have the greatest replication fitness as well as maximum drug resistance in patients with multi-drug resistant HBV.

This study was conducted to determine whether mutations conferring resistance to multiple antiviral agents are present on the same HBV genome *in vivo* and to describe the evolution of these mutations.

Patients and Methods

Materials. Serum samples from six patients with chronic HBV infection were studied. These patients were participants in studies on HBV that included collection of blood samples for surveillance of drug-resistant HBV mutations. Institutional Review Board approval of the studies and written consent from patients were obtained. All patients had a history of viral breakthrough during treatment with lamivudine. Adefovir or entecavir was started after the diagnosis of lamivudine-resistant HBV and HBIG was added at the time of liver transplantation to prevent HBV reinfection post-transplantation. Serum samples at the time of viral breakthrough from these six patients were directly sequenced. Samples found to have mutations that are resistant to more than one HBV therapy were cloned. Serial samples from three patients (nos. 1, 2, and 3) were analyzed additionally to examine the evolution of HBV mutations.

HBV Quantification. HBV DNA was quantified using COBAS Amplicor HBV Monitor Assay (Roche, Branchburg, NJ), which has a lower detection limit of 200 copies/mL.

Nested Polymerase Chain Reaction and Direct Sequencing. DNA extraction was carried out with QIAamp® DNA Mini Kit (Qiagen Inc., Valencia, CA) following the manufacturer's instructions. Nested polymerase chain reaction (PCR) was performed as described previously.[7] The amplicons spanned domains A through F of the reverse transcriptase region of the HBV polymerase gene (rt1-rt280). PCR products were purified by QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. Purified HBV DNA was directly sequenced by the DNA sequencing core facility at the University of Michigan Medical Center, using the standard protocol for the ABI 3730xl DNA Analyzer (Applied Biosystems Co., Foster City, CA). The DNA sequences were aligned using Seqman™ II and EditSeq™ software (DNASTAR Inc., Madison, WI).

Cloning. To determine whether the various drug-resistant mutations detected by direct sequencing co-locate on the same HBV genomes, PCR-amplified HBV DNA was cloned into pGEM T Easy Vector (Promega Co., Madison, WI) according to the manufacturer's instructions. Eighteen to 20 colonies with HBV insert were selected for each sample. The colonies were grown overnight at 37°C in 3 mL Luria-Bertoni broth mixed with ampicillin (100 μg/mL). Recombinant plasmid DNA was purified using the Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, WI) following the manufacturer's instructions. Purified plasmids were electrophoresed after digestion with restriction enzymes, *Xba* I and *Pst* I (Roche Diagnostics Co., Indianapolis, IN), and sequenced using primers, SP6 (5′-ATT TAG GTG ACA CTA TAG-3′) or T7 (5′-TAA TAC GAC TCA CTA TAG GG-3′). The sequences of all the clones from each sample were compared using MegAlign™ software (DNASTAR Inc., Madison, WI).

Results

Clinical Characteristics of Patients. Clinical characteristics of the six patients are described in Table 1; mean age was 45.3 ($\pm$10.8) years, and five were men. Two patients (nos. 1 and 2) received entecavir after viral breakthrough during lamivudine treatment. Patient 1 received combination of lamivudine and entecavir (initially 0.5 mg/d for 20 months and then 1 mg/d), whereas patient 2 stopped lamivudine when he was switched to entecavir (1 mg/d). Maximum viral suppression to 4.3 $\log_{10}$ copies/mL was achieved at month 23 of entecavir therapy in patient 1, and to 2.9 $\log_{10}$ copies/mL at month 32 in patient 2. Dual resistance to lamivudine and entecavir was

Table 1. Characteristics of Patients

Patients	1	2	3	4	5	6
Age/sex	36/F	61/M	36/M	35/M	42/M	62/M
Race	Asian	Caucasian	Caucasian	Asian	Middle Eastern	Caucasian
HBV genotype	C	A	D	B	D	D
Liver disease	Chronic hepatitis	Cirrhosis	Liver transplant recipient	Liver transplant recipient	Cirrhosis	Liver transplant recipient
Duration of prior lamivudine treatment (mo)	18	36	7	11	18	27
HBeAg/Ab at the start of 2nd course of antiviral treatment	+/−	+/−	−/−	−/−	−/+	−/−
Second antiviral treatment	Entecavir+ lamivudine	Entecavir	HBIG+ lamivudine	HBIG+lamivudine	Adefovir	Adefovir+ lamivudine
Interval between onset of second course of treatment and first detection of multi–drug-resistant mutation by direct sequencing (months)	26	36	15	20	19	25
Third antiviral treatment	No	No	Adefovir	Adefovir	Tenofovir	No
Resistance to third treatment	NA	NA	Yes*	No	No	NA

Abbreviation: NA, not applicable.
*Detected 41 months after start of 3rd treatment.

detected by direct sequencing 26 and 36 months after initiation of entecavir in patients 1 and 2, respectively (Table 2). Patient 1 discontinued both therapies at month 34 because she wished to be pregnant. Patient 2 continued entecavir monotherapy and had increasing HBV DNA level to 5.0 $\log_{10}$ copies/mL at month 45.

Patient 3 had received lamivudine for 7 months before transplantation. Despite combination prophylaxis of lamivudine and intravenous HBIG, this patient was diagnosed as having HBV reinfection 15 months after transplantation. Resistant mutations to both lamivudine and HBIG were detected at this time (Table 2). HBIG was stopped and adefovir was added, serum HBV DNA became undetectable, and lamivudine was discontinued after an overlapping period of 7 months. Viral breakthrough was detected 41 months after adefovir was initiated. Lamivudine was reintroduced after adefovir resistance was confirmed. However, mutations resistant to both adefovir and lamivudine were detected within 4 months of resumption of lamivudine.

Patient 4 had received lamivudine for 11 months before liver transplantation. Post-transplantation, this patient continued to receive lamivudine; HBIG was administered intravenously during the first week and intramuscularly thereafter. HBV reinfection was diagnosed at month 20 after liver transplantation. Resistant mutations to both lamivudine (methionine to isoleucine at codon 204 in the reverse transcriptase region of HBV polymerase [M204I]) and HBIG were detected (Table 3). HBIG was stopped, and lamivudine was switched to adefovir. Serum HBV DNA levels have decreased and remained suppressed.

Patient 5 had viral breakthrough after 18 months of lamivudine treatment. Lamivudine was stopped, and the patient was switched to adefovir. Viral breakthrough occurred after 19 months of adefovir monotherapy; at this time, resistant mutations to both lamivudine (leucine to methionine at codon 180 of the reverse transcriptase region of HBV polymerase [L180M] +M204I) and adefovir (alanine to valine at codon 181 and glutamine to serine at codon 215 [A181V+Q215S]) were detected (Table 3).[32] Adefovir was switched to tenofovir, and liver transplantation was performed subsequently. This patient was maintained on HBIG, lamivudine, and tenofovir and had undetectable hepatitis B surface antigen and serum HBV DNA at the last follow-up, 18 months post-transplantation.

Patient 6 had received lamivudine for 3 months before liver transplantation. Post-transplantation, he received a combination of lamivudine and intravenous HBIG during the first year and lamivudine monotherapy in the second year. At month 22 post-transplantation, HBV reinfection was diagnosed, and lamivudine-resistant mutation was detected. Lamivudine was continued and adefovir was added. Serum HBV DNA levels decreased from 9.7 to 4.7 $\log_{10}$ copies/mL after 12 months of lamivudine and adefovir combination therapy. Twenty-five months after the addition of adefovir, lamivudine-resistant mutations (leucine to isoleucine at codon 80 and valine to leucine at codon 173 [L80I+V173L] +M204I]) remained detectable and adefovir-resistant mutation (N238T, asparagine to threonine at codon 238) emerged (Table 3).[32] Serum HBV DNA level increased from a nadir of 3.2 to 4.1 $\log_{10}$ copies/mL after the emer-

Table 2. Evolution of Multi-Drug Resistant Mutations: Clonal Analyses of Serial Samples From Three Patients

Second Course of Treatment	Mutations on Direct Sequencing	Mutations on Clonal Analysis					Number of Clones (total)
PATIENT 1		**LAM- resistant mutations + ETV-resistant mutations**					
Mo 0	L180M/L+M204V/I	L180M	M204V				12
			M204V				2
LAM+ETV			M204I				4 (18)
Mo 12	L180M+M204V	L180M	M204V				18
		L180M	M204I				1
LAM+ETV			M204I				1 (20)
Mo 20	L180M+M204V	L180M	M204V				14
		L180M	M204V		T184L		2
LAM+ETV		L180M	M204V			S202G	2 (18)
Mo 26	L180M+M204V+T184L/T	L180M	M204V		T184L		14
		L180M	M204V				4 (18)
LAM+ETV							
Mo 32	L180M+M204V+T184L	L180M	M204V		T184L		20 (20)
LAM+ETV							
Follow-up	L180M+M204V+T184L/T	L180M	M204V		T184L		12
Mo 6		L180M	M204V				6
			M204I				1
No treatment		WT					1 (20)
PATIENT 2		**LAM- resistant mutations + ETV-resistant mutations**					
Mo 0	L180M+M204V	L180M	M204V				20 (20)
ETV							
Mo 36	L180M+M204V	L180M	M204V	I169T	T184A		14
ETV	+I169T/I+T184A/T+S202S/G	L180M	M204V	I169T		S202G	1
		L180M	M204V			S202G	3
		L180M	M204V				2 (20)
Mo 41	L180M+M204V	L180M	M204V			S202G	11
ETV	+I169I/T+T184T/A+S202G	L180M	M204V		T184I	S202G	5
		L180M	M204V	I169T	T184A		3
		L180M	M204V	I169T	T184A	S202G	1 (20)
Mo 45	L180M+M204V+ T184I +S202G	L180M	M204V		T184I	S202G	18
ETV		L180M	M204V			S202G	1
		L180M	M204V	I169T	T184A		1 (20)
PATIENT 3		**HBIG + LAM-resistant mutations and LAM-resistant mutations +ADV-resistant mutations**					
Mo 15	sG145R+M204I	sG145R		M204I			18 (18)
LAM+HBIG							
Mo 56	N236T				N236T		17
ADV		sG145R			N236T		1 (18)
Mo 60	V173L/V+L180M+M204V	V173L	L180M	M204V			10
ADV+LAM	+P237P/H		L180M	M204V			6
		V173L	L180M	M204V		P237H	2
			L180M	M204V		P237H	2 (20)

Abbreviations: A, alanine; G, glycine; H, histidine; I, isoleucine; L, leucine; M, methionine; N, asparagine; P, proline; R, arginine; S, serine; T, threonine; V, valine; ADV, adefovir; ETV; entecavir; HBIG; hepatitis B immunoglobulin; LAM, lamivudine; Mo, month; WT, wild type.

gence of N238T despite continued treatment with lamivudine and adefovir.

Clonal Analysis. Clonal analysis was performed on 16 samples from these six patients (Fig. 1). The timing of the samples studied are indicated in Tables 2 and 3.

Eleven samples showed dual-resistant mutations to lamivudine + adefovir, lamivudine + HBIG, or lamivudine + entecavir by direct sequencing. A total of 215 clones from these 11 samples were analyzed to determine whether the dual-resistant mutations co-locate, that is, were present in the same clone. Of the 215 clones analyzed, 183 (85%) clones had mutations resistant to both therapies in the same clone, 31 clones had mutations resistant to lamivudine only, and one clone from a follow-up sample 6 months after cessation of antiviral treatment (patient 1) showed wild-type sequence.

Five samples showed lamivudine-resistant mutations only on direct sequencing. Eighty-nine of 94 clones (95%) from these five samples confirmed the presence of lamivudine-resistant mutations only whereas the remaining five clones (from patients 1 and 3) had multi–drug-resistant mutations.

Evolution of Multi-Drug Resistant Mutations. Evolution of multi-drug resistant mutations was exam-

Table 3. Clonal Analysis of Samples From Three Patients With Multi-Drug Resistant Mutations by Direct Sequencing

Second Course of Treatment	Mutations on Direct Sequencing	Mutations on Clonal Analysis					Number of Clones (total)	
Patient 4		**HBIG-resistantmutation+LAM-resistantmutation**						
Mo 20	sG145R+M204I	sG145R		M204I			20	(20)
LAM+HBIG								
Patient 5		**LAM-resistant mutations +ADV-resistant mutations**						
Mo 19	L180M/L		L180M		A181V	Q215S	16	
ADV	+A181V/A+Q215S			M204I		Q215S	1	
						Q215S	2	(19)
Patient 6		**LAM-resistant mutations + ADV-resistant mutations**						
Mo 25	L80I/L+V173V/L+M204I	L80I		M204I	N238T		7	
LAM+ADV	+N238T	L80I	V173L	M204I	N238T		4	
			V173L	M204I	N238T		6	
				M204I	N238T		2	
				M204I	N238T	M250V	1	(20)

Abbreviations: A, alanine; G, glycine; I, isoleucine; L, leucine; M, methionine; N, asparagine; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; ADV, adefovir; HBIG; hepatitis B immunoglobulin; LAM, lamivudine; Mo, month.

ined in three patients (Table 2). Patient 1 received a combination of lamivudine and entecavir after lamivudine breakthrough (Fig. 2). At the start of entecavir therapy (month 0), all 18 clones had lamivudine-resistant mutations. Clonal analysis first detected entecavir-resistant mutations in 4 of 18 clones at month 20, 6 months earlier than direct sequencing. At this time, two clones had threonine to leucine substitutions T184L and two had serine to glycine substitutions S202G together with L180M and methionine to valine substitutions at codon 204 (M204V) (Table 2). As treatment continued, T184L became predominant whereas S202G was no longer detected. By month 32, all 20 clones had both entecavir- (T184L) and lamivudine-resistant (L180M+M204V) mutations. Six months after cessation of both treatments, T184L remained detectable in 12/20 clones in association with L180M+M204V whereas seven clones had lamivudine-resistant mutations only, and one clone had wild-type HBV sequence.

Patient 2 was switched to entecavir monotherapy after lamivudine breakthrough (Fig. 3). At the start of entecavir therapy (month 0), all 20 clones had lamivudine-resistant mutations (L180M+M204V). Entecavir-resistant mutations were first detected after 36 months of entecavir treatment. At that time, 14 clones had isoleucine to threonine substitutions at codon 169 and threonine to alanine substitutions at codon 184 (I169T+T184A), three clones had S202G, and one clone had I169T+S202G, in association with lamivudine-resistant mutations. Two clones had lamivudine-resistant mutations only (Table 2). After 45 months of continued treatment with entecavir monotherapy, S202G became predominant, being present in 19 of 20 clones together with T184I in 18 clones; only one clone had I169T along with T184A. Lamivudine-resistant mutation L180M+ M204V persisted in all clones 45 months after lamivudine had been discontinued.

Patient 3 developed HBV recurrence after liver transplantation despite receiving lamivudine and HBIG (Fig. 4). At the time HBV reinfection was diagnosed, all 18 clones had lamivudine- (M204I) and HBIG-resistant mutations (sG145R) (Table 2). Adefovir was added and lamivudine was stopped 7 months later. After 41 months of adefovir treatment, viral breakthrough was diagnosed, and all 18 clones had adefovir-resistant mutation asparagine to threonine substitution at codon 236 (N236T). HBIG-resistant mutation (sG145R) remained detectable in one clone, but lamivudine-resistant mutation was not detected. Four months after reintroduction of lamivudine, lamivudine-resistant mutations (L180M+M204V) that were different from those found at the time of HBV reinfection were detected in all 20 clones. Twelve clones had additional compensatory mutation V173L, a valine-to-leucine substitution. However, adefovir-resistant mutation became less prominent, being present in only four clones. N236T was replaced by a different adefovir-resistant mutation P237H, a proline-to-histidine substitution, which has been described previously.[32]

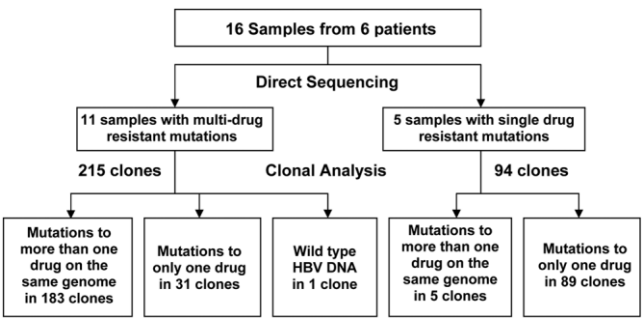

Fig. 1. Flow chart of samples and clones studied.

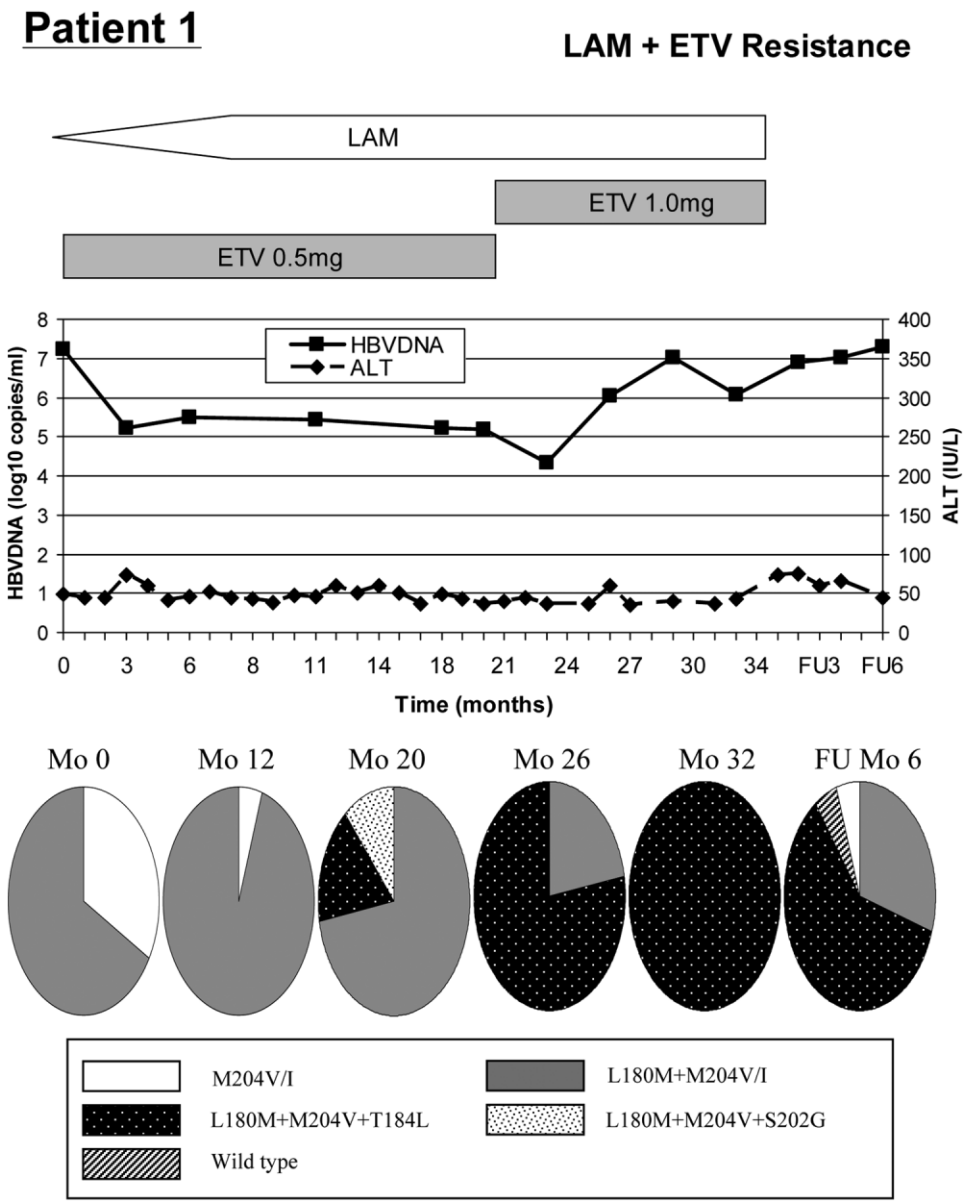

Fig. 2. Clinical course and evolution of antiviral resistant mutations in patient 1, who had lamivudine-resistant HBV, and then was treated with combination of lamivudine and entecavir. HBV DNA levels in solid line and ALT levels in dashed line are plotted against time in months from the start of lamivudine and entecavir combination treatment. Proportions of clones at each time with various patterns of mutations are depicted as pie charts. Antiviral therapies are shown above each graph. HBV, hepatitis B virus; ALT, alanine aminotransferase; LAM, lamivudine; ETV, entecavir; Mo, month; FU, follow-up.

Corresponding Changes in Overlapping Polymerase/S Genes. Because the S gene of HBV overlaps the polymerase gene, mutations in one gene may result in changes in the other gene.[33,34] Table 4 shows the nonsynchronous changes affecting the overlapping gene. Several corresponding changes are genotype specific because of HBV DNA sequence polymorphisms.[35]

Discussion

Multi-drug resistant HBV mutants are becoming increasingly prevalent with sequential NA monotherapy.[11,13,14,36] Information on whether multi–drug-resistant mutations co-locate on the same viral genome is important in implementing appropriate rescue therapy. In this study, we demonstrated that among patients with multi-drug resistant HBV on direct sequencing, mutations conferring resistance to multiple antiviral agents co-locate on the same viral genome in more than 80% of clones; the remaining clones with resistance to only one drug had mutations to lamivudine only. A recent *in vitro* study showed that an HBV construct with resistant mutations to lamivudine and adefovir (L180M+M204V+N236T) was associated with a 58.8-fold decrease in susceptibility to combination of the two drugs, whereas con-

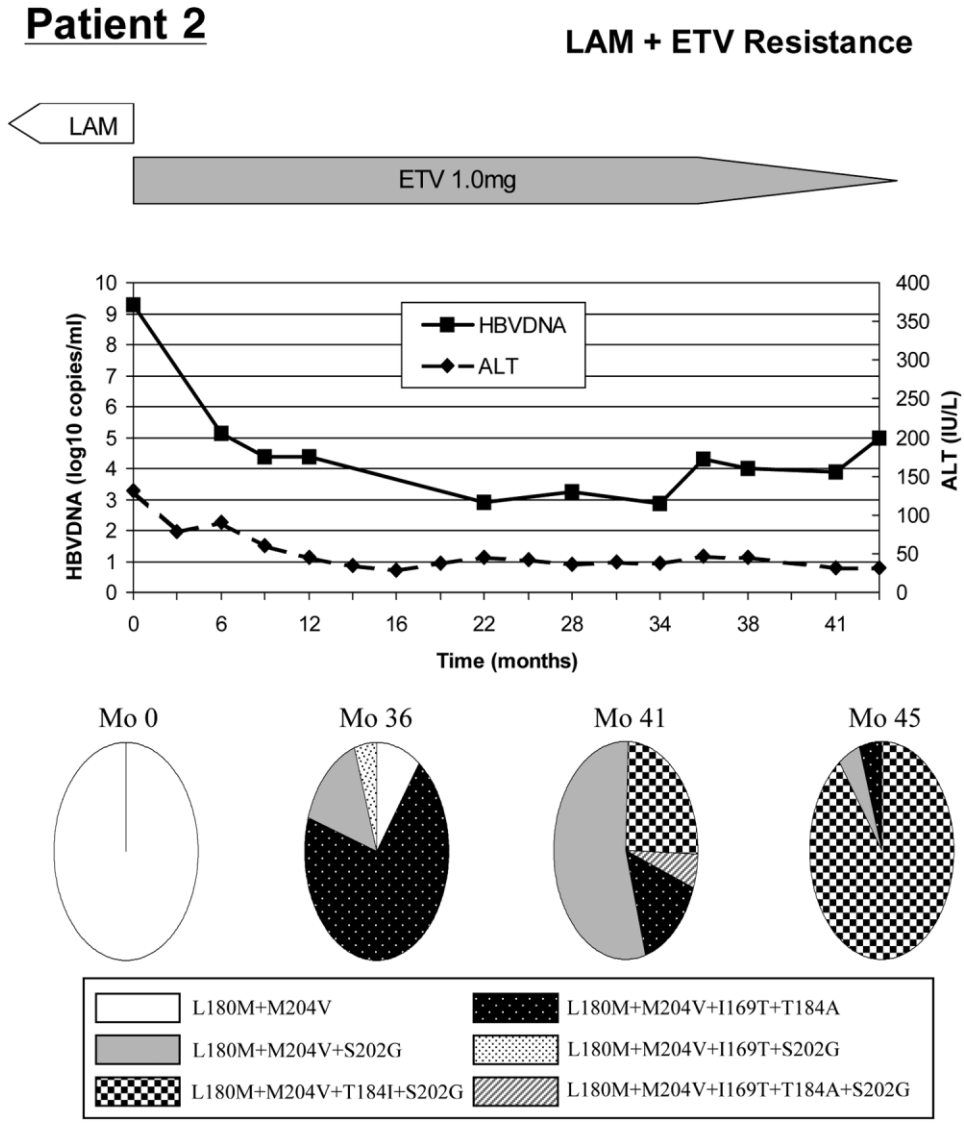

Fig. 3. Clinical course and evolution of antiviral resistant mutations in patient 2, who had lamivudine-resistant HBV and was switched to entecavir. HBV DNA levels in solid line and ALT levels in dashed line are plotted against time in months from the start of entecavir treatment. Proportions of clones at each time point with various patterns of mutations are depicted as pie serially. HBV, hepatitis B virus; LAM, lamivudine; ETV, entecavir; Mo, month.

structs with mutations to lamivudine (L180M+M204V) or adefovir (N236T) alone were associated with only 11- and 1.58-fold decreases in susceptibility.[12] Our data suggest that the combination of two antiviral agents with activity against mutants resistant to each treatment may not be adequate in suppressing dual-resistant HBV.

The multi-drug resistant mutants were first detected by direct sequencing after a mean of 23.5 months (17.5 months for lamivudine- and HIBG- resistance and 26.5 months for resistance to two NA) of the second antiviral treatment. Our clonal analysis was expected to have a sensitivity of approximately 5% (1/20) based on an average of 20 clones analyzed from each sample. Using this method, multi-drug resistant mutations could be detected up to 6 months earlier than with direct sequencing, indicating that selection of multi-drug resistant HBV can occur very rapidly during sequential NA therapies. We acknowledge that the rapid selection of multi–drug-resistant HBV observed in this study may be related to the fact that three of the six patients had undergone liver transplantation. Enhanced HBV replication associated with immunosuppression as well as increase of replication space in the previously uninfected allograft liver may have facilitated the selection of drug-resistant mutations.[37] We also included Q215S, P237H and N238T as adefovir-resistant mutations. These changes had been reported to be associated with adefovir resistance in one study.[32] Although these mutations have not yet been validated by other investigators, both patients 3 and 6 had an increase of serum HBV DNA

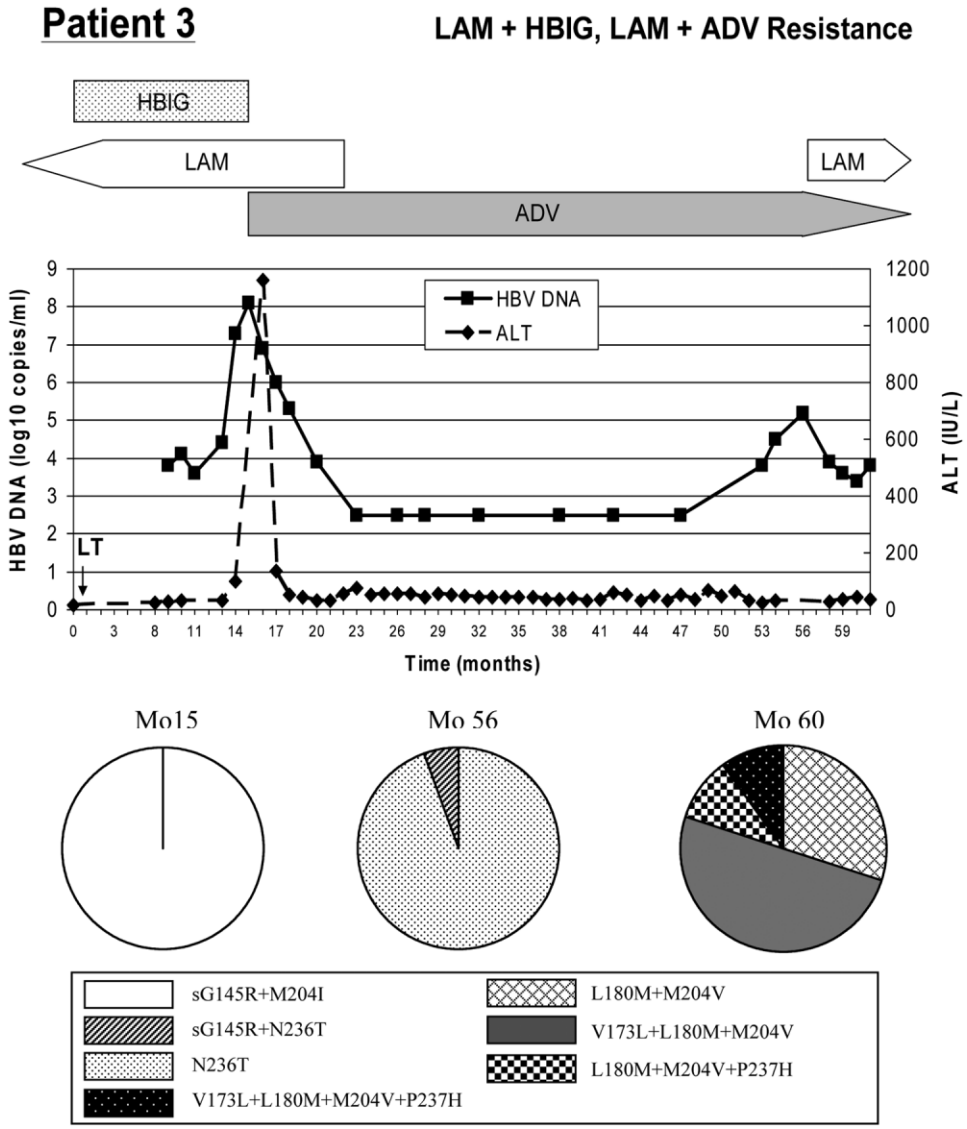

Fig. 4. Clinical course and evolution of antiviral + HBIG–resistant mutations in patient 3, who received lamivudine before liver transplantation and developed HBV reinfection despite receiving lamivudine and HBIG post-transplantation. HBIG was stopped, adefovir was added, and lamivudine was continued for 7 months. The patient subsequently was detected to have adefovir-resistant HBV. On reintroduction of lamivudine, mutations to lamivudine rapidly re-emerged. HBV DNA levels in solid line and ALT levels in dashed line are plotted against time in months from the time of liver transplantation. Proportions of clones at each time with various patterns of mutations are depicted as pie charts. HBV, hepatitis B virus; LAM, lamivudine; ADV, adefovir; HBIG, hepatitis B immune globulin; Mo, month; LT, liver transplantation.

levels by approximately 1 $\log_{10}$ copies/mL when these mutations emerged.

Evolution of resistant mutations with progressive selection for mutants that confer greater degree of resistance or mutants that restore replication fitness has been reported in patients receiving long-term lamivudine therapy.[30, 31] In this study where patients received sequential therapies, we demonstrated progressive evolution from all clones with lamivudine-resistant HBV mutations only to mixtures of clones that have multidrug resistant mutations and clones that have lamivudine-resistant HBV mutations only, and ultimately all clones having multi-drug resistant mutations. In addition, we found that the predominant mutation to a specific antiviral therapy also evolves during the course of treatment. For example, patient 1, who was receiving lamivudine and entecavir initially, had two different entecavir-resistant mutations: T184L and S202G on different clones, but T184L became dominant during continued treatment. Conversely, patient 2, who was receiving entecavir monotherapy, had predominantly I169T and T184A mutations initially, but T184I and S202G became dominant during continued treatment. Whether the subsequent mutation pattern conferred

Table 4. Reciprocal Changes of P-gene and S-gene Observed During Antiviral Treatment

Location in P-gene	Location in S-gene	Observed Genotypes in This Study	Related Antiviral Therapy
R153Q*	sG145R†	B, D	HBIG
I169T	sY161H*	A	Entecavir
V173L	sE164D	D	Lamivudine
A181V	sL173F	D	Adefovir
T184L	sL175F	C	Entecavir
M204V	sI195M	A, C, D	Lamivudine
M204I	sW196L	B, D	Lamivudine
Q215S	sS207R*	D	Adefovir

Abbreviations: A, alanine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; L, leucine; M, methionine; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine; HBIG, hepatitis B immunoglobulin; P-gene, polymerase gene; S-gene, surface gene.

*These mutations involve polymorphic sites and vary according to hepatitis B virus genotypes.

†This mutation is in the a determinant of the S gene.

greater resistance to entecavir or improved replication fitness of the mutant remain to be determined. Whether the difference in dominant entecavir-resistant mutations between patients 1 and 2 is related to the continued presence or absence of lamivudine treatment is also unclear. Studies are ongoing to compare the replication capacity and drug susceptibility of these mutants.

Cessation of antiviral therapy has been suggested to lead to disappearance of drug-resistant mutations.[38] However, other investigators have cautioned that drug-resistant mutations are archived and are rapidly selected upon re-introduction of antiviral therapy.[39] In this study, lamivudine-resistant HBV mutation was not detected in patient 3 by clonal analysis 34 months after lamivudine was stopped. Nevertheless, lamivudine-resistant mutations re-emerged within 4 months after reintroduction of lamivudine, indicating that treatment to which the virus had been previously resistant has limited efficacy even after it had been withheld for several years. Persistence of lamivudine-resistant mutations in patients who are switched to entecavir is worrisome because resistance to entecavir is greatly enhanced in the presence of lamivudine-resistant mutations.[13] In this study, lamivudine-resistant mutations remained detectable in all 20 clones from patient 2 up to 4 years after withdrawal of lamivudine. Our finding raises concerns about the long-term efficacy of entecavir in patients with lamivudine-resistant HBV.

Because of overlap of the HBV surface and polymerase genes, antiviral-resistant polymerase gene mutations can result in amino acid changes in the surface protein, leading to alteration of S epitope and decreased binding to HBIG.[20,40] We found that mutations conferring resistance to lamivudine, adefovir, and entecavir all can result in amino acid changes in the HBV surface protein (Table 4). Whether all these changes decrease binding to HBIG has not been determined. However, some of the S gene changes such as glutamic acid to aspartic acid sE164D (attributable to lamivudine-resistant V173L in the P gene) are located just downstream of the immunodominant "a" determinant and may affect binding to HBIG.[20,40] Decreased efficacy of HBIG in a patient with lamivudine- (or other antiviral) resistant mutations is of grave concern in liver transplant patients. Other studies have found that HBIG-resistant S gene mutations result in changes in the P gene that restore replication fitness of lamivudine-resistant HBV mutants, leading to severe hepatitis and rapid progression to liver failure.[22,23] Although adefovir and entecavir are associated with lower rates of drug resistance compared with lamivudine, close monitoring of serum HBV DNA levels during antiviral treatment is crucial, particularly in liver transplant patients. With the availability of more antiviral therapies for hepatitis B, confirmation of genotypic resistance and characterization of resistant mutations are of increasing importance in the selection of rescue therapy.

In conclusion, we showed that mutations conferring resistance to multiple antiviral agents are present on the same viral genome, suggesting that the combination of two antiviral agents with activity against mutants resistant to each treatment may not be adequate in suppressing dual-resistant HBV. Sequential antiviral therapy leads to selection of multi-drug resistant HBV and evolution of mutations during continued treatment may select for mutants with increased replication fitness or maximal viral resistance. Thus, antiviral therapy should only be administered after careful balance of benefits versus risks, *de novo* combination of potent antiviral agents with low risk of resistance should be encouraged, and viral response should be carefully monitored.

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