The High Incidence of the Emergence of Entecavir-Resistant Mutants among Patients Infected with Lamivudine-Resistant Hepatitis B Virus

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Nagasaki, F., Niitsuma, H., Ueno, Y., Inoue, J., Kogure, T., Fukushima, K. and Shimosegawa, T. *The High Incidence of the Emergence of Entecavir-Resistant Mutants among Patients Infected with Lamivudine-Resistant Hepatitis B Virus.* Tohoku J. Exp. Med., 2007, **213** (2), 181-186 —— Hepatitis B virus (HBV) infection remains to be one of the most prevailing infection in the world, causing chronic liver diseases. Although lamivudine has been effective to suppress HBV replication, longer durations of administration can lead to the emergence of drug-resistant mutant viruses, followed by reactivation of hepatic inflammation (breakthrough hepatitis). Moreover, the optimal period of administration as well as the effects of anti-viral nucleot(s)ide such as lamivudine, adefovir, and entecavir, has not been established. To evaluate the efficacy of the anti-viral effects of entecavir for lamivudine-resistant HBV, we administered entecavir sequentially in four patients with chronic HBV infection, who demonstrated the emergence of lamivudine-resistant HBV and histological active hepatitis. The antiviral effects were evaluated by the serum viral loads and biochemical laboratory data. After follow-up periods of more than 36 months, we found high incidence in the emergence of entecavir resistant mutants (3/4, i.e., 75%). An additional mutation at the 184th amino acid, different from the previously reported lamivudine-resistant mutations (80th, 180th, and 204th), seemed to have a close relationship with the induction of entecavir-resistant mutants at least for Japanese HBV genotype C. Our observation draws attention to the possibility that the usage of entecavir for lamivudine-resistant HBV could promptly induce entecavir-resistant mutations in addition to lamivudine-resistance. ———— HBV; lamivudine; entecavir; antiviral resistant virus

Chronic hepatitis B virus (HBV) infection remains one of the major serious health problems in the world (Lee 1997). Its clinical manifestation is affected by various factors, particularly the genotype (Maddrey 2000). Eight different genotypes of HBV, designated A to H, have been already determined (Okamoto et al. 1987, 1988; Norder et al. 1992; Stuyver et al. 2000; Arauz-Ruiz et al. 2002). Furthermore, it has been reported that a genotype is composed of various subgenotypes (Norder et al. 2004). For example, genotype B causes seroconversion more frequent-

ly than genotype C (HBV/C), and those infected with HBV/B appear to have a better prognosis (Kikuchi 2000; Orito et al. 2001). At present, HBV genotype B (HBV/B) has been clustered into five subgenotypes (Nagasaki et al. 2006a).

Lamivudine, the first approved orally administered nucleotide analogue, is well known for its safety and effectiveness in the treatment of HBV infections, including HBV cirrhosis, regardless of the HBV genotype (Yao and Bass 2000; Yao et al. 2001; Yuen et al. 2003). On the other hand, long durations of lamivudine therapy could lead to the evolution of drug-resistant mutants, which can emerge in 15% in one year, and in 50% in three years after the initiation of therapy (Lee 1997; Dienstag et al. 1999; Kapoor et al. 2000; Yao et al. 2001), and could be followed by hepatic failure, i.e., breakthrough hepatitis (Liaw et al. 2000; Santantonio et al. 2000; Lok and McMahon 2001). As we previously reported, some patients could discontinue lamivudine administration successfully (Nagasaki et al. 2006b), but it is usually considered to be very exceptional (Wang et al. 2002).

Recently, other antiviral agents, such as adefovir, entecavir, and tenofovir have revealed usefulness in suppressing the serum HBV-DNA levels. In Japan, the use of adefovir was approved in 2004 and that of entecavir in 2006. However, their long term efficacies have not been established. Especially, the usage of entecavir for lamivudine resistant HBV has been considered questionable since both lamivudine and entecavir show similar mechanisms in the development of resistant mutations.

In this study, we administered entecavir to 4 patients with chronic HBV infection, who had been previously administered lamivudine in whom the emergence of a resistant mutant had been confirmed.

MATERIALS AND METHODS

We administered entecavir monotherapy treatment for more than 36 months in 4 patients with chronic HBV infection, who had been previously treated with lamivudine 100 mg daily and developed breakthrough hepatitis due to the emergence of lamivudine-resistant mutant virus after the cessation of lamivudine. The inclusion criteria of these patients strictly followed those of the phase III clinical trial by Bristol-Myers Japan Inc. (Tokyo); briefly, both i) the presence of active HBV viremia ($> 10^5$ copy/mL), and ii) the presence of pathologi-

TABLE 1. The clinical backgrounds of the four patients.

Patients	1	2	3	4
Sex	M	M	F	M
Age	51	43	51	27
Duration of prior lamivudine administration	31	13	20	16
T. Bil (mg/dL)	0.9	0.5	0.7	0.4
AST (IU/L)	61	34	39	37
ALT (IU/L)	74	62	61	102
HBeAg (RIA)	+	+	+	+
Anti-HBe (RIA)	−	−	−	−
HBV-DNA (Log copy/ml)	7.5	5.4	> 7.6	> 7.6
Genotype (RFLP)	C	C	C	C
YMDD motif	YIDD	YMDD/YIDD	YIDD/YVDD	YIDD

T-Bil, total bilirubin; AST, asparate aminotransferase; ALT, alanine aminotransferase; HBeAg, hepatitis B envelope antigen; Anti-HBe, hepatitis B envelope antibody; RIA, radioimmunoassay; RFLP, restriction fragment length polymorphism; Y, tyrosine; M, methionine; D, aspartic acid; I, isoleucine; V, valine; M, male; F, female.

cally proven chronic hepatitis. The study protocol was approved by institutional review board (IRB) in Tohoku University Hospital. For this clinical trial, 4 patients were initially screened and all of these patients were considered as eligible patients for this study. All of these four patients had been administered entecavir 0.5 or 1.0 mg daily for 52 weeks in a coded manner, and thereafter 1.0 mg daily by the protocol of our present study. Their clinical data are shown on Table 1. The genotype of HBV detected in all 4 patients was C, and the serum HBV-DNA level and alanine aminotransferase (ALT) were 7.5 Log copy/mL and 70.8 IU/L on average, respec-

tively. The quantitative range of HBV-DNA by polymerase chain reaction (PCR)-assay (Roche Ampricor HBV, Roche Japan Inc., Tokyo) was 2.6-7.7 Log copy/mL.

We defined antiviral resistance as a more than 1 log10 increase in the HBV-DNA level in the serum from a patient who had an initial virologic response (Lok and McMahon 2007). We conducted HBV-DNA direct sequence analyses in the polymerase region before the administration of entecavir, and at the time antiviral resistance emerged.
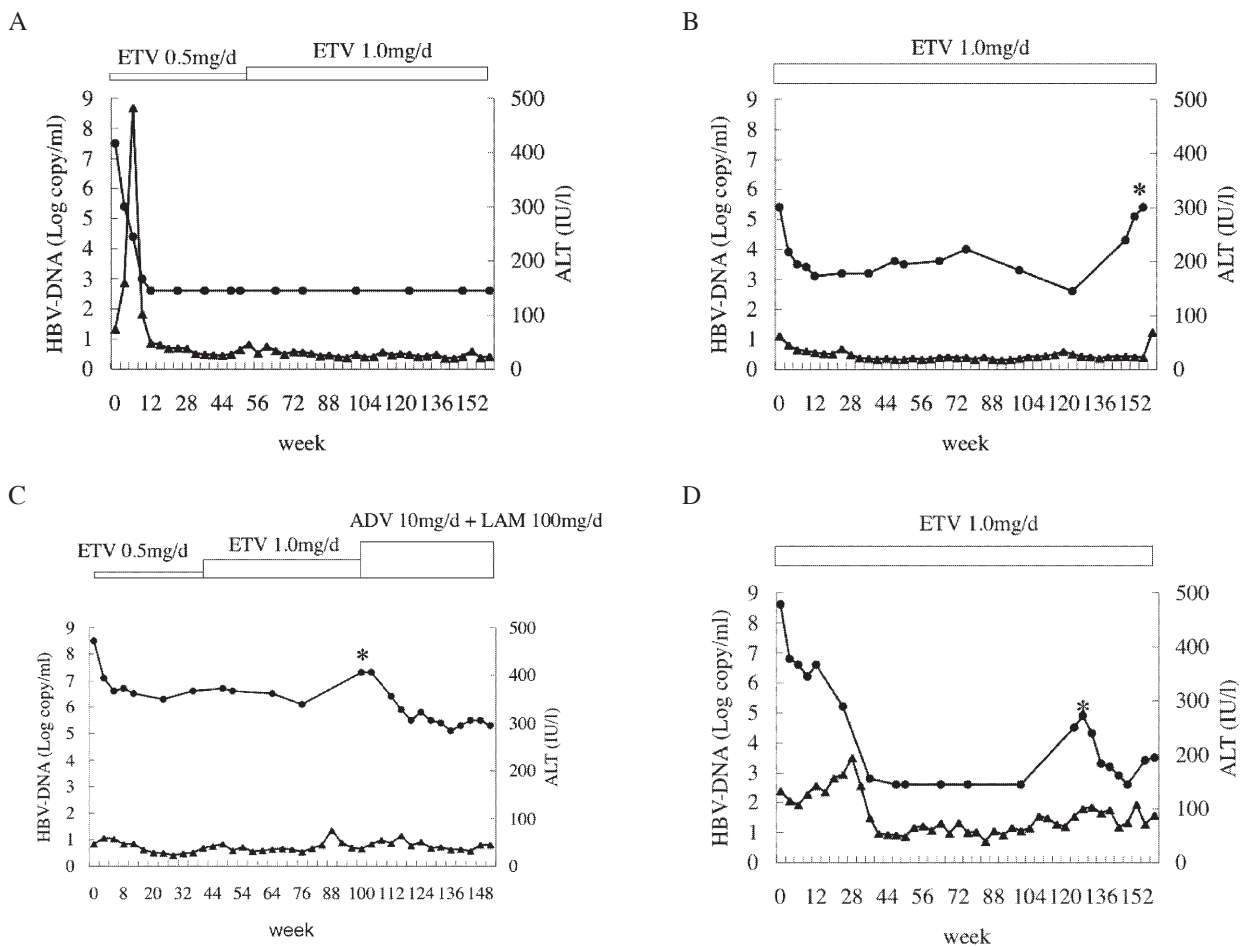

Fig. 1. Clinical course of the four patients treated with entecavir.
    A: Clinical course of Patient No. 1. An entecavir-resistant mutant was not found in this patient.
    B: Clinical course of Patient No. 2. An entecavir-resistant mutant was detected at the 152nd week (*).
    C: Clinical course of Patient No. 3. Entecavir was administered 0.5 mg/day for 52 weeks, thereafter 1.0 mg/day. An entecavir-resistant mutant was detected at the 104th week (*), and the treatment was then changed to adefovir and lamivudine combination therapy.
    D: Clinical course of Patient No. 4. An entecavir-resistant mutant was detected at the 130th week (*).
    ETV, entecavir; ADV, adefovir; LAM, lamivudine; ALT, alanine aminotransferase.
    ━●━, HBV-DNA; ━▲━, ALT.

RESULTS

Clinical evaluation

Patient No. 1 had a good response to entecavir without the emergence of a resistant mutant virus (Fig. 1A). On the other hand, the other three patients (75%) experienced the emergence of entecavir mutant viruses during the therapy. Although patient No. 2 at first showed continuously suppressed serum HBV-DNA levels of 2.6 to 4.0 Log copy/mL, at the 52nd week the patient showed an increase of the serum HBV-DNA levels followed by an elevation of ALT and was found to have entecavir-resistant mutant viruses (Fig. 1B). Patient No. 3 was administered entecavir 0.5 mg daily and the serum HBV-DNA level decreased from 8.5 Log copy/mL but did not decrease further below 6.0 Log copy/mL even after an increase of the entecavir dosage as shown in Fig. 1C. We changed the entecavir monotherapy to lamivudine and adefovir combination therapy at the 104th week. Thereafter, this patient showed decreases in both the serum HBV-DNA level and ALT. Patient No. 4 had been administered entecavir 1.0 mg daily which resulted in the suppression of HBV-DNA to below a detectable level by PCR assay. At the 130th week, the serum HBV-DNA level increased to 4.9 Log copy/mL (ALT 100 IU/L) and entecavir-resistant mutant viruses were revealed. Later, the serum HBV-DNA level decreased without any change in treatment (Fig. 1D).

The analysis of HBV sequences regarding mutations after entecavir administration

HBV amino acids (aa) in polymerase region analyses from the three patients with entecavir-resistant mutants are shown in Table 2. The HBV nucleotide sequences in the polymerase regions obtained at the initiation of the therapy and during the week entecavir-resistant mutants had emerged were determined and amino acids were analyzed by them. The drug resistant mutations as shown in the table were confirmed as follows. In the three patients the 80th aa in the A domain was substituted to leucine, and the 180th aa in the B domain was methionine. Thus, the lamivudine resistant 80th aa mutation found at the initiation of entecavir treatment had been returned to be wild type. In two patients the 184th aa was leucine, and in one patient there was a mixed pattern of threonine, isoleucine, proline, and leucine. The 204th aa in the C domain of the three patients was valine. Other domains, F, D and E showed no nucleotide and amino acid mutations.

DISCUSSION

We administered entecavir monotherapy for more than 3 years in 4 patients with chronic HBV infection who had previously showed break-

TABLE 2. The analysis of amino acids in the HBV polymerase region by the nucleotide sequences of HBV detected from three patients with entecavir resistance at the start of the therapy and at the time resistant viruses emerged. Patient No. 1 did not have entecavir resistance and thus sequencing was not performed.

		HBV-DNA Polymerase region							
	Weeks when resistant mutation confirmed	A Domain		B Domain				C Domain	
Patient		80		180		184		204	
(No.)		0w	Mutant	0w	Mutant	0w	Mutant	0w	Mutant
2	152	I/L	L	L	M	T	L	I/M	V
3	104	I/L	L	L	M	T	T/I/P/L	M/I/V	V
4	130	I	L	L	M	T	L	I	V

I, isoleucine; L, leucine; M, methionine; P, proline; T, threonine; V, valine.

through hepatitis induced by prior lamivudine administration. The administration of entecavir for these patients caused the emergence of entecavir-resistant viruses in three of the four patients, although severe liver dysfunction did not yet occur. Breakthrough hepatitis can often be fatal, especially for patients with progressed liver diseases, so we administered entecavir when virologic breakthrough was confirmed.

It was reported that lamivudine-resistant mutant HBV nucleotide sequences showed changes of methionine to isoleucine or valine at the 204th aa, leucine to methionine at the 180th aa, leucine to isoleucine at the 80th nucleotide and valine to leucine at the 173rd aa (Stuyver et al. 2001).

In this study, we determined the HBV nucleotide sequences obtained from three patients, and analyzed the amino acids in the polymerase region both before the administration of entecavir and at the 152nd, 104th and 130th week, respectively. As a result, we found at first that in all of them the resistance to lamivudine was related to the change from leucine to isoleucine at the 80th aa. They then showed the lamivudine resistant mutations after the administration of entecavir monotherapy (180th and 204th aa). It has been reported that susceptibility to entecavir decreases when mutations at both the 180 and the 204th aa emerge (Tenney et al. 2004), which is in accord with our present results.

At the 184th aa, as previously reported concerning its relation to entecavir resistance (Tenney et al. 2004), all of the patients showed the mutation from threonine to leucine, although patient No. 4 showed a mixed pattern.

Additionally, we found a new change at the 80th aa, which showed an inverse pattern of the lamivudine-resistant mutant (Yim et al. 2006). Actually, 80th mutation found at the initiation of entecavir treatment return to be wild type when entecavir-resistant mutants developed (Table 2). The results might suggest that some of the lamivudine-related mutations could disappear with the cessation of lamivudine.

We suppose that the emergence of resistance to antiviral agents in patients with sequential

administration of entecavir monotherapy for chronic HBV after the emergence of a lamivudine-resistant mutant virus might have the closest relationship with the 184th aa and, additionally, the 80th aa, at least in HBV genotype C, the most common genotype in Japan.

In addition to lamivudine, adefovir has also been reported to develop resistant mutants (Fung et al. 2005). Sequential therapy for patients with chronic HBV infection could result in the emergence of multi-drug resistant mutants (Yim et al. 2006).

The discontinuation of lamivudine before the emergence of a mutant virus might be useful to prevent breakthrough hepatitis; nevertheless, the reactivation of hepatitis is sometimes fatal, especially for patients with progressed liver diseases, such as liver cirrhosis (Honkoop et al. 2000). These findings underscore the difficulties in determining the best therapy for patients with chronic HBV infection.

The results of our study suggested the possibility that entecavir monotherapy should not be the first choice for the treatment of patients with chronic HBV infection who have developed a lamivudine-resistant virus.

Further studies regarding optimal patient selection, treatment duration, and the dosages of antiviral agents including entecavir are warranted.

References

Arauz-Ruiz, P., Norder, H., Robertson, B.H. & Magnius, L.O. (2002) Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J. Gen. Virol.*, **83**, 2059-2073.

Dienstag, J.L., Schiff, E.R., Wright, T.L., Perrillo, R.P., Hann, H.W., Goodman, Z., Crowther, L., Condreay, L.D., Woessner, M., Rubin, M. & Brown, N.A. (1999) Lamivudine as initial treatment for chronic hepatitis B in the United States. *N. Engl. J. Med.*, **341**, 1256-1263.

Fung, S.K., Andreone, P., Han, S.H., Rajender Reddy, K., Regev, A., Keeffe, E.B., Hussain, M., Cursaro, C., Richtmyer, P., Marrero, J.A. & Lok, A.S. (2005) Adefovir-resistant hepatitis B can be associated with viral rebound

and hepatic decompensation. *J. Hepatol.*, **43**, 937-943.

Honkoop, P., de Man, R.A., Niesters, H.G., Zondervan, P.E. & Schalm, S.W. (2000) Acute exacerbation of chronic hepatitis B virus infection after withdrawal of lamivudine therapy. *Hepatology*, **32**, 635-639.

Kapoor, D., Guptan, R.C., Wakil, S.M., Kazim, S.N., Kaul, R., Agarwal, S.R., Raisuddin, S., Hasnain, S.E. & Sarin, S.K. (2000) Beneficial effects of lamivudine in hepatitis B virus-related decompensated cirrhosis. *J. Hepatol.*, **33**, 308-312.

Kikuchi, K., N.H., Ishii, M., Julieta, G.C., Shang, H. & Ojima, T. (2000) Genoepidemiology and its relationship to clinical features in patients infected chronically with hepatitis B virus (HBV). *Hepatology Res.*, **17**, 43-55.

Lee, W.M. (1997) Hepatitis B virus infection. *N. Engl. J. Med.*, **337**, 1733-1745.

Liaw, Y.F., Leung, N.W., Chang, T.T., Guan, R., Tai, D.I., Ng, K.Y., Chien, R.N., Dent, J., Roman, L., Edmundson, S. & Lai, C.L. (2000) Effects of extended lamivudine therapy in Asian patients with chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. *Gastroenterology*, **119**, 172-180.

Lok, A.S. & McMahon, B.J. (2001) Chronic hepatitis B. *Hepatology*, **34**, 1225-1241.

Lok, A.S. & McMahon, B.J. (2007) Chronic hepatitis B. *Hepatology*, **45**, 507-539.

Maddrey, W.C. (2000) Hepatitis B: an important public health issue. *J. Med. Virol.*, **61**, 362-366.

Nagasaki, F., Niitsuma, H., Cervantes, J.G., Chiba, M., Hong, S., Ojima, T., Ueno, Y., Bondoc, E., Kobayashi, K., Ishii, M. & Shimosegawa, T. (2006a) Analysis of the entire nucleotide sequence of hepatitis B virus genotype B in the Philippines reveals a new subgenotype of genotype B. *J. Gen. Virol.*, **87**, 1175-1180.

Nagasaki, F., Ueno, Y., Yamamoto, T., Nakagomi, Y., Kido, O., Kakazu, E., Matsuda, Y., Kogure, T., Yamagiwa, Y., Kikuchi, K., Fukushima, K., Kanno, N., Niitsuma, H. & Shimosegawa, T. (2006b) Sustained clinical improvement of a patient with decompensated hepatitis B virus-related cirrhosis after treatment with lamivudine monotherapy. *Tohoku J. Exp. Med.*, **210**, 29-36.

Norder, H., Hammas, B., Lofdahl, S., Courouce, A.M. & Magnius, L.O. (1992) Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains. *J. Gen. Virol.*, **73**, 1201-1208.

Norder, H., Courouce, A.M., Coursaget, P., Echevarria, J.M., Lee, S.D., Mushahwar, I.K., Robertson, B.H., Locarnini, S. & Magnius, L.O. (2004) Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. *Intervirology*, **47**, 289-309.

Okamoto, H., Imai, M., Tsuda, F., Tanaka, T., Miyakawa, Y. & Mayumi, M. (1987) Point mutation in the S gene of hepatitis B virus for a d/y or w/r subtypic change in two blood

donors carrying a surface antigen of compound subtype adyr or adwr. *J. Virol.*, **61**, 3030-3034.

Okamoto, H., Tsuda, F., Sakugawa, H., Sastrosoewignjo, R.I., Imai, M., Miyakawa, Y. & Mayumi, M. (1988) Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J. Gen. Virol.*, **69**, 2575-2583.

Orito, E., Mizokami, M., Sakugawa, H., Michitaka, K., Ishikawa, K., Ichida, T., Okanoue, T., Yotsuyanagi, H. & Iino, S. (2001) A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. Japan HBV Genotype Research Group. *Hepatology*, **33**, 218-223.

Santantonio, T., Mazzola, M., Iacovazzi, T., Miglietta, A., Guastadisegni, A. & Pastore, G. (2000) Long-term follow-up of patients with anti-HBe/HBV DNA-positive chronic hepatitis B treated for 12 months with lamivudine. *J. Hepatol.*, **32**, 300-306.

Stuyver, L., De Gendt, S., Van Geyt, C., Zoulim, F., Fried, M., Schinazi, R.F. & Rossau, R. (2000) A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J. Gen. Virol.*, **81**, 67-74.

Stuyver, L.J., Locarnini, S.A., Lok, A., Richman, D.D., Carman, W.F., Dienstag, J.L. & Schinazi, R.F. (2001) Nomenclature for antiviral-resistant human hepatitis B virus mutations in the polymerase region. *Hepatology*, **33**, 751-757.

Tenney, D.J., Levine, S.M., Rose, R.E., Walsh, A.W., Weinheimer, S.P., Discotto, L., Plym, M., Pokornowski, K., Yu, C.F., Angus, P., Ayres, A., Bartholomeusz, A., Sievert, W., Thompson, G., Warner, N., Locarnini, S. & Colonno, R.J. (2004) Clinical emergence of entecavir-resistant hepatitis B virus requires additional substitutions in virus already resistant to Lamivudine. *Antimicrob. Agents Chemother*, **48**, 3498-3507.

Wang, J.H., Lu, S.N., Lee, C.M., Lee, J.F. & Chou, Y.P. (2002) Fatal hepatic failure after emergence of the hepatitis B virus mutant during lamivudine therapy in a patient with liver cirrhosis. *Scand. J. Gastroenterol.*, **37**, 366-369.

Yao, F.Y. & Bass, N.M. (2000) Lamivudine treatment in patients with severely decompensated cirrhosis due to replicating hepatitis B infection. *J. Hepatol.*, **33**, 301-307.

Yao, F.Y., Terrault, N.A., Freise, C., Maslow, L. & Bass, N.M. (2001) Lamivudine treatment is beneficial in patients with severely decompensated cirrhosis and actively replicating hepatitis B infection awaiting liver transplantation: a comparative study using a matched, untreated cohort. *Hepatology*, **34**, 411-416.

Yim, H.J., Hussain, M., Liu, Y., Wong, S.N., Fung, S.K. & Lok, A.S. (2006) Evolution of multi-drug resistant hepatitis B virus during sequential therapy. *Hepatology*, **44**, 703-712.

Yuen, M.F., Tanaka, Y. & Lai, C.L. (2003) Hepatitis B genotypes in chronic hepatitis B and lamivudine therapy. *Intervirology*, **46**, 373-376.