Eastern Africa and Western Asia (A and D, respectively) where the prevalence of other genotypes was less than 5%. On the other hand, in countries with high levels of immigration, a variety of genotypes are being reported; all of the known genotypes can be found in the Europe and North America. In Australia, genotypes A, B, C and D were reported in equal prevalence. Two genotypes, A and D are prevalent in European Union (except for the Mediterranean where D predominates), and in Central/Southern Asia. Genotypes B and C are the major variants in South and South East Asia and the Pacific region, while genotypes A and F are the most common in South America or E and A in Central Africa. Genotype E is restricted to Central and West Africa however, its prevalence tends to increase in Europe. Genotype F is subdivided into 4 subgenotypes, and is prevalent in Central and South America and Alaska. In recent reports the subgenotypes of genotype F were further subdivided into clades (Table 2).

A recent study from Peru described a full genome analysis for three strains from Peru that belonged to subtype F1 and suggested they should be considered as clade 1c within subgenotype F1.[281] Genotype G was found in Europe and the United States. A few cases of genotype G infection have been reported from Asia,[227,283] and more recently from Brazil.[284] Despite the geographical dispersion of the reported G strains, they show a very low genetic diversity. Genotype H is frequent in and restricted to Central America where it was also reported in co-infection with genotype G.[133] The pattern of genotype distribution changes according to the pattern of global migration.

EVOLUTIONARY HISTORY OF THE GENOTYPES

THE FIRST ATTEMPT to date the evolutionary history of HBV was carried out by the phylogenetic analysis based on synonymous substitutions in the polymerase coding gene of hepadnavirus family strains isolated from the human, chimpanzee, woodchuck, ground squirrel and duck.[7] The substitution rate estimated in the study was 4.57×10^{-5} substitutions/site/year. This study concluded that the duck strain was the most divergent and shared the most recent common ancestor with other strains approximately 30 000 years ago, whereas different human HBV genotypes emerged about 3000 years ago.[7] However, the overlapping composition of Open Reading Frames (ORFs) in the HBV genome complicates an estimation of the synonymous substitutions, as the same mutation considered synonymous in one of the ORF may cause an aminoacid change in overlapping ORF.[285] The mutation rate of HBV estimated in the serial specimens collected at distant periods of time from genotype B infected carriers, was 7.9×10^{-5} substitutions/site/year.[271] Another study carried on genotype D strains representing localised epidemic in Western Japan, have set the mutation rate to 5.4×10^{-5}.[277] A study aiming to estimate the substitution rate using two independent data sets of non-overlapping ORF coding core protein, concluded that a reliable molecular clock does not exist.[286] Phylogenetic topology of the genotypes heavily depends on the genomic region and substitution model used in analysis, thus hinder any attempt to reconstruct the past spread of this virus.[286] In addition to the complex overlapping structure of the genome, a recombination of HBV severely hampers an assessment of its evolution.[287] New methodological approach is required to explore rules of the HBV evolution.

HBV RECOMBINATION

ONE OF THE most comprehensive analyses of occurrence and composition of HBV intergenotype recombinants indicated the existence of 24 phylogenetically independent recombinant forms of HBV involving all human genotypes as well as both chimpanzee and gibbon variants.[288] Further reports are constantly extending this number.[18,118,283,289] It has been shown that 60% of the intergenotype recombinants have the breakpoints within nucleotides 1640–1900.[289] It was also concluded that recombination sites often localise to gene boundaries.[288,289] Further, using a newly developed approach ("TreeOrder Scan") the authors could demonstrate that analysed in different parts of the HBV genome, genotypes are interchangeably shifting the relative phylogenetic topology. This consists with changes in the overall phylogenetic topology of the HBV genotypes that can be observed in trees reconstructed from different parts of the genome. Genotype G strains in particular demonstrate evidence of recombination with genotype A in the Small S fragment (nucleotides: 250–350) as well as genotype E with genotype D in the core gene (nucleotides: 1950–2500) and genotype H with genotype F within the *Small S* gene (nucleotides: 350–500).[288] It was hypothesised that some of the genotypes that are conventionally regarded as "nonrecombinant," demonstrate evidence of recombination, that is, during evolution in some cases, one or other of the ancestral HBV variants that might have been involved in recombination are virtually replaced by a more viable

Table 2 Hepatitis B virus (HBV) subgenotypes

Genotype	Subgenotype	*n*	Complete genome Nucleotides diversity (complete genome)			Geography	Ref
			Clustering	Intra-subgenotype mean + SD (max)	Next closest negbour mean + SD (min)		
HBV/A							
	A1/Aa	78	yes	2.6 + 0.8 (5.5)	4.4 + 0.4 (3.3) for A4	Africa, Asia, South America	15,261,262
	A2/Ae	94	yes	1.7 + 0.9 (5.5)	4.7 + 0.7 (3.6) for A4	Europe, North America	15,261,262
	A3/Ac	8	yes	3.0 + 0.9 (4.1)	4.7 + 0.4 (3.8) for A1	Western Africa	19,21,22
	A4	3	no	2.9 + 0.9 (3.5)	3.8 + 0.2 (3.4) for A3	Western Africa	21,263
	A5	0	?	?	?	Western Africa	21
HBV/B							
	B1/Bj	38	yes	2.4 + 0.6 (4.1)	4.6 + 0.5 (3.6) for B2	Japan	264–266
	B2	173	yes	1.7 + 0.8 (4.0)	4.4 + 0.5 (2.9) for B4	China, Taiwan	190,200,264–268
	B3	5	yes	1.6 + 0.6 (2.7)	3.6 + 0.5 (2.9) for B5	Indonesia	269
	B4	21	yes	2.7 + 0.6 (4.4)	5.0 + 0.5 (4.3) for B3	Vietnam, Cambodia	269
	B5	7	yes	2.8 + 1.5 (4.5)	5.2 + 0.6 (4.0) for B2	the Philippines	166,167
	B6	27	yes	2.7 + 0.7 (4.2)	5.7 + 0.6 (4.6) for B3	Native populations in Arctic	270,271
	B7	2	no			Indonesia	161
HBV/C							
	C1/Cs	97	yes	2.4 + 0.7 (5.1)	4.4 + 0.5 (3.1) for C2	South and South East Asia	272–274
	C2/Ce	295	yes	2.5 + 0.6 (4.7)	4.9 + 0.5 (3.8) for C3	Eastern Asia (Korea, Japan) and North China	
	C3	3	yes	4.2 + 1.2 (5.2)	5.8 + 0.6 (4.6) for C1	Pacific	269
	C4	2	yes	0.9	6.6 + 0.6 (6.0) for C3	Australia	256
	C5	8	yes	2.0 + 1.0 (3.4)	6.2 + 0.5 (5.0) for C1	Philippines, Vietnam	167
HBV/D							
	D1	88	yes	2.3 + 0.8 (5.2)	3.1 + 0.6 (1.7) for D2	North Africa, Europe, Central Asia	84,269,275
	D2	53	yes	3.0 + 0.8 (5.8)	4.2 + 0.6 (2.6) for D3	North Europe, Russia, Japan (Ehime)	269,276–278
	D3	66	yes	2.9 + 1.1 (5.9)	4.1 + 0.7 (2.3) for D1	South Africa, Europe	
	D4	7	yes	2.6 + 1.2 (4.9)	4.6 + 0.6 (3.5) for D1	Australia	256
	D5	2	yes	2.4	5.2 + 0.5 (4.9) for D4	Eastern India	73
HBV/F							
	F1a	4		1.1 + 0.2 (1.4)	2.0 + 0.2 (1.6) for 1b	Central America: Costa Rica	279,280
	F1b	7		0.4 + 0.1 (0.6)	1.9 + 0.3 (1.5) for 1d	Venezuela, Argentina, Alaska	154,279,281,282
	F1d	2		2.2	2.8 + 0.3 (2.4) for 1a	Japan	279,281
	F2a	9		1.1 + 0.3 (1.4)	3.2 + 0.2 (2.8) for 2b	Brasil, Venezuella, Nicaragua	24,154
	F2b			0.5 + 0.1 (0.6)	4.1 + 0.9 (2.8) for F4		
	F3	23	yes	1.1 + 0.9 (4.2)	4.5 + 0.3 (3.9) for F2	Venezuella	
	F4	6		1.9 + 0.9 (3.7)	4.6 + 0.6 (3.8) for F3	Argentina, Bolivia	142

product of the recombination. Discovery of "pure" genotypes E, G or H strains would confirm this hypothesis. Most of the studies that have found a high prevalence of both D and E were reported in Europe, in particular France.[92–95,103] The only country less affected by recent migration is Cameroon, where these two variants might have been endemic for a longer period of time. However, sequencing of a number of Cameroonian HBV/E strains to date did not reveal any evidence of the presence of a "pure" genotype E that is not "sharing" its core gene sequence with genotype D.[21,22] Genotypes G and H have the highest prevalence in Mexico,[133,134] a country where genotypes A and F are also prevalent.[132,290] Hence further molecular epidemiological studies in Cameroon and Mexico may reveal traces of "pure" non-recombinant ancestors of currently known genotypes.

GENOTYPES COINFECTION

AS MORE THAN one genotype is predominant in most of the geographic regions, coinfection between the predominating genotypes is not a rare finding; especially for B and C,[169,176,179,202,223,251,291] or A and D.[19,21,27,63,69,70,77,79,105,110,138,139] Co-infections with different genotypes of HBV are being reported with various frequencies. The frequency, however, seems to have a stronger association with the genotyping method rather than a geographic region or genotype endemic in a studied population. Most of the reported cases of co-infection with different genotypes were detected by using multiplex PCR or hybridisation assays and are rarely confirmed by conventional cloning and sequencing.[20,81,95,206] However, genotyping based on PCR with specific primers, probes, and/or restriction enzymes may produce misleading non-specific results due to single nucleotide polymorphisms. This is particularly important in case the PCR-based genotyping assays are applied when studying populations with only a few representing HBV sequences in the database, which means that the local variability of HBV strains was not considered when the assay was designed. In some reports, coinfections detected between genotypes not endemic in the studied population. A cross-sectional international population study using PCR-RFLP genotyping, reported 10/47 cases of genotype C in African cohorts and 6 of the 10 cases were found in coinfection (mainly with genotype G.)[20] The same study detected genotypes E to be more frequent in Asian cohorts compared to European and African ones.[20] However these findings are discrepant with previous reports on the geographical distribution of the genotypes therefore the result obtained by PCR-based genotyping assay requires confirmation by cloning and sequencing.

SUBGENOTYPES

GENETIC VARIABILITY WITHIN the genotypes is being extensively investigated since the concept of subgenotyping has evolved from studies on genotype A[15,261] and B.[266] All genotypes except for E, G and H are currently subdivided into subgenotypes (Table 2). Analysis of accumulated sequence data of HBV indicated that nucleotide sequence divergence exceeding 4% but less than 7.5% in the entire genome sequence should be used as criterion for identifying subgenotypes, whereas divisions within the subgenotypes showing less than 4% divergence should be referred to as "clades."[262] In this view, the recently proposed subgenotype A4[21] with a mean and minimal nucleotide divergence from subgenotype A3; 3.8% and 3.4%, respectively, is a clade rather than a subgenotype (Table 2). This can be further supported by a phylogenetic tree constructed on the complete genome of the strains, showing the "A4" strains to group along with the A3 strains (Fig. 2). Similar geographic distribution of the strains (West Africa) concurs that "A4" and A3 strains represent the same subgenotype. The small genetic distance and similar endemicity of the recently proposed subgenotypes B3, B5 and B7 can also suggest that these can be considered as clades representing the same subgenotype of genotype B (Table 2).

EVOLVING OF GENOTYPING CRITERIA

AN EXTENSIVE ANALYSIS of accumulated HBV genome sequence data indicated that the nucleotide diversity of genotype H strains is less than 8% from its closest neighbour; genotype F.[279] It was further proposed to use 7.5% of nucleotide divergence in the complete genome as a cut off for designation of new genotypes.[262] A new genotype "I" was recently reported to be circulating in Vietnam.[294] However, the conclusions of the paper on the new genotype and on the complex intergenotypic recombination did not correspond with existing genotyping criteria.[295] First of all, the complete genome genetic diversity of the strain was lower than 7.5% from the closest neighbour; genotype C (7.0%). Second, the genetic recombination with other genotypes was evident.[288,295,296] And finally, the epidemiological significance of the aberrant variant in terms of a new genotype was questionable as only three strains

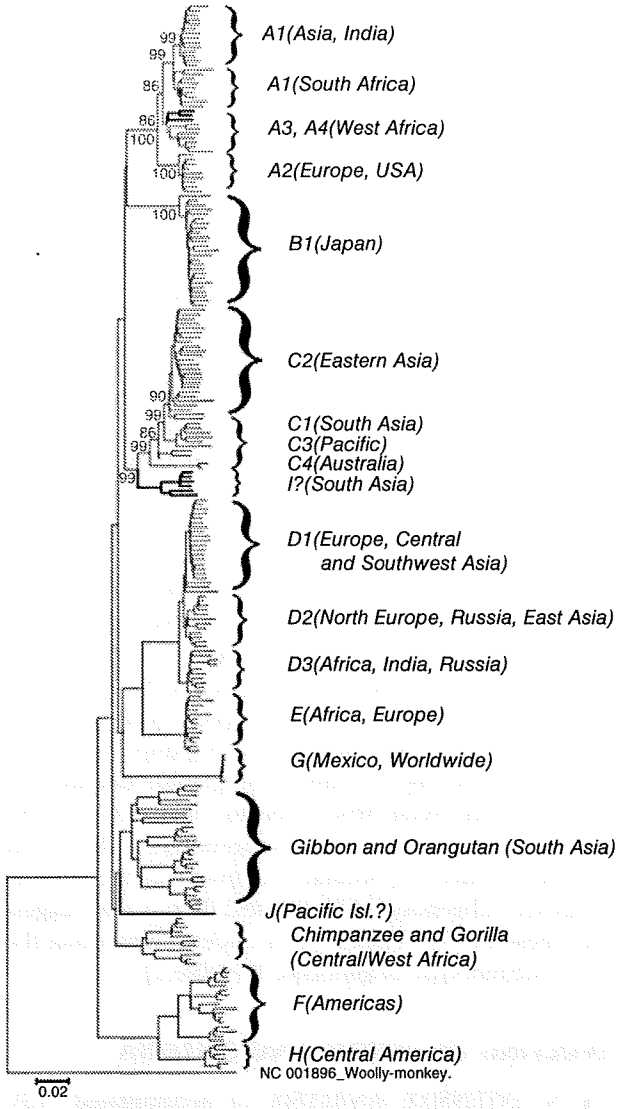

Figure 2 Phylogenetic tree constructed on selected complete genome strain references, which had no evidence of recombination. The neighbour joining tree was constructed using online aligning and tree drawing tools.[292,293]

have been reported during the 8 years since the first report describing this variant.[296] More recent study carried in Laos have revealed more strains that are genetically similar to the three Vietnamese isolates, providing evidence for the epidemiological value of the specific designation of the variant.[297] However, further studies are required to justify the classification of the variant into a separate genotype (HBV-I) or to consider it as a subgenotype of the existing genotype (HBV-C) (Fig. 2.) Another recent study from Japan, based on a strain isolated from an hepatocellular carcinoma patient who had a history of travelling to Borneo, revealed a novel genetic variant of HBV phylogenetically positioned between clusters of human and primate isolates.[298] The tentative genotype J strain show no evidence of recombination with any of known genotypes, and it is phylogenetically close to strains previously isolated from Gibbons and Orangutan (Fig. 2.) Epidemiological, virologic and clinical features of both provisional genotypes I and J require further studies to justify their classification.

Alternative approaches for the genotyping of HBV were recently proposed, suggesting that known variants of HBV can be grouped into 4 groups[299] or 3 groups,[300] however, the relevance of these classifications still needs to be substantiated from epidemiological and clinical points of view.

GENOTYPING CRITERIA

INTENSIVE INVESTIGATIONS HAVE indicated an uneven geographical distribution and epidemiology of distinct HBV genotypes and subgenotypes, however, many questions remain unanswered in terms of their virologic and clinical features. Further investigations in this field require standardised criteria for genotyping and subgenotyping, and these criteria need to be updated regularly in the context of new findings. Currently we propose the following check list of minimal requirements for defining genotypes and subgenotypes:

1 A complete genome sequence analysis is required to identify a new genotype or subgenotype.
2 Nucleotide divergence in a complete genome should exceed 7.5% to distinguish a genotype or 4% to distinguish a subgenotype. Variability below 4% confirmed by specific phylogenetic clustering can be used as a criterion to identify clades within subgenotypes.
3 Genotypes and subgenotypes should be identified by robust independent clustering on molecular evolutionary analysis based on complete HBV genomes.
4 Evidence of recombination with other known or unknown genotypes should be used as a criterion for identifying a subgenotype or clade of genotype involved in recombination rather than a new independent genotype.
5 Identification of a new genotype should be substantiated by its epidemiological, virological or clinical characteristics.

In conclusion, HBV demonstrates significant genetic and geographical divergence. Further studies are required to investigate genetic characteristics of the virus

in less studied developing countries, especially those with a high endemicity. Updated unified criteria are required to resolve future issues in genotype assignment.

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Mechanism of Entecavir Resistance of Hepatitis B Virus with Viral Breakthrough as Determined by Long-Term Clinical Assessment and Molecular Docking Simulation▽†

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The mechanism by which entecavir resistance (ETVr) substitutions of hepatitis B virus (HBV) can induce breakthrough (BT) during ETV therapy is largely unknown. We conducted a cross-sectional study of 49 lamivudine (LVD)-refractory patients and 59 naïve patients with chronic hepatitis B. BT was observed in 26.8% of the LVD-refractory group during weeks 60 to 144 of ETV therapy. A line probe assay revealed ETVr substitutions only in the LVD-refractory group, i.e., in 4.9% of patients at baseline, increasing to 14.6%, 24.4%, and 44.8% at weeks 48, 96, and 144, respectively. Multivariate logistic regression analysis adjusted for age, gender, HBV DNA levels, and LVD resistance (LVDr) (L180M and M204V, but not M204I) indicated that T184 substitutions and S202G (not S202C) were a significant factor for BT (adjusted odds ratio [OR], 141.12, and 95% confidence interval [CI], 6.94 to 2,870.20; OR, 201.25, and 95% CI, 11.22 to 3608.65, respectively). Modeling of HBV reverse transcriptase (RT) by docking simulation indicated that a combination of LVDr and ETVr (T184L or S202G) was characterized by a change in the direction of the D205 residue and steric conflict in the binding pocket of ETV triphosphate (ETV-TP), by significantly longer minimal distances (2.2 Å and 2.1 Å), and by higher potential energy (−117 and −99.8 Kcal/mol) for ETV-TP compared with the wild type (1.3 Å; −178 Kcal/mol) and LVDr substitutions (1.5 Å; −141 Kcal/mol). Our data suggest that the low binding affinity of ETV-TP for the HBV RT, involving conformational change of the binding pocket of HBV RT by L180M, M204V plus T184L, and S202G, could induce BT.

Infection with hepatitis B virus (HBV) is extremely widespread and affects more than 350 million people worldwide. Chronic HBV infection leads to the development of complications, such as liver cirrhosis (LC) and hepatocellular carcinoma (HCC) (12). HBV has been classified into 8 geographically, genetically, and clinically diverse genotypes, designated alphabetically from A to H according to their order of discovery (14). Genotypes B and C are prevalent in Asia, and genotype C is associated with more serious liver disease, including LC and HCC, and a poorer response to interferon therapy than genotype B (5). The ultimate therapeutic goal when treating chronic HBV infection is to prevent the development of LC and HCC by eliminating or producing sustained suppression of HBV replication. However, lamivudine resistance (LVDr) was reported to occur in 24% of patients treated for 1 year and in 74% of those treated for 5 years (16, 26). The rate of adefovir resistance (ADVr) in nucleoside-naive hepatitis B e antigen (HBeAg)-negative patients has been reported to be 0% after 1 year, but after 5 years of treatment, the rate increases to 28% to 42% (13). Entecavir (ETV) has been shown to be more potent *in vitro* than either LVD or ADV. Results from clinical studies showed that the efficacy of ETV was superior to that of the direct comparator, LVD, in both nucleoside-naive and LVD-refractory patients (6, 11, 15, 18).

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TABLE 1. Patient characteristics of naïve and LVD-refractory patients

Characteristic	Value	
	Naïve (n = 59)[a]	LVD refractory (n = 41)[a]
Male/female no.	41/18	34/7
Mean age (yr)	46.5 ± 8.4	48.6 ± 8.3
HBeAg positive (%)	33 (55.9)	23 (56.1)
Mean ALT (U/liter)	118.9 ± 108.6	119.8 ± 99.0
Mean HBV DNA ($\log_{10}$ copies/ml)	6.7 ± 1.8	6.8 ± 1.0
Genotypes (no. A/B/C/D/E)	3/11/43/1/1	5/7/28/1/0

[a] Values are means ± standard deviations.

The persistence of LVDr substitutions in patients switched to ETV is worrisome, because LVDr was shown to enhance the risk of developing ETVr and treatment failure, defined as viral breakthrough (BT) (an increase in serum HBV DNA of at least 1 $\log_{10}$ copy/ml compared with the nadir value as observed during ETV therapy) (20). A recent *in vitro* study showed that LVDr (L180M and M204V) substitutions confer an ~8-fold reduction in susceptibility to ETV and that additional substitutions at residues T184, S202, and M250 are needed to confer high levels of ETVr and BT (2, 3).

These analyses, however, used a limited number of patient isolates and/or laboratory HBV clones, and there has been a paucity of community-based data derived from long-term trials regarding the clinical outcomes of ETVr variants in naïve or LVD-refractory patients. Therefore, the aim of this study was to evaluate the incidence of ETVr and BT by comparing outcomes following 3-year ETV treatment in treatment-naïve patients and LVD-refractory patients. ETVr was assessed by using a recently reported line probe assay (HBV DR v.3) (7). Importantly, as the mechanism by which ETVr substitutions can induce BT during ETV therapy is largely unknown, changes in the conformation of HBV reverse transcriptase (RT) arising from LVDr and ETVr substitutions were modeled by using 3-dimensional (3D) docking simulation.

MATERIALS AND METHODS

Study design. We conducted a cross-sectional study of 100 patients (Tables 1 and 2), 45 of whom were from Japan, 25 from the United States, and 30 from Hong Kong. The patients were subdivided into two groups; treatment-naïve (n = 59) and LVD-refractory (n = 41) patients, whose gender, age, HBeAg status, and mean HBV DNA levels are summarized in Table 1. The patients received 0.5 mg or 1.0 mg ETV. The 1.0-mg ETV once-daily (QD) dosage has been approved for use in LVD-refractory patients, and only patients treated with 1.0 mg per day were included in resistance assessments. The study protocol conformed to the 1975 Declaration of Helsinki and was approved by the Ethics Committees of the institutions, and written informed consent was obtained from each participant.

Screening for drug-resistant substitutions. Simultaneous detection of wild-type HBV and drug-induced substitutions was performed using HBV DR v.3 and v.2 (Innogenetics, Ghent, Belgium) according to the manufacture's protocol. HBV DR v.3 and v.2 were developed for detection of ETVr-specific substitutions (T184SCGA/ILFM, S202G/C/I, and M250V/I/L), TDFr-specific substitutions (A194T), and newly reported ADVr (I233V) substitutions, as well as LVDr (L80V/I, V/G173L, L180M, and M204V/I) and ADVr (A181T/V and N236T) substitutions. The HBV DR assay consistently detected ETVr-specific substitutions present in ≥5% of the virus population when the HBV DNA concentration was ≥4 $\log_{10}$ copies/ml (7). The AUTOLiPA (Innogenetics, Ghent, Belgium) was used for the automated test procedure. An 867-bp-long fragment of the polymerase gene (domains A to F) was amplified using biotinylated PCR primers (HBV DR v.3 and v.2). PCR products were directly sequenced.

Statistical analyses. The statistical significance of observed differences was assessed using the chi-square test and the Mann-Whitney U test, where appropriate. In the 67 patients (38 naïve and 29 LVD refractory) with 3 years of ETV treatment (Fig. 1), the logistic regression model was used to assess the factors associated with BT. STATA 10 (Statacorp LP, TX) and the Statistical Program for Social Sciences (SPSS 12.0 for Windows; SPSS Inc., Chicago, IL) were used for all analyses.

HBV polymerase sequencing. HBV DNA was extracted from serum samples using a Qiagen QIAamp DNA blood minikit (Qiagen GmbH, Germany), and an 867-bp-long fragment of the polymerase gene (domains A to F) was amplified using biotinylated PCR primers (INNO-LiPA). PCR products were directly sequenced. Nucleotide mixtures were reliably detected when they were mixed at a ratio of approximately 25% or greater.

Three-dimensional-structure-based docking simulation methods. The amino acid sequence of HBV RT was retrieved from GenBank (gene Pol product of accession no. X75665), and the 323rd to 697th residues, which correspond to the finger, palm, and thumb domains, were extracted. The sequence and that of HIV RT, retrieved from the Protein Data Bank (accession no. 1RTD), were aligned using BLASTP (1), and then the resulting alignment was modified manually to obtain a match of the RT-specific motifs in both sequences. The main-chain structure of HBV RT was built from the alignment and the 3D structure of HIV RT (accession no. 1RTD) (8) by the use of the "nest" module (17) in the JACKAL package (19), where global energy minimization was done to find the most stable backbone structure. The loop and secondary-structure regions were then refined (24), after which the side chain structure was refined by the use of the "scap" module in the package (23). The 3D structures of HBV RT containing three sets of substitutions, L180M plus M204V, L180M plus S202G plus M204V, and L180M plus T184L plus M204V, were also designed in the same manner.

The binding site of ETV was searched on the wild-type HBV RT molecule by docking simulation. First, the structure of ETV triphosphate (ETV-TP) was designed by a small-molecule-editing function in the SYBYL 8.0 package (Tripos Inc., St. Louis, MO). Then, the possible binding sites of the ligand were searched from the surface of the protein by the use of the "Surflex-Dock" (9) module in the package. Here, the docking candidate area was restricted to the surfaces of the residues that were within 3 Å from L180, T184, S202, Y203, M204, D205, or D206. The binding potential was estimated from the GOLD score calculated by

TABLE 2. Three-year assessment (HBeAg loss, ALT normalization, and HBV-DNA 2.6) of naïve and LVD-refractory patients

Parameter	Value for follow-up week:					
	Naïve (n = 59)[a]			LVD refractory (n = 41)[a]		
	48	96	144	48	96	144
Follow-up [n (%)]	59 (100)	39 (66.1)	38 (64.4)[b]	41 (100)	40 (97.6)[c]	26 (63.4)[d]
HBeAg loss [n (%)]	5 (15.2)	7 (24.1)	9 (32.1)	4 (17.4)	7 (31.8)	4 (22.2)
ALT normalization [n (%)]	24 (40.7)	25 (64.1)	27 (71.1)	16 (39.0)	20 (50.0)	13 (50.0)
HBV DNA loss [n (%)]	24 (40.7)	28 (71.8)	28 (73.7)	15 (36.6)	19 (47.5)	12 (46.2)

[a] Values are means ± standard deviations.

[b] One naïve patient (J44) stopped ETV therapy at week 80 (ALT, 119 U/liter, and HBV DNA, 7.6 $\log_{10}$ copies/ml at baseline; ALT, 17, and HBV DNA, <2.6 at week 80) due to severe headache during therapy. Twenty patients in Hong Kong stopped ETV therapy between weeks 48 and 72.

[c] One LVD-refractory patient (J37) switched from ETV therapy to LVD plus adefovir due to BTH with ETVr before week 96.

[d] Two patients (J33 and J40) switched from ETV therapy to LVD plus adefovir due to BTH with ETVr before week 144. Twelve patients in the United States were treated with ETV for <120 weeks.

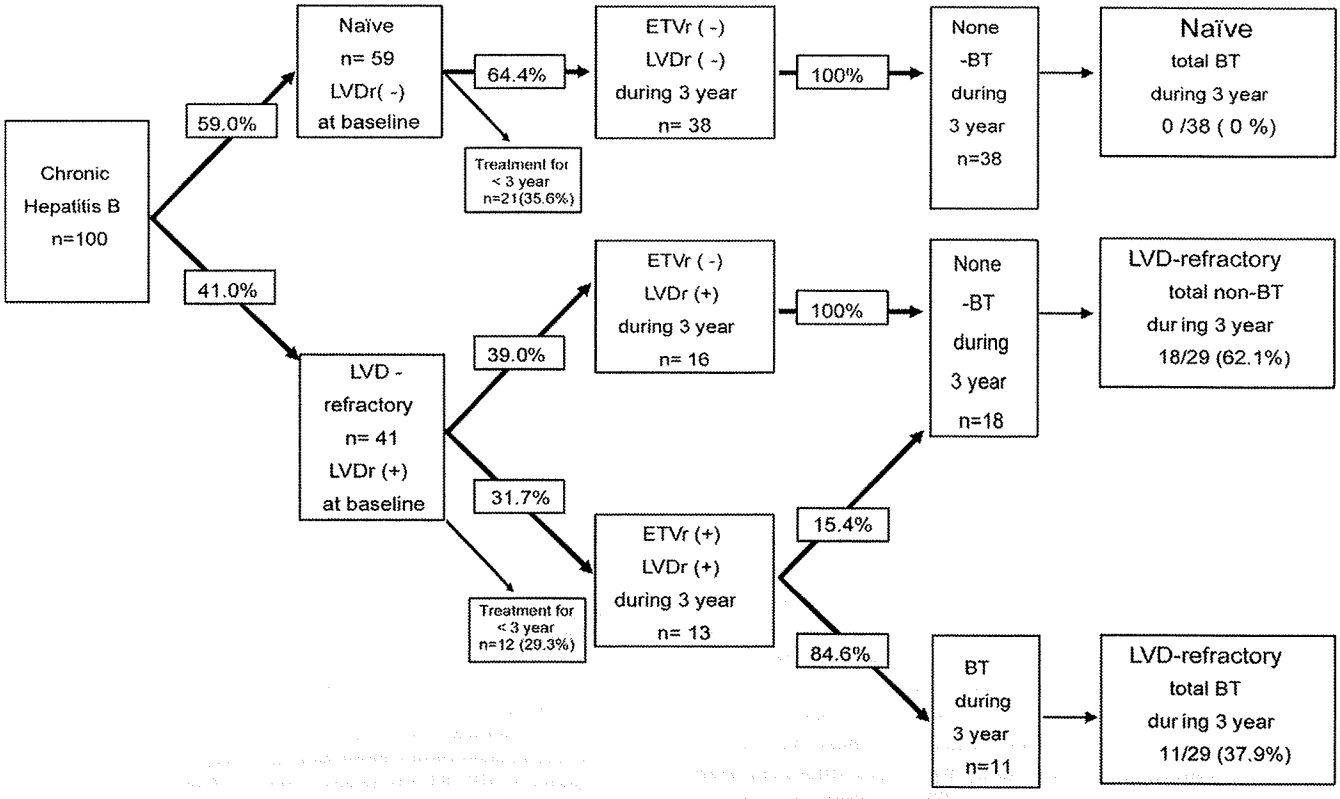


FIG. 1. Flowchart of 100 naïve/LVD-refractory patients during ETV therapy.

the "CScore" module (4) in the package. The score was evaluated based on hydrogen bond energy, the internal energy of molecules, and complex energy between ligand and protein. The minimal distance between their molecular surfaces was also calculated.

RESULTS

Clinical efficacy. The clinical backgrounds and the percentages of LVD-naïve and LVD-refractory patients who achieved HBeAg loss, alanine aminotransferase (ALT) normalization, and non-PCR-detectable HBV DNA levels (<2.6 $\log_{10}$ copies/ml) during the ETV treatment course are summarized in Table 2. There were no significant differences in clinical data at entry between the 2 groups. The rates of HBeAg loss, ALT normalization, and HBV DNA loss were significantly higher in naïve patients than in LVD-refractory patients.

Detection of substitutions responsible for ETV resistance in naive and LVD-refractory patients during treatment for 144 weeks. The characteristics of patients who had ETVr substitutions detected by HBV DR v.3 are summarized in Table 3. The percentage of the typical LVDr (L180M, M204V, and M204I) or ETVr observed in naïve patients was 0% (0/38) during the 144-week treatment period.

Among the patients examined at entry prior to treatment with ETV, in 41 LVD-refractory patients, M204V (30/41; 72.4%), M204I (24/41; 58.5%), L180M (38/41; 92.7%), L80V (6/41; 14.6%), L80I (18/41; 43.9%), and V173L (4/41; 9.76%) substitutions were detected. In the 41 LVD-refractory patients, the cumulative ETVr substitutions were detected in 2/41 (4.9%) at baseline and increased to 6/41 (14.6%), 10/41 (24.4%), and 13/29 (44.8%) at weeks 48, 96, and 144, respectively (Fig. 2). In the 29 patients treated with ETV for 3 years, T184SCGA, T184ILMF, S202G, and S202C were found in 5 (17.2%), 4 (13.8%), 9 (31.0%), and 1 (3.4%), respectively. Neither S202I nor M250V/I/L substitutions were detected in this population.

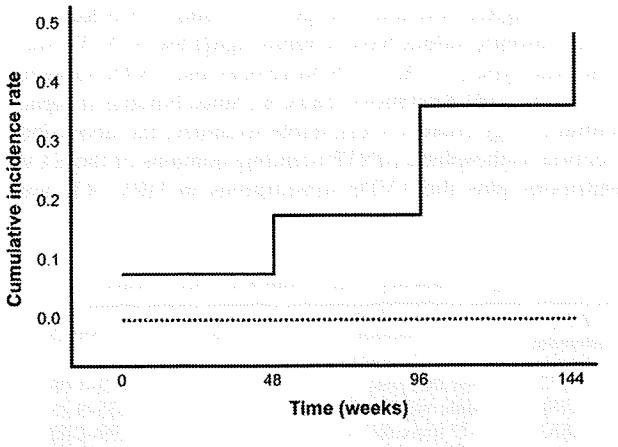
A comparative summary of the ETVr substitutions, detected by HBV DR v.3 and direct sequencing, during week −8 (8 weeks before the start of treatment) and week 144 is presented in Table 4. HBV DR v.3 revealed ETVr substitutions earlier (up to 48 weeks) than did direct sequencing. In addition, HBV DR v.3 allowed the detection of mixed quasispecies containing different substitutions.

Viral BT during the 144 weeks on treatment. The rates of BT among 59 naive and 41 LVD-refractory patients treated with ETV for 144 weeks are summarized in Fig. 1. There were no cases of BT in the LVD-naïve group during the 144-week treatment period, whereas in the LVD-refractory group treated with 1.0 mg ETV, 11 of 13 patients with genotypic ETVr had evidence of BT after 60 to 144 weeks of treatment, followed by 7 breakthrough hepatitis (BTH) (defined as a flare up of ALT) patients (median interval, 11.4 weeks after BT). The LVDr substitutions (L180M and M204V/I) were detected in all of the BT patients in specimens obtained at baseline (Table 3). Among the 11 patients with BT, 8 (72.7%) had an additional S202G substitution and 7 (63.6%) had a T184SCGA or T184ILMF substitution, indicating that the T184 and/or S202 substitution emerged before BT during ETV treatment (Table 3 and Fig. 2). Seven patients with BTH had LVDr

TABLE 3. Characteristics of ETVr detected by HBV DR v3 among 13 patients at week 0, 24, 48, 72, 96, and 144

Case	Age (yr)	Sex	HBV genotype	ALT (IU/liter)			HBV DNA (copies/ml)			Wk			No. of wks ETVr to BT	LVDr at baseline	ETVr at baseline	ETVr emerged during therapy
				Baseline	Nadir	Peak	Baseline	Nadir	Peak	ETVr	BT	BTH				
J20	34	M	C	82	22	131	7.6	4.5	7.4	<92[a]	92	100	<22	M180, V204	ND[f]	ILMF184, G202
J27	61	M	C	79	38	150	7.4	3.9	7	<128[b]	128	144	<32	M180, V204	ND	SCGA184
J30	43	M	C	95	21	108	7.6	4.6	6.8	48	76	92	28	I80, M180, V/I204	ND	G202
J33	43	M	C	69	21	199	7.6	5.1	7.1	96	116	128	18	I80, L173, M180, VI204	ND	(ILFM184),[c] G202
J37[d]	48	M	C	465	43	76	7.6	4.7	6	48	60	60	12	I80, M180, V204, I204	ND	G202
J39	59	M	C	35	22	82	7	4.6	8	72	108	128	36	M180, V204	ND	SCGA/ILFM184, G202
J40	28	M	C	149	15	398	5.3	3.6	6.6	−8	64	72	72	M180, V204	(G+C) 202	G202
J22	44	M	C	240	17	24	7.6	3.5	5.6	<140[e]	144	NO	<44	M180, I204	ND	G202
J28	45	M	B	43	15	23	6.9	5.5	6.5	0	144	NO	144	M180, V204	SCGA184, V233	SCGA184, V233
U72	47	F	C	29	25	44	7.5	3.8	5	<144	144	NO	<48	L + V80, M180, V204	ND	SCGA184, (S+G)202
H55	47	F	C	102	19	20	6.1	3	4.2	48	88	NO	40	V + I80, M180, V204	ND	ILFM184
J19	57	M	B	233	29	43	7.4	2.8	3.3	24	NO	NO	NO	V + I80, M180, I204	ND	G202
U42	52	M	A	135	36	50	>7.6	5.3	5.8	<80	NO	NO	NO	M180, V204	ND	SCGA184

[a] Not tested during weeks 72 and 92.
[b] Not tested during weeks 96 and 128.
[c] ILFM184 was detected at week 144.
[d] Switch to ADV/LVD at week 60.
[e] Not tested during weeks 96 and 140.
[f] ND, not detected.



LVD-refractory patients

Weeks	0	48	96	144
No.	41	41	41	29*
ETVr	2 (4.9%)	6 (14.6%)	10 (24.4%)	13 (44.8%)
T184SCGA	1	1	3	5
T184ILFM	0	1	4	4
S202G	1	4	7	9
S202C	1	1	1	1
S202I	0	0	0	0
M250V/L	0	0	0	0

*Twelve patients in the US have ETV treatment for <120 weeks.

FIG. 2. Kaplan-Meier plot and tabulated data for time to ETVr and cumulative ETVr patterns over 144 weeks. Pretreatment variables (solid line, LVD refractory; broken line, naïve) were analyzed in relation to the occurrence of ETVr. A previous LVD treatment was associated with a more rapid occurrence of ETVr (Breslow analysis; $P < 0.001$). Among the patients examined at entry prior to treatment with ETV, for the 41 LVD-refractory patients, the cumulative ETVr substitutions were detected in 2/41 (4.9%) at baseline and increased to 6/41 (14.6%), 10/41 (24.4%), and 13/29 (44.8%) at weeks 48, 96, and 144, respectively. Neither the S202I nor the M250V/I/L substitution was detected in this population.

(100% for both M180 and V204) at baseline and ETVr substitutions (S202G, 85.7%; T184SCGA/ILFM, 57.1%) during 3-year ETV treatment. Representative cases with BTH during ETV therapy are shown in the supplemental material.

Pretreatment status (LVD refractory or naïve) was analyzed in relation to the occurrence of ETVr, BT, and BTH during the 144-week course of ETV therapy. The log rank analysis of pretreatment variables showed that prior refractoriness to LVD was associated with more rapid occurrence of ETVr (Fig. 2), BT, and BTH ($P \leq 0.001$, $P < 0.001$, and $P = 0.0039$, respectively). Additionally, among the 67 patients receiving 3-year ETV treatment, BT occurred in 10 of 52 (19.2%) patients with HBV genotype C and 1 of 8 (12.5%) with genotype B, whereas no BT was observed in patients with genotypes A, D, and E. No significant association between BT and HBV genotypes was found.

Baseline characteristics and factors associated with viral breakthrough during 3-year ETV therapy. When non-BT and BT groups within the 67 patients treated with ETV for 144 weeks were compared, no significant baseline differences were observed in mean age, gender, serum ALT levels, HBV DNA, or HBeAg status (Table 1), while 2-$\log_{10}$-unit reductions in HBV DNA levels or undetectable (<2.6) HBV DNA levels at the end of year 1 were significantly higher in the non-BT group (Table 5). Interestingly, the proportion of patients refractory to LVD with both the L180M and M204V substitutions at baseline ($P < 0.001$) and the incidence of S202G or T184SCGA/ILMF substitutions during the 3-year ETV treatment ($P < 0.001$) were significantly higher in the BT group.

None of the BT cases reached undetectable HBV DNA levels at the end of the first year of ETV treatment (BT, 0%, versus non-BT, 58.9%), but all were refractory to LVD (BT, 100%, versus non-BT, 32.1%) and had the L180M substitution

TABLE 4. Detection of ETVr mutations by HBV DR v3 and direct sequencing at weeks 0, 24, 48, 72, 96, and 144

Case	INNO-LiPA detection		Direct-sequencing detection	
	Wk	ETVr	Wk	ETVr
J20	96	ILFM184 + G202	96	L184 (L184 and G202 detected at wk 144)
J27	144	SCGA184 + T184	144	S184 + T184
J30	48	G202	96	G202 (ND[b] at wk 48)
J39	72	SCGA/ILFM184 + G202	96	I184, G202 (ND at wk 72)
J40	−8	G202	0	G202 (ND at wk −8)
J33	96	G202	144	G202 (ND at wk 96)
J19	24	G202 + C202	48	G202 (ND at wk 24)
J22	144	S202 + G202	144	S202
J28	0	SCGA184, V233	0	A184, V233
J37[a]	48	S202 + G202	48	S202
U42	80	SCGA184 + T184	80	T184 + A184
U72	144	T184 + SCGA184, S202 + G202	144	T184 + A184, S202
H55	48	T184 + ILFM184	48	T184

[a] Switched from ETV to LVD plus ADV therapy at week 60.
[b] ND, ETVr was not detected.

at baseline (BT, 100%, versus non-BT, 28.6%) (Table 5). For other factors, additional analysis showed that ETVr substitutions (i.e., S202G, T184SCGA/ILFM, and M204V) were strongly associated with BT during the 3-year ETV treatment (OR, 146.67 [95% CI, 13.55 to 1,587.24], 96.25 [95% CI, 9.38 to 987.41], and 10.91 [95% CI, 4.72 to 354.28]) (Table 5).

After adjustment for age, gender, baseline HBV DNA, and reduction in HBV DNA, we found that ETVr substitutions (i.e., T184SCGA/ILFM and S202G) significantly increased the risk of BT among patients with LVDr (OR, 141.12 [95% CI, 6.94 to 2,870.20] and 201.25 [95% CI, 11.22 to 3,608.65], respectively).

Mechanism of ETVr assessed by 3D docking simulation. Modeling of the DNA binding cleft of HBV RT by docking simulation indicated that ETVr substitutions (T184L and S202G), which are located in the palm, were found to change the direction of the D205 residue (YMDD domain) and to narrow the binding pocket in comparison with the wild type and LVDr substitutions (M204V and L180M) (Fig. 3). The results of docking simulation showed that ETVr substitutions (T184L and S202G) plus LVDr substitutions (M204V and L180M) have significantly longer minimal distances between the molecular surfaces of the protein and the drug (2.2 Å and 2.1 Å) and higher potential energy (−118 and −99.8 Kcal/mol [smaller absolute values have a minus sign]) for ETV-TP than for the wild type (1.3 Å; −178 Kcal/mol) and LVDr substitutions (1.5 Å; −141 Kcal/mol) (Table 6). Since binding at higher potential energy creates a less stable structure, the deoxyribonucleotide triphosphate (dNTP)-binding domains of the ETVr substitutions plus the LVDr substitutions in HBV RT have

TABLE 5. ORs and 95% CIs of BT according to baseline characteristics among 67 patients treated with ETV for 3 years

Characteristic	Non-BT (n = 56)[a]	BT (n = 11)[a]	P for difference	Contrast	OR	95% CI
Age (mean)	45.4 ± 8.1	45.4 ± 9.4	0.932	1-yr increase	1	0.92–1.08
Male (%)	71.4	81.8	0.481	Male vs. female	1.8	0.35–9.26
ALT (mean)	115.9 ± 105.6	126.2 ± 127.3	0.832	1-U increase	1	0.99–1.00
HBeAg (%)	66.1	81.8	0.307	Positive vs. negative	2.31	0.45–11.78
HBV-DNA						
Level (mean)	6.8 ± 1.6	7.1 ± 0.8	0.959	1-U increase	1.16	0.72–1.89
2-log-unit reduction at 1 yr (%)	98.2	63.6	<0.001	With vs. without	0.03	0.00–0.33
DNA <2.6 at 1 yr (%)	58.9	0.0	<0.001	With vs. without	NA[b]	
LVD refractory (%)	32.1	100.0	<0.001	With vs. without	NA	
Amino acid substitutions at baseline						
V80 (%)	3.6	18.2	0.063	With vs. without	6	0.74–48.17
I80 (%)	17.9	36.4	0.171	With vs. without	2.63	0.64–10.72
L173 (%)	3.6	18.2	0.063	With vs. without	6	0.75–48.18
M180 (%)	28.6	100.0	<0.001	With vs. without	NA	
V204 (%)	19.6	90.9	<0.001	With vs. without	10.91	4.72–354.28
I204 (%)	23.2	36.4	0.363	With vs. without	1.89	0.48–7.49
Amino acid substitutions during ETV therapy (3 yr)						
SCGA/ILFM184 (%)	1.8	72.7	<0.001	With vs. without	96.25	9.38–987.41
G202 (%)	1.8	63.6	<0.001	With vs. without	146.67	13.55–1,587.24

[a] Values are means ± standard deviations.
[b] NA, not applicable.