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HBV MUTATIONS ASSOCIATED WITH LAMIVUDINE THERAPY

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ABSTRACT

Lamivudine (LMV) is still the most commonly used nucleoside analogue in majority of the world. Its administration rapidly leads to resistance associated with mutations in HBV polymerase.

THE AIM of the study was to assess the prevalence, nature and the time of LMV resistant variants appearence during a long term therapy.

PATIENTS AND METHODS. Study was carried out among 175 chronic hepatitis B patients treated with LMV. HBsAg, HBeAg as well as anti-HBe antibodies were detected by enzyme immunosorbent assay and HBV-DNA quantification was performed by RT-PCR. Mutations in HBV polymerase gen were detected by PCR using specific primers and direct sequencing. Liver biopsies were performed in 138 patients to evaluate grading and staging of chronic hepatitis by Scheuer's classification.

RESULTS. Mean pre-treatment viral load was comparable among HBeAg-positive and negative patients (4.24×10^8 vs. 1.26×10^8 IU/ml). Mutations in HBV polymerase gen were detected in 96 patients. After 5 years of LMV therapy the prevalence of mutations was 51.9% in HBeAg-positive and 56.1% in HBeAg-negative. The most common mutations were observed at position 180, followed by 204, 202, and 169 of HBV polymerase gen. After average treatment period of 25 months in HBeAg-positive and 35 months in HBeAg-negative additional mutation 204 was observed in 81% and 77% respectively.

CONCLUSIONS. Large majority of patients develop point mutations at positions 180 and 204 of HBV polymerase gene after 2 years of treatment with LMV. These mutations limit the efficacy of LMV but also yield cross-resistance with entecavir.

Key words*: HBV, Lamivudine therapy, HBV polymerase gene mutation*

INTRODUCTION

Approximately two billion people in the world are HBV-infected and nearly 350 million are diagnosed with chronic hepatitis B (1). The primary goal of antiviral treatment in these patients is to achieve sustained immune control with possible HBsAg seroconversion, and if it is not possible acceptable according to current guidelines is to achieve viral control demonstrated trough stable suppression of viral replication. Inhibition of HBV replication seems sufficient to decrease likelihood of progression to liver cirrhosis and hepatocellular carcinoma (HCC). Therefore application of any antiviral therapy decreasing HBV replication is beneficial for patient. Currently, either pegylated interferon alpha (PegIFN-alfa) or nucleos(t)ide analogues (AN) are standard options in the treatment of chronic hepatitis B. Therapy with PegIFN-alfa provides the best opportunity to achieve sustained viral suppression possible even after treatment termination, but this therapy might be associated with adverse events and the optimal end-point – HBsAg clearance occurs in up to 15% patients only, usually many years after treatment termination (2). AN effectively inhibit the replication of HBV and lamivudine (LMV) is the oldest compound in this group. However, relatively low antiviral activity and low genetic barrier result in rapid selection of LMV resistant HBV variants (3).

The most common natural mutation found in the HBV genome is related to changes in the preC/C re-

gion. PreC/C variants are unable to synthesize HBeAg. The presence of this mutation is especially common in HBV genotype D and B (4). Other types of mutation are variants in the region of preS/S HBV genome. These mutants present altered HBsAg structure, which may be clinically significant causing infection regardless of active and passive prophylaxis against HBV infections (5). Additionally, these mutations cause reduction of HBeAg expression on the cell surface, and weakening the effectiveness of immune responses. Mutations in the X region of HBV genome, usually affect HBeAg and/or HBsAg synthesis (6). These mutations modify the structure of HBx protein which, than exhibit pro-proliferative and anti-apoptotic properties. Such modifications increase the likelihood of HCC development (7).

The resistance to AN is an effect of mutations in different domains of the HBV polymerase gene. Among AN used in the therapy of chronic hepatitis B, the most potent are entecavir (ETV) and tenofovir (TDF), in contrast to less potent - LMV, telbivudine (LDT) and adefovir (ADV). The use of anti-HBV agents with lower antiviral activity contributes to the faster selection of HBV variants. Suboptimal HBV suppression inhibit wild-type HBV replication, but allows arising of primary substitution and further replication of drug resistant viruses. LMV therapy which is still the most common AN administered for HBV chronic infection, often contributes to the development of such mutations usually in the form of changes in position 204 of the HBV polymerase gen. Conversion of amino acids in this position is sufficient to produce a partial resistance to LMV. Presence of mutations in positions 180, 173 and 80 of polymerase gene is less often. Mutations in position 204 and / or 180 and the appearance of additional mutations at positions 202, 169, 184, 250 causes the resistance to both LMV and ETV (8). Although the resistant variants are characterized by a lower HBV replication as compared to a wild-type virus, the systematic increase in the number of mutants leads to a total replecement of wild-type resulting with possible more aggressive course of the disease and higher risk of HCC development (9, 10).

Aim of the study was to evaluate frequency of and the type of mutations in HBV polymerase gen during LMV treatment. Moreover, the time sequence of these mutations and their incidence in respect to HBeAg status were analyzed.

MATERIALS AND METHODS

The study was conducted in 175 patients (48 females and 127 males, mean age 44 years, range: 18-77 years) diagnosed with chronic hepatitis B (52 HBeAg (+) and 123 HBeAg (-). All patients were treated with LMV until the development of LMV-resistance. Liver biopsy was performed prior to AN therapy in 138 patients (61 HBeAg-positive and 77 HBeAg-negative) for hepatitis activity and liver fibrosis evaluation according to the Scheuer classification[1] (11)

Serum HBsAg, HBeAg, anti-HBe were detected by enzyme immunosorbent (EIA, Abbott, Germany).

DNA from blood plasma specimens was isolated using automatic magnetic extraction method. In brief, 500 ml of blood plasma was incubated with Proteinase K for 2 hours in 56^0C for protein digestion. Nucleic acids from deproteinated lysate were then extracted automatically with magnetic beads on EasyMag machine (Biomerieux, France) according to the producer's protocol. The resulting 25 ml of DNA solutions in Buffer 3 were obtained.

Standard polymerase chain reaction was used to amplify a HBV DNA polymerase gene. The primers applied were described by Allen i in. (12) and were as follows: forward primer 12F 5'-aga ctc gtg gtg gac ttc tct-3', and reverse primer 5RC 5'- caa aag aaa att ggt aac agc ggt a-3'. In the case of low amplification output, the nested PCR reaction was performed with the primers: forward primer 377 5'-gga tgt gtc tgc ggc gtt t-3', and reverse primer 840 5'-acc cca tct ttt tgt ttt gtt agg-3'. Direct sequencing of PCR products was performed using BigDye Terminator Sequencing Kit (v.3.1) and ABI PRISM 3500 Sequencer (Applied Biosystems, Foster City, CA). Obtained sequences were compared with those stored in an NCBI GenBank database using an NCBI application BLAST (12).

Among the patients with HBV genotype was not evaluated because in Poland with 86.6% of patients present genotype A (13).

Informed consent was obtained from each patient and the Bioethics Committee at the Medical University of Białystok approved the study protocol.

Statistical analysis was performed on Statistica software, version 10.0, using the U Mann – Whitney and Chi – square tests; p value below 0.05 was considered statistically significant.

RESULTS

All patients were performed every 3 months ALT assessment and evaluation of HBV-DNA levels at every 6 – 12 months. **Among** patient in an increase of ALT activity was determined HBV viral load and mutation. The largest increase in ALT levels during the occurrence of a mutation does not exceed three times the norm. No patients in the lamivudine treatment ALT flares

1 Morphological evaluation of liver biopsy specimens was carried-out by Prof. A Panasiuk in the Department of Infectious Diseases and Hepatology, Medical University of Bialystok

Table I. Association between baseline viral load and frequency of mutations occurring in consecutive years of LMV therapy.

	HBeAg-positive, n=52		HBeAg-negative, n=123		p
year of mutation development	mutations prevalence n (%)	mean (±SE) baseline viral load of HBV (IU/ml)	mutations prevalence n(%)	mean (±SE) baseline viral load of HBV (IU/ml)	HBeAg-positive versus negative
0	-	424±232 x 10^6	-	126±66 x 10^6	p=0.12
1	6 (11.5)	7.6 ±6 x 10^6	11 (8.9)	181 ±140x 10^6	p=0.82
2	10 (19.2)	950 ±498 x 10^6	20 (16.)	200 ±81 x 10^6	p=0.18
3	5 (9.6)	62 ±37 x 10^6	11 (8.9)	31 ±14 x 10^6	p=0.36
4	3 (5.8)	4.3 ±2 x 10^6	10 (8.1)	320 ±320 x 10^6	p=0.93
5	3 (5.8)	6.4 ±6 x 10^6	17 (13.8)	20 ±14 x 10^6	p=0.53

were observed. Mean (±SE) baseline ALT levels was 142±62 IU/ml and after and at the time of occurrence of the virus 52±24 IU/ml.

Mean HBV-DNA concentration in HBeAg-positive patients before treatment (4.24 ± 2.32 x10^8 IU/mL) was comparable to HBV viral load in HBeAg-negative (1.26 ± 0.66 x10^8 IU/mL). There was no association between initial viral load and the frequency or timing of HBV polymerase mutations (tab.I). Mean HBV-DNA concentration during the mutation in patients with HBeAg (+) was 1.21 ± 0.42 x108 IU/mL and in patients with HBeAg (-) 2.38 ± 1.36 x107 IU/mL.

HBV polymerase mutations were detected in 96 patients (29 females and 67 males, 27 patients were HBeAg-positive and 69 HBeAg-negative). There were no statically significant differences in the incidence of mutation between HBeAg-positive and HBeAg-negative (51.9% vs. 56.1%). In both HBeAg-positive and negative patients the majority of mutations developed within initial two years of treatment. Lower frequency of new mutations was observed in following years of therapy. (fig. 1).

Among patients confirmed as infected with resistant HBV variants, liver biopsy was performed in 24 HBeAg-positive and in 53 HBeAg-negative patients. There were no significant association between HBV viral load and the severity of inflammation and fibrosis (tab.II). The selection of HBV mutants was faster among patients with lower fibrosis score and less severe inflam-

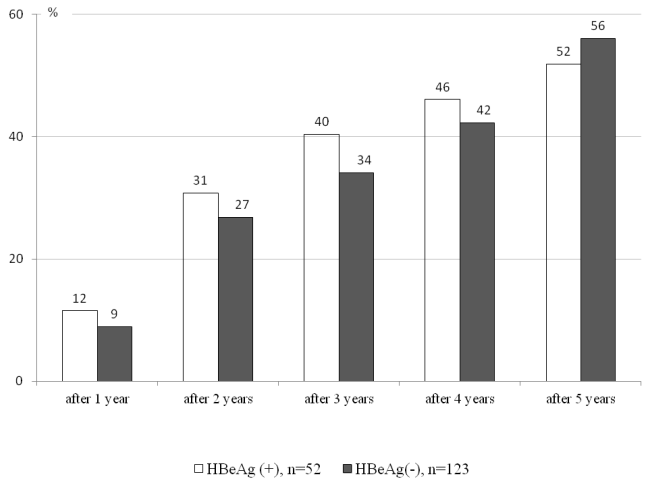

Figure 1. Cummulative prevalence of HBV mutations in consecutive years of lamivudine treatment

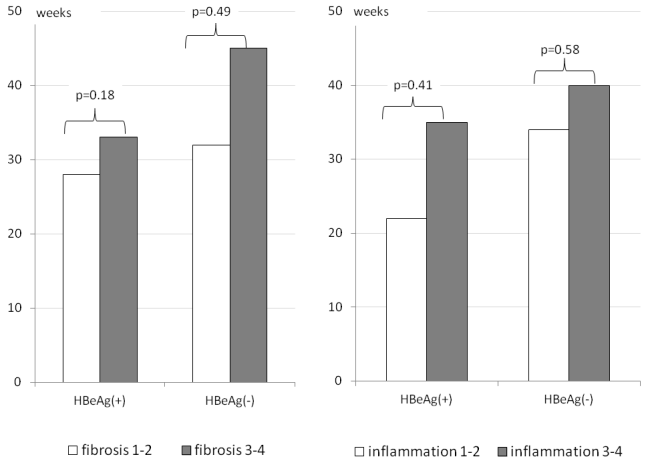

Figure 2. The time of mutations occurence in relation to the severity of inflammation and fibrosis evaluated according to the Scheuer's classification

Table II. Comparison of baseline viral load in respect to degree of inflammation and fibrosis in patients who developed HBV mutations.

		viral load (IU/ml)			
	degree of histological changes	n	HBeAg-positive mean (±SE)	n	HBeAg-negative mean (±SE)	p
inflammation	1-2	18	395 ±282 x 10^6	41	138 ±103 x 10^6	p=0.25.
	3-4	6	504 ±431 x 10^6	12	108 ±62 x 10^6	p=0.50
	p		p=0.64		p=0.41	
fibrosis	1-2	11	5.6 ±2.8 x 10^6	31	109 ±78 x 10^6	p=0.63
	3-4	13	746 ±395 x 10^6	22	176 ±105 x 10^6	p=0.34
	p		p=0.40		p=0.40	

Table III. Combinations of HBV mutations and their frequency (%) among HBeAg-positive versus HBeAg -negative patients.

type of mutation	Mutation at position of HBV polymerase gen							HBeAg-positive n(%)	HBeAg-negative n(%)	Chi – square p
	204	202	184	181	180	173	169			
A	+							1 (4)	3 (4)	p=0.89
B	+				+			13 (48)	37 (55)	p=0.63
C	+		+						1 (1)	
D	+				+		+	5 (18)	8 (12)	p=0.37
E	+	+			+		+		1 (1)	
F	+	+			+			2 (7)		
G					+		+	4 (15)	8 (12)	p=0.67
H		+		+				1 (4)		
I	+				+	+		1 (4)	2 (3)	p=0.84
J		+							2 (3)	
K					+	+			2 (3)	
L	+		+		+		+		1 (1)	
M		+			+		+		3 (4)	
N	+				+	+	+		1 (1)	

mation, although the differences were not statistically significant (fig.2).

Depending on the location of nucleoside analogue substitution, 14 types of mutations leading to treatment modification were identified. The most frequent were mutations at positions 204, 202, 180 and 169. The time at which the mutation occurred during LMV therapy was related to HBeAg status (tab.III). The earliest point mutation occurred at position 204, after mean 25 ±4.1 months of treatment in HBeAg-positive patients (81%) and 35 ±3.6 months in HBeAg-negative patients (71%). Mutations at position 180 usually were identified 3 months later in HBeAg-positive and 1 month later in HBeAg-negative (tab.IV).

In patients with HbeAg-positive, (n = 52), after 5 years of treatment occurred in 15% (n=10) elimination of HBeAg as well as in 8% (n=4) of seroconversion to anti-HBe.

DISCUSSION

The primary goal of anti-HBV therapy is viral eradication, which is difficult to reach, therefore suppression of HBV replication and undetectable HBV DNA serve as an acceptable goal reducing the progression of the disease. During the treatment with lamivudine early selection of drug resistant mutants is observed in numerous patients. In our study, after 3 years of therapy with LMV, polymerase HBV mutations were found in 31% HBeAg-positive and 26% HBeAg-negative patients.

After 5 years it was demonstrated in 52 and 56% patients respectively. Baseline viral load was comparable in both HBeAg-positive and HBeAg-negative patients, which probably explains a comparable incidence of mutations in the studied population. Our findings indicate a lower incidence of HBV mutations than demonstrated by other authors showing emergence of up to 80% LAM-resistant variants after five years of LMV treatment (14). The occurrence of mutations depends on the HBV genotype, viral load, external factors, comorbidities, additional medication, as well as cultural behavior. It is difficult to determine which of these factors are the most important. *Akuta* et al (15) showed a greater impact of geographic residence on frequency of mutations than HBV genotypes (A, B, C). Furthermore, there was considerable variation in the frequency of mutation depending on the presence of HBeAg and anti-HBe in patients infected with the same genotype (C) (15). In our study faster selection of HBV polymerase mutants was observed in HBeAg-positive patients.

HBV mutations are present at a frequency of approximately 1 per 10^5 copied polymerase genes with (mean about 10^{11} virus replication copies/day) (14). Treatment with less potent AN enhances selection of mutants, particularly in patients with higher viral load

Table IV. Comparison of time to particular mutations development (p) and its frequency among HBeAg-positive versus HBeAg-negative patients on LMV treatment.

Mutation at position	HBeAg-positive		HBeAg-negative		
	time to mutations development mean ±SE (months)	prevalence of mutations (%)	time to mutations development mean ±SE (months)	prevalence of mutations (%)	p
204	25 ±4.1	22/27 (81)	35 ±3.6	53/69 (77)	p=0.08
202	45 ±16.7	3/27 (11)	48 ±12.7	7/69 (10)	p=0.21
180	28 ±3.9	25/27 (93)	36 ±3.3	63/69 (91)	p=1.0
169	30 ±4.7	9/27 (33)	44 ±5.3	24/69 (35)	p=0.28

(16). However, in our study there was no correlation between mutations frequency and the baseline HBV-DNA level.

Preiss et al, (17) showed no association between HBV viral load and fibrosis. We also did not demonstrate association between viral load and inflammation and fibrosis score, but a trend of faster resistant variants selection in patients with less advanced fibrosis and inflammation was observed.

We demonstrated the earliest mutations after about 2 years of treatment in HBeAg-positice and 3 years in HBeAg-negative and it was usually observed at position 204 by substitution of methionine, valine or isoleucine. Observations of other authors confirm that such mutations occur early in the treatment with AN (18, 19). Mutation occurring at position 204 is sufficient to reduce the genetic barrier to LMV. This mutation decreases LMV activity, although the drug still has some antiviral activity (20). However, this mutation initiates the cross-resistance to ETV. This is especially disadvantageous because the next occurring point mutation during LMV therapy causes the total loss of antiviral activity. Cross-resistance to ETV limit the possible further options of anti-HBV therapy. Therefore *Hige* et al. (20) recommend strict monitoring of patients treated with LMV who selected mutation at position 204, to avoid missing of mutation at position 180. Our findings demonstrated the appearance of a mutation at position 180 in 93% of HBeAg-positive patients after 28 months of therapy (4 months after mutation at position 204), and in 91% HBeAg-negative patients treated for 36 months (1 month after mutations at position 204). According to this observation it seems advantageous to replace LMV immediately after identification of mutation at position 204. Another important observation is that after just a few months of mutations appearance at position 204 also mutations at position 169 and 204 may arrise, resulting the loss of antiviral efficacy of LMV and enhanced risk of cross-resistance to other AN. Selection of LMV for long-term treatment in patients with chronic hepatitis B seem to be hazardous. Therefore LMV is no longer recommended as a first-line drug for HBV treatment if other AN are available. Rapid emergence of mutations in the course of such a therapy also requires administration of higher dose of ETV (1.0 mg) instead of 0.5 mg which is sufficient for patients infected with wild-type HBV. However even such an action will not prevent the occurrence of further mutations, which can be found in 36% patients after three years of treatment with ETV (21). In contrast LMV treatment does not cause cross-resistance with ADV and TDF which are nucleotide analogues. Switching to ADV in LMV-resistant patients is not the best choice because ADV is not potent enough. Combined treatment with LMV and ADV may provide higher efficiacy and can increase genetic barrier compared to LMV alone, but unfortunately ADV can cause nephrotoxicity (22). Development of mutations at position 194 during treatment with TDF has been shown in HIV infected patients only. No such a mutations have been described in HBV monoinfection (23). TDF seems to be the best alternative for treatment of patients with LMV resistance.

It is interesting that the mutations at position 202, 184 and 173 will probably not cause resistance to LMV. However, in our study revealed a mutation as unique. It is possible that such patients, there is not an amino acid substitution mutations, but the degeneracy of the genetic code (24).

Mutations in the HBV polymerase gene can also lead to resistance to currently used anti-HBV vaccines (antiviral drug-associated potential vaccine escape mutant, ADAP-VEM). The most common mutation is an effect of the long-term LMV monotherapy (5, 24). VEM emergence of mutants and their spread can reduce success of effective anti-HBV prophylaxis in the world. Furthermore, a mutation at position 204 of polymerase gene is common in patients with HCC. (7)

CONCLUSION

Lamivudine treatment affects the selection of HBV mutants in positions 180 and 204 of the HBV polymerase gene in approximately 80% of HBeAg-positive patients after 2 years and HBeAg-negative patients after 3 years. These mutations reduce antiviral efficacy and create the risk of cross-resistance to entecavir. Currently there are no reasonable background for long-term lamivudine monotherapy in patients with chronic HBV infection.

REFERENCES

1. Lavanchy D. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. J Viral Hepat 2004; 2: 97-107.
2. Marcellin P, Bonino F, Lau GK i in. Peginterferon alfa-2a in HBeAg-negative Chronic Hepatitis B Study Group. Sustained response of hepatitis B e antigen-negative patients 3 years after treatment with peginterferon alpha-2a. Gastroenterology. 2009; 7: 2169-2179.
3. EASL Clinical Practice Guidelines: Management of chronic hepatitis B virus infection. J Hepatol. 2012; 1: 167-85.
4. Malik A, Singhal DK, Albanyan A i in. Hepatitis B virus gene mutations in liver diseases: a report from New Delhi. PLoS One. 2012; 6: e39028.
5. Locarnini SA, Yuen L. Molecular genesis of drug-resistant and vaccine-escape HBV mutants. Antivir Ther. 2010; 15: 451-61.

6. Rybicka M, Stalke P, Charmuszko U i in. The influence of hepatitis B virus polymorphism on the progression of chronic liver disease. Postepy Hig Med Dosw (online), 2011; 65: 244-254.
7. Li D, Cheng H, Gong W i in. Detection of primary YMDD mutations in HBV-related hepatocellular carcinoma using hybridization-fluorescence polarization. J Virol Methods. 2013; 2: 259-63.
8. Reijnders JG, Deterding K, Petersen J i in. Antiviral effect of entecavir in chronic hepatitis B: influence of prior exposure to nucleos(t)ide analogues. J Hepatol. 2010; 4: 493-500.
9. Lai MW, Yeh CT. The oncogenic potential of hepatitis B virus rtA181T/ surface truncation mutant. Antivir Ther 2008; 7: 875-9.
10. Warner N, Locarnini S. The antiviral drug selected hepatitis B virus rtA181T/sW172* mutant has a dominant negative secretion defect and alters the typical profile of viral rebound. Hepatology. 2008; 1: 88-98.
11. Scheuer PJ. Classification of chronic viral hepatitis: A need for reassessment. J Hepatol, 1991, 13, 372-74.
12. Allen MI, Gauthier J, DesLauriers M i in. Two sensitive PCR-based methods for detection of hepatitis B virus variants associated with reduced susceptibility to lamivudine. J Clin Microbiol 1999; 37: 3338-47.
13. Ślusarczyk J, Białkowska J, Bucholc B i in. HBV genotypes among patients with chronic hepatitis B in the area of central Poland. Przegl Epidemiol. 2006; 3: 555-61.
14. Zoulim F, Locarnini S. Hepatitis B Virus Resistance to Nucleos(t)ide Analogues. Gastroenterology. 2009; 5: 1593-608.
15. Akuta N, Suzuki F, Kobayashi M i in. The influence of hepatitis B virus genotype on the development of lamivudine resistance during long-term treatment. J Hepatol 2003; 3: 315-21.
16. Chun J, Kim W, Kim BG i in. High viremia, prolonged Lamivudine therapy and recurrent hepatocellular carcinoma predict posttransplant hepatitis B recurrence. Am J Transplant. 2010; 7: 1649-59.
17. Preiss S, Littlejohn M, Angus P i in. Defective hepatitis B virus DNA is not associated with disease status but is reduced by polymerase mutations associated with drug resistance. Hepatology. 2008; 3: 741-9.
18. Mello FC, Lago BV, Lewis-Ximenez LL i in. Detection of mixed populations of wild-type and YMDD hepatitis B variants by pyrosequencing in acutely and chronically infected patients. BMC Microbiol. 2012; 1: 96.
19. Murata M, Furusyo N, Unno M i in. Long-term effects of lamivudine treatment in Japanese chronic hepatitis B patients. World J Gastroenterol. 2011; 24: 2945-52.
20. Murata M, Furusyo N, Unno M i in. Sensitive Assay for Quantification of Hepatitis B Virus Mutants by Use of a Minor Groove Binder Probe and Peptide Nucleic Acids. J Clin Microbiol. 2010; 12: 4487-94.
21. Wong VW, Wong GL, Tse CH i in. Antiviral drug resistance testing in patients with chronic hepatitis B. Dig Dis Sci. 2012; 1: 221-31.
22. Wang LC, Chen EQ, Cao J i in. De novo combination of lamivudine and adefovir versus entecavir monotherapy for the treatment of naïve HBeAg-negative chronic hepatitis B patients. Hepatol Int. 2011; 2: 671-6.
23. Amini-Bavil-Olyaee S, Herbers U, Sheldon J i in. The rtA194T polymerase mutation impacts viral replication and susceptibility to tenofovir in hepatitis B e antigen-positive and hepatitis B e antigen-negative hepatitis B virus strains. Hepatology. 2009; 4: 1158-65.
24. Clements CJ, Coghlan B, Creati M i in. Global control of hepatitis B virus: does treatment-induced antigenic change affect immunization? Bull World Health Organ. 2010; 1: 66-73.

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