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## Mutations outside the YMDD motif in the P protein can also cause DHBV resistant to Lamivudine

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Received: 2004-12-22 Accepted: 2005-01-12

### Abstract

**AIM:** To observe the Lamivudine resistance character of a DHBV strain *in vitro* and *in vivo*, and to analyze if the Lamivudine resistance character is caused by gene mutation or by abnormality of the Lamivudine metabolism.

**METHODS:** Congenitally DHBV-negative Guangdong brown ducks and duck embryo liver cells were respectively taken as animal and cell model. The Lamivudine-susceptible DHBV and Lamivudine-resistant DHBV (LRDHBV) were infected and Lamivudine was administrated according to the divided groups. The changes of DHBV quantity in the animal and cell model were tested. Three Lamivudine-resistant and two Lamivudine-susceptible DHBV complete genomes were successfully amplified, sequenced and then submitted to GenBank. All the DHBV complete sequences in the GenBank at present were taken to align with the three LRDHBV to analyze the mutational points related to the Lamivudine-resistant mutation.

**RESULTS:** Both the animal and cell model showed that the large and the small dosage Lamivudine have no significant inhibitory effect on the LRDHBV. Five sequences of DHBV complete genomes were successfully cloned. The GenBank accession numbers of the three sequences of LRDHBV are AY521226, AY521227, and AY433937. The two strains of Lamivudine-susceptible DHBV are AY392760 and AY536371. The correlated mutational points are KorR86Q and AorE591T in the P protein.

**CONCLUSION:** The Lamivudine resistance character of this DHBV strain is caused by genome mutation; the related mutational points are KorR86Q and AorE591T and have no relations with the YMDD motif mutation.

He JY, Zhu YT, Yang RY, Feng LL, Guo XB, Zhang FX, Chen HS. Mutations outside the YMDD motif in the P protein can also cause DHBV resistant to Lamivudine. *World J Gastroenterol* 2005; 11(27): 4261-4267  
<http://www.wjgnet.com/1007-9327/11/4261.asp>

### INTRODUCTION

More than 400 million people worldwide are chronically infected by HBV<sup>[1]</sup>. HBV infections, the 10<sup>th</sup> leading cause of death worldwide, result in 500 000 to 1.2 million deaths per year caused by chronic hepatitis, cirrhosis, and hepatocellular carcinoma<sup>[2]</sup>. Lamivudine had been considered to be a great progress in the area of anti-virus drug. It can make the blood HBV DNA of more than 90% of patients changed to negative when administrated for 1 year by 100 mg/d<sup>[3,4]</sup>. But it can also lead to YMDD mutant and cause Lamivudine resistance. YMDD mutations developed in 12.1%, 49.7% and 70.5% of the patients respectively at year 1, 2, and 3<sup>[5]</sup>. So the searching of Lamivudine-resistant problem is very important. It is believed consistently that the cause of Lamivudine resistance is HBV gene mutation. The main mutant motif is the YMDD in the P protein of HBV<sup>[6-8]</sup>. But there are no animal and cell models of Lamivudine-resistant HBV which can be conveniently used, which cause very little progress that has been the problem of Lamivudine-resistant HBV.

After the finding of the DHBV by Summers *et al.*<sup>[9,10]</sup>, the process of replication and genome sequence of DHBV had been discovered rapidly. These facilitated the understanding of the related aspects of HBV. Because of the similarity of the DHBV and HBV in the replicating manner and pathogenesis, the duck hepatitis B model had been used as animal model of anti-HBV drug screening and HBV pathogenesis searching. When we persistently used the congenitally infected duck as animal model to screen anti-HBV drug, one congenitally infected duck had been found to have the character of Lamivudine resistance. We named it Lamivudine-resistant duck (LRD). The LRD-infected virus was named as Lamivudine-resistant DHBV (LRDHBV). We proved the character of Lamivudine resistance of LRDHBV in embryo duck liver cell model and postnatally infected duck model. Then the complete genome of LRDHBV and Lamivudine-susceptible DHBV were cloned and sequenced. The LRDHBV-related mutant points were analyzed.

### MATERIALS AND METHODS

#### Animals

One-day-old Guangdong brown ducks were obtained from

a duck factory on the Shi-Jing town of Guangzhou city. Congenitally DHBV-negative ducks were chosen by dot blot assay to be experimental animals. The ducks were fed a standard duck diet and water according to the guidelines approved by the China Association of Laboratory Animal Care.

### Primary hepatocytes

Guangdong brown duck eggs were purchased from a commercial supplier. The eggs were incubated for 20 d in the environment of 37.6 °C and 50-60% humidity. The eggs were opened and were proved to be congenitally DHBV negative using PCR method. The duck embryo liver was plucked out and digested with 0.2% collagenase (type II, Gibco) in 3 mL of serum-free William E medium (Sigma) supplemented with 2 mol/L L-glutamine, 15 mol/L HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid [pH 7.2]), 100 U of penicillin per mL, 100 µg of streptomycin, 10<sup>-5</sup> mol/L hydrocortisone, 1 µg of insulin per mL, and 1.5% dimethyl sulfoxide (all from Sigma, Germany) for 30 min at 37 °C. After washing twice with 5 mL of medium, the cells from one liver were suspended in 20-24 mL of medium, seeded onto 10-12 tissue culture dishes (60-mm diameter; 2 mL per dish, the density is 5×10<sup>5</sup> cells per well), and cultivated at 37 °C and 50 mL/L CO<sub>2</sub>. The medium was first changed 30 min after seeding, and further medium changes were done daily. Cellular toxicity was tested daily by light microscope examination and MTT assay of Mosmann<sup>[11]</sup> as described by Alley *et al.*<sup>[12]</sup>.

### Serum and DHBV infection

Lamivudine-susceptible DHBV serum was from congenitally infected ducks that had been proved to be DHBV positive by dot blot method. LRDHBV serum was from LRD at the time point of 10-d Lamivudine administration. In the animal model, 0.2 mL of Lamivudine-susceptible DHBV or LRDHBV was injected to the shank vein of every duck that were 2 d old. The primary hepatocytes were infected between 24 and 48 h after seeding by adding 50 µL Lamivudine-susceptible DHBV or LRDHBV per dish directly to the medium. After 3-h incubation at 37 °C, the inoculum was removed, and 2 mL of fresh medium was added.

### Groups and dot blot assay

Twelve ducks infected with Lamivudine-susceptible DHBV and 18 ducks infected with LRDHBV were chosen and randomly divided into five groups. Group A were infected with Lamivudine-susceptible DHBV; group B were infected with LRDHBV; group C were infected with LRDHBV and fed with large dosage Lamivudine (100 mg/kg, b.i.d.); group D were infected with LRDHBV and fed with small dosage Lamivudine (20 mg/kg, b.i.d.); group E were infected with Lamivudine-susceptible DHBV and fed with small dosage Lamivudine (20 mg/kg, b.i.d.). The serum samples were collected at time points of 1 d before drug administration (D0), 5<sup>th</sup> d of drug administration (D5), 10<sup>th</sup> d of drug administration (D10) and 3<sup>rd</sup> d post stopping of drug administration (P3). The primary hepatocytes were also divided into five groups similar to the animal experiment. The hepatocytes were also

treated with two concentrations of Lamivudine (100 and 1 000 µmol/L) starting from 3 d after virus inoculation. The hepatocytes were collected and DNA was extracted at the time points of 3, 6, 9, and 12 d after virus inoculation respectively. Forty microliters of serum or twenty-five microliters of hepatocytes DHBVDNA extraction dissolution was spotted, and DHBVDNA was detected with a full-length DHBV genomic DNA probe labeled with [α-<sup>32</sup>P] dCTP as described previously<sup>[13]</sup>. A quantity analysis was carried out using enzyme photometer. The limit of detection of serum viral DNA by this assay is 100 pg/mL.

### Amplification of the DHBV complete genome

Three samples of LRDHBV DNA were extracted from the serum of LRD which was the same as the LRDHBV serum used to infect the congenitally DHBV-negative ducks and duck embryo liver cells. Two samples of Lamivudine-susceptible DHBVDNA were extracted from serum of two ducks that were susceptible to Lamivudine. One pair of primers was used to test if there was DHBVDNA in the extraction solutions. The primer sequences<sup>[14]</sup> are: P1 -5'-GCG CTT TCC AAG ATA CTG GAG CCC AA-3' (sense) and P2-5'-CTG GAT GGG CCG TCA GCA GGA TTA TA-3' (anti-sense). The PCR condition is: 94 °C 30 s, 55 °C 30 s, 72 °C 1 min, 30 cycles. One pair of primers was designed to amplify the complete genome of DHBV. The primer sequences are Q1-5'-ACC CCT CTC TCG AAA GCA ATA-3' (sense) and Q2-5'-GTG TAT GTA AGA GCC GTC CAA TC-3' (anti-sense). The LAPCR condition is: 94 °C 30 s; 52 °C 1 min; 72 °C 3.5 min, after 30 cycles, another 72 °C 5 min was supplemented. One percent agarose gel was used to analyze the LAPCR product.

### Cloning and sequencing of DHBV complete genome

The 3.0-kb PCR product was inserted into the pMD18-T vector using the TAKARA Ligation kit. JM109 competent cells were made with the method of CaCl<sub>2</sub> which was found by Mandel *et al.*<sup>[15,16]</sup>. Three LRDHBV DNA recombinants and two Lamivudine-susceptible DHBV DNA recombinants were transformed into the JM109 competent cells. The five kinds of *E. coli* containing the five kinds of recombinants were conserved and sent to TAKARA to do bidirectional sequencing. The sequencing results were analyzed with related software.

### Statistical analysis

Results were expressed as mean±SD. Statistical comparisons between the groups were done using Nemenyi method. *P* values less than 0.05 were considered statistically significant.

## RESULTS

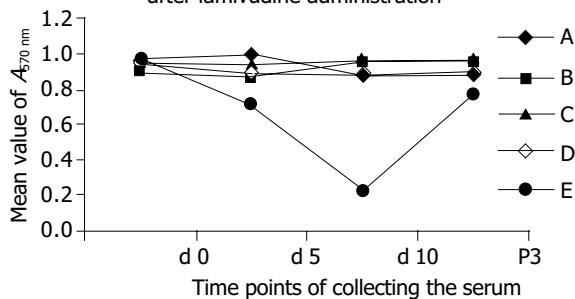
### Lamivudine could not inhibit LRDHBV in duck animal model

We first examined if the LRDHBV could be inhibited in body of the other duck. We injected the LRDHBV-positive serum to the congenitally DHBV-negative ducks. These ducks were infected with LRDHBV and treated with Lamivudine. Lamivudine were used in large and small dosage groups. The feeding was maintained for 10 d. Serum samples were collected at four time points from 1 d before administration to 3<sup>rd</sup> d post stopping of administration. The DHBV in the

serum samples were quantified by dot blot assay. After 10 d of Lamivudine administration, the quantity of Lamivudine-susceptive DHBV in the duck blood markedly got down. But 3 d after stopping of administration, the quantity of susceptible DHBV rose up to former level. No significant effect was observed when the large and small dosage of Lamivudine was fed to the LRDHBV-infected ducks (Table 1 and Figure 1). So it can be concluded that the LRDHBV can also show the character of Lamivudine resistance in the ducks postnatally infected with the LRDHBV.

Table 1 and Figure 1 showed that no significant effect was observed when the large and small dosage of Lamivudine was fed to the LRDHBV-infected ducks. While the 10-d Lamivudine administration could make the Lamivudine-susceptive DHBV markedly get down in the duck model.

The dynamic change of the DHBV quantity in the blood before and after lamivudine administration



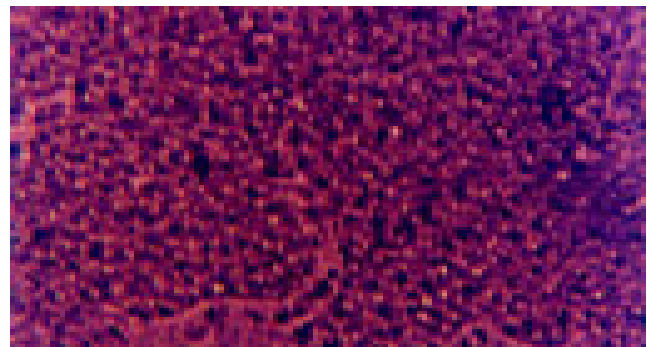
**Figure 1** A: Lamivudine-susceptive DHBV control group; B: LRDHBV control group; C: LRDHBV-infected group administrated by large dose Lamivudine group; D: LRDHBV-infected group administrated by small dose Lamivudine group; E: Lamivudine-susceptive DHBV-infected group administrated by small dose Lamivudine group.

### Lamivudine could not inhibit LRDHBV in the duck embryo liver cells

We inoculated the LRDHBV to the primary duck embryo liver cells and administrated Lamivudine according to the divided groups to observe if the LRDHBV have the Lamivudine-resistant character in the liver cells. Lamivudine administration was started from 3<sup>rd</sup> d post DHBV inoculation and maintained for 9 d. The liver cells were collected at four time points from 3 to 12 d post DHBV inoculation. The total DHBV in the cells was extracted immediately after cell collection. The DHBV quantity was tested by dot blot assay. The results showed that the Lamivudine-susceptive DHBV got down markedly at the time points of 6<sup>th</sup>, 9<sup>th</sup>, and 12<sup>th</sup> d post DHBV inoculation, while both the small and large dosage Lamivudine have no significant effect on the LRDHBV in the duck embryo liver cells (Figure 3). So

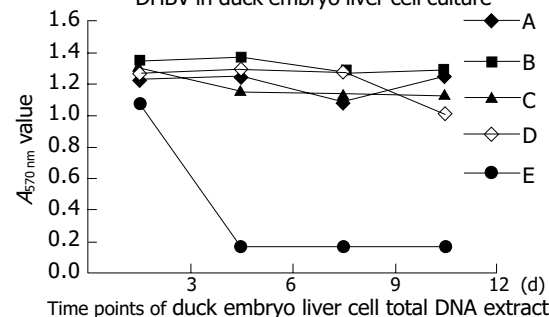
the LRDHBV showed Lamivudine-resistant character in the duck embryo liver cells too. We also tested the cytotoxicity of Lamivudine to the embryo liver cells. However, no significant cytotoxicity was detected in liver cells cultured with different concentrations of Lamivudine for 9 d by daily microscope examination (Figure 2) and by MTT method.

Lamivudine-susceptive DHBV was inhibited markedly at the time points of 6<sup>th</sup>, 9<sup>th</sup>, and 12<sup>th</sup> d post DHBV inoculation, while both the small and large dosage Lamivudine have no significant effect on the LRDHBV in the duck embryo liver cells.



**Figure 2** Duck embryo liver cells (5 d after DHBV inoculation, optic microscope ×40). They have no significant effect of Duck embryo liver cells infected with DHBV or LRDHBV.

Effects of lamivudine to the lamivudine susceptible and resistant DHBV in duck embryo liver cell culture



**Figure 3** A: Lamivudine-susceptive DHBV control; B: LRDHBV control; C: LRDHBV-infected cells administrated by large dosage Lamivudine; D: LRDHBV-infected cells administrated by small dosage Lamivudine; E: Lamivudine-susceptive DHBV-infected cells administrated by small dosage Lamivudine.

### DHBV complete genomes were successfully amplified and cloned

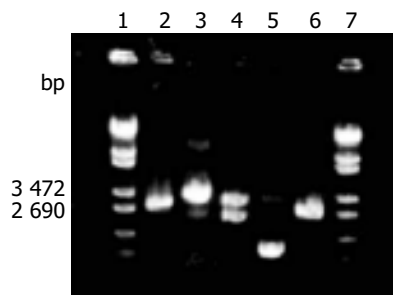
We designed two pairs of primer to amplify the DHBV complete genome. One was designed according to the method

**Table 1** DHBV quantity in the duck blood of various groups expressed by  $A_{570\text{ nm}}$  value (mean±SD)

Groups	D0	D5	D10	P3
Lamivudine-susceptive DHBV control	0.96±0.31	1.00±0.06	0.87±0.05	0.87±0.02
LRDHBV control	0.91±0.03	0.86±0.01	0.95±0.01	0.95±0.04
LRDHBV-infected group administrated by large dose Lamivudine	0.96±0.03	0.94±0.02	0.97±0.05	0.97±0.03
LRDHBV-infected group administrated by small dose Lamivudine	0.96±0.02	0.88±0.02	0.87±0.01	0.90±0.05
Lamivudine-susceptive DHBV-infected group administrated by small dose Lamivudine	0.96±0.05	0.73±0.05 <sup>b</sup>	0.22±0.03 <sup>b</sup>	0.69±0.02 <sup>b</sup>

<sup>b</sup> $P<0.01$  group A vs group E.

of Gunther to amplify the HBV complete genome<sup>[17]</sup>. It covered the DR1 of DHBV genome. Another pair of primer covered the DR2 of DHBV genome and LAPCR was used to amplify. We found that the primer covering the DR1 could not amplify the DHBV genome after optimizing the LAPCR condition. However, when we used the primer covering the DR2, the complete genome of DHBV was easily amplified (Figure 4). So we amplified three strains of LRDHBV and two strains of Lamivudine-susceptible DHBV complete genome. Then we inserted these DHBV complete genomes into the pMD18-T vector and transformed them to the JM 109 competent cells (Figure 4). Then the *E. coli* that included the five DHBV complete genomes were sent to the TAKARA to sequencing. The five complete sequences of DHBV complete genomes were submitted to the GenBank. Three strains of LRDHBV are AY521226, AY521227, and AY433937. Two strains of Lamivudine-susceptible DHBV are AY392760 and AY536371.



**Figure 4** Electrophoresis graph of the amplification of DHBV complete genome and enzyme cutting of the recombinant. Lanes 1 and 7:  $\lambda$ -EcoT14I digest marker; lane 2: the LAPCR product of DHBV complete genome; lane 3: the cloning product of DHBV complete genome; lane 4: *EcoRI* enzyme cutting product of DHBV complete genome recombinants; lane 5: loop product of pMD18-T vector; lane 6: *EcoRI* enzyme cutting product of loop product of pMD18-T vector.

#### Analyze the related mutant points in the LRDHBV genome

Firstly, we compared the complete nucleotide sequences of the AY521226, AY521227, and AY433937, and the identity is the same 98%. The identity of the AY392760 and the AY536371 is also 98%. But comparing the nucleotide sequences of the LRDHBV (AY521226, AY521227, and AY433937) with the Lamivudine-susceptible DHBV (AY392760 and AY536371), the identity in the nucleotide level is the same 92%. So it seems that there are marked differences between LRDHBV and the Lamivudine-susceptible DHBV in nucleotide level. As the HBV or DHBV P protein is the target of Lamivudine, we turned to the P protein sequences of these DHBV. We initially aligned the P protein sequences of AY521226, AY521227, AY433937 with AY392760, AY536371. There were too many mutant points to analyze which points were related to the Lamivudine-resistant character. As the identities of AY392760 and AY433937 is only 88%, alignment should be done in a wider range. We downloaded all the DHBV protein sequences and aligned with the three Lamivudine-resistant sequences AY521226, AY521227, and AY433937. We found that there were two mutational points in the P protein. The two mutational points are KorR86Q and AorE591T (Figures 5A and B). We have not found any

significant mutational points in S or C protein sequences. The KorR86Q is located in the TP (terminal protein) domain, and the AorE591T is located in the RT (reverse transcriptase) domain. So we guessed that these two mutational points were related to the character of Lamivudine resistance.

## DISCUSSION

We referred to Gunther's method<sup>[17]</sup> when we designed the primers of amplifying the DHBV complete genome. This method noted that only the primers cover the DR1 sequence of HBV, the HBV genome can be wholly amplified. We first used one pair of primers that cover the DR1 of DHBV to amplify DHBV complete genome. Though all kinds of methods of optimizing the PCR condition were tested, the DHBV complete genome could not be amplified. So we guessed that the DR2 of DHBV sequence is also an important obstacle of amplifying the DHBV complete genome. In our experience, it is very hard to get the target products when the primers are located at both sides of the DR2. Additionally, the DR2 is located at the terminal of DHBV-positive strand. There is nick part followed by DR2 in the positive strand of DHBV. So we designed one pair of primers to cover the DR2 to amplify the whole DHBV genome. To our surprise, the complete genomes of DHBV were easily amplified. This phenomenon may be because the genome structure of DHBV is different to the HBV.

If Lamivudine-resistant character of LRDHBV was caused by unusual metabolism of Lamivudine in the duck body and did not correlate with virus genome mutation, it should not have the Lamivudine-resistant character in duck embryo liver cells and other ducks infected with the LRDHBV. Our results showed that the LRDHBV resistant to the Lamivudine appeared both in duck embryo liver cells and in duck body. So we concluded that the Lamivudine-resistant character is caused by DHBV mutation.

To our present knowledge, most of the Lamivudine-resistant phenomena of HBV are related to the YMDD motif mutation<sup>[6-8,18]</sup>. Similar to HBV, mutagenesis *in vitro* in the YMDD motif of DHBV can also cause Lamivudine resistance<sup>[19]</sup>. So we speculate that the mutational points were also located in the YMDD motif of LRDHBV. But the sequencing results denied this speculation.

After we obtained the five DHBV complete sequences, we found that there are no YMDD motif mutations in the P protein sequences of the three LRDHBV sequences. Then we aligned the P protein sequences of three LRDHBV with the two Lamivudine-susceptible DHBV P protein sequences. There were too many different points to analyze the mutational points related to the character of Lamivudine resistance. So we planned to align these three LRDHBV P protein sequences with more DHBV P protein sequences. There should be a prerequisite that the DHBV P protein sequences aligned with the three LRDHBV P protein sequences come from the DHBV which are susceptible to Lamivudine. Because it was never reported that there was a naturally occurred LRDHBV and we found only one strain of DHBV that have the Lamivudine-resistant character in so many years of laboratory work, we believed that the LRDHBV that occurred naturally is very few. So we

<b>A</b>	AF493986	61	VRAPLSHVRAATIDLRLGNKLP	<b>B</b>	538	ELGIRINFDKTTPSPVNEIRFLGYQID
	AY294656		RHHLGKLSGLYQMGCTFNPEWKVPDISDTHFNLD			ENFMKIEESRWKELRTVIKKIKVGEWYDWKCIQ
			VRAPLSHVRAATIDLRLGNKLP			ELGIRINFDKTTPSPVNEIRFLGYQID
	AF047045		RHHLGKLSGLYQMGCTFNPEWKVPDISDTHFNLD			ENFMKIEESRWKELRTVIKKIKVGEWYDWKCIQ
			VRAPLSHVRAATIDLRLGNKLP			ELGIRINFDKTTPSPVNEIRFLGYQID
	AF505512		RHHLGKLSGLYQMGCTFNPEWKVPDISDTHFNLD			ENFMKIEESRWKELRTVIKKIKVGEWYDWKCIQ
			VRAPLSHVRAATIDLRLGNKLP			ELGIRINFDKTTPSPVNEIRFLGYQID
	AY294029		RHHLGKLSGLYQMGCTFNPEWKVPDISDTHFDLD			ENFMKIEESRWKELRTVIKKIKVGEWYDWKCIQ
			VRAPLSHVRAATIDLRLGNKLP			ELGIRINFDKTTPSPVNEIRFLGYQID
	K01834		RHHLGKLSGLYQMGCTFNPEWKVPDISDTHFNLD			ENFMKIEESRWKELRTVIKKIKVGEWYDWKCIQ
			VRAPLSHVRAATIDLRLGNKLP			ELGIRINFDKTTPSPVNEIRFLGYQID
	AY250901		RHHLGKLSGLYQMGCTFNPEWKVPDISDTHFNLD			ENFMKIEESRWKELRTVIKKIKVGEWYDWKCIQ
			VRAPLSHVRAATIDLRLGNKLP			ELGIRINFDKTTPSPVNEIRFLGYQID
	AY250902		RHHLGKLSGLYQMGCTFNPEWKVPDISDTHFNLD			ENFMKIEESRWKELRTVIKKIKVGEWYDWKCIQ
			VRAPLSHVRAATIDLRLGNKLP			ELGIRINFDKTTPSPVNEIRFLGYQID
	AY250903		RHHLGKLSGLYQMGCTFNPEWKVPDISDTHFNLD			ENFMKIEESRWKELRTVIKKIKVGEWYDWKCIQ
			VRAPLSHVRAATIDLRLGNKLP			ELGIRINFDKTTPSPVNEIRFLGYQID
	AY250904		RHHLGKLSGLYQMGCTFNPEWKVPDISDTHFNLD			ENFMKIEESRWKELRTVIKKIKVGEWYDWKCIQ
			VRAPLSHVRAATIDLRLGNKLP			ELGIRINFDKTTPSPVNEIRFLGYQID
	AY294028		RHHLGKLSGLYQMGCTFNPEWKVPDISDTHFNLD			ENFMKIEESRWKELRTVIKKIKVGEWYDWKCIQ
			VRAPLSHVRAATIDLRLGNKLP			ELGIRINFDKTTPSPVNEIRFLGYQID
	M60677		RHHLGKLSGLYQMGCTFNPEWKVPDISDTHFNLD			ENFMKIEESRWKELRTVIKKIKVGEWYDWKCIQ
			VRAPLSHVRAATIDLRLGNKLP			ELGIRINFDKTTPSPVNEIRFLGYQID
	X74623		RHHLGKLSGLYQMGCTFNPEWKVPDISDTHFNLD			ENFMKIEESRWKELRTVIKKIKVGEWYDWKCIQ
			VRAPLYHVRAATIDLRLGNKLP			ELGIRINFDKTTPSPVNEIRFLGYQID
	X12798		RHHLGKLSGLYQMGCTFNPEWKVPDISDTHFNLD			ENFMKIEESRWKELRTVIKKIKVGEWYDWKCIQ
			VRAPLSHVRAATIDLRLGNKLP			ELGIRINFDKTTPSPVNEIRFLGYQID
	X58567		RHHLGKLSGLYQMGCTFNPEWKVPDISDTHFDLE			ENFMKIEESRWKELRTVIKKIKVGEWYDWKCIQ
			VRAPLSHVRAATIDLRLGNKLP			ELGIRINFDKTTPSPVTEIRFLGYQID
	AY494850		RHHLGKLSGLYQMGCTFNPEWKVPDISDTHFKSE		539	EQFMKIEESRWKELRTVIKKIKIGEWYDWKCIQ
			VRAPLSHVRAATIDLRLGNKLP			ELGVRINFDKTTPSPVNEIRFLGYQID
	AY494851		RHHLGKLSGLYQMGCTFNPEWKVPDISDTHFKSE		539	QKYLKIEDDRWKELRTVIKKIKVGEWYDWKCIQ
			VRAPLSHVRAATIDLRLGNKLP			ELGVRINFDKTTPSPVNEIRFLGYQID
	AY521226		RHHLGKLSGLYQMGCTFNPEWKVPDISDTHFKSE			QRYMKIEESRWKELRTVIKKIKIGEWYDWKCIQ
			VRAPLSHVRAATIDLRLGNRLPA		540	ELGIRINFDKTTPSPVTDIRFLGNQID
	AY521227		QHHLGKLSGLYQMGCSFNPEWKVPDISDTHFDLQ			EKYMKIEESRWKELRTVIKKIKVG 7WYDWKCIQ
			VRAPLSHVRAATIDLRLGNRLPA			ELGIRINFDKTTPSPVNDIRFLGYQID
	AY433937		QHMGKLSGLYQMGCSFNPEWKVPDISDTHFDLQ			EKYMKIEESRWKELRTVIKKIKVG 7WYDWKCIQ
			VRAPLSHVRAATIDLRLGNRLPA			ELGIRINFDKTTPSPVTDIRFLGYQID
	NC_001344		QHMGKLSGLYQMGCSFNPEWKVPDISDTHFDLQ			EKYMKIEESRWKELRTVIKKIKVG 7WYDWKCIQ
			VRAPLSHVRAATIDLRLGNKLP			ELGIRINFDKTTPSPVNDIRFLGYQID
	X60213		KHHLGKLSGLYQMGCTFNPEWKVPDISDTHFDLQ			QKFMKIEESRWIELRTVIKKIKIGAWYDWKCIQ
			VRAPLSHVRAATIDLRLGNKLP			ELGIRINFDKTTPSPVNDIRFLGYQID
	M32990		KHHLGKLSGLYQMGCTFNPEWKVPDISDTHFDLQ			QKFMKIEESRWIELRTVIKKIKIGAWYDWKCIQ
			VRAPLSHVRAATIDLRLGNKLP			ELGIRINFDKTTPSPVNDIRFLGYQID
	M32991		KHHLGKLSGLYQMGCTFNPEWKVPDISDTHFDLQ			QKFMKIEESRWKELRTVIKKIKIGAWYDWKCIQ
			VRAPLSHVRAATIDLRLGNKLP			ELGIRINFDKTTPSPVNDIRFLGYQID
	AJ006350		KHHLGKLSGLYQMGCSFNPEWKVPDISDTHFDLQ			QKFMRIEESRWKELRTVIKKIKIGAWYDWKCIQ
			VRAPLSHVRAATIDLRLGNKLP			ELGVRINFDKTTPSPVNDIRFLGYQID
	AF404406		KHHLGKLSGLYQMGCTFNPEWKVPDISDTHFDLQ			QKFMKIEESRWKELRTVIKKIKIGAWYDWKCIQ
			VRAPLSHVRAATIDLRLGNKLP		539	ELGIRINFDKTTPSPVTEIRFLGYQID
	M21953		KHHLGKLSGLYQMGCTFNPEWKVPDISDTHFNLD			QKFMKIEESRWKELRTVIKKIKIGAWYDWKCIQ
			VRAPLSHVRAATIDLRLGNKLP		539	ELGIRINFDKTTPSPVTEIRFLGYQID
	AY392760		KHHLGKLSGLYQMGCTFNPEWKVPDISDTHFDLQ			QKFMKIEEDRWKELRTVIKKIKVGEWYDWKCIQ
			VRAPLSHVRAATVDLRLGNRLPA		540	ELGIRINFDKTTPSPVNEIRFLGYQID
	AY536371		RHHLGKLSGLYQMGCTFNPEWKVPDISDTHFDMQ			QKYMKIEESRWSELRTVIKKIKVGEWYDWKCIQ
			VRAPLSHVRAATVDLRLGNRLPA		540	ELGIRINFDKTTPSPVNEIRFLGYQID
			RHHLGKLSGLYQMGCTFNPEWKVPDISDTHFDMQ			QKYTKIEESRWSELRTVIKKIKIGEWYDWKCIQ
	X58568		VRAPLSHVRAATEDLRLGNKLP		539	ELGIRINFDKTTPSPVNEIRFLGYQID
			RHHLGKLSGLYQMGCTFNPDWKVPDISDTHFDMQ			HRFMKIEESRWKELRTVIKKIKIGEWYDWKCIQ
	X58569		VRAPLSRVRAATEDLRLGNRLPA		539	ELGIRINFDKTTPSPVNEIRFLGYQID
			RHHLGKLSGLYQMGCTFNPEWKVPDISDTHFDMQ			QRFMKIEESRWKELRTVIKKIKIGEWYDWKCIQ

Figure 5 A: KorR86Q mutant point in the P protein of the LRDHBV;

mutant point in the P protein of the LRDHBV.

presumed that the DHBV sequences in the GenBank up to now come from DHBV that are susceptible to Lamivudine. So we downloaded all the DHBV sequences in the GenBank and aligned the P protein sequences of these DHBV. The results showed that there were two mutational points that occurred in the P protein sequences of LRDHBV. It is KorR86Q in the TP domain and AorE591T in the RT domain. The TP domain of DHBV P protein mainly acts as a primer to originate the synthesis of DHBV-negative strand<sup>[20]</sup>. But it is the tyrosine residue in 96aa of TP domain that primed the reverse transcription of negative strand<sup>[21,22]</sup>. AorE591T is located in the lower reaches of YMDD motif. So the roles of KorR86Q and AorE591T in the Lamivudine-resistant phenomenon need deeper research.

For both hepadnaviruses and HIV, the mechanism of action of Lamivudine requires phosphorylation to 3TC-5'-triphosphate (3TC-TP), which in turn specifically inhibits the viral polymerase<sup>[23-26]</sup>. The specificity is conferred by the much lower affinity of 3TC-TP for the cellular  $\alpha$ - and  $\beta$ -polymerase<sup>[25,27]</sup>. The mechanism of inhibition of hepadnavirus involves inhibition of the viral polymerase<sup>[26,28,29]</sup>. Acting as a chain terminator for the DNA polymerase activities, the Lamivudine inhibit the reverse transcriptase in a manner that resemble competitive inhibition with respect to dCTP<sup>[30]</sup>. The side groups of isoleucine and valine of the YMDD mutants sterically prevent Lamivudine from appropriately configuring into the nucleotide binding site of the reverse transcriptase<sup>[31]</sup>. This can cause Lamivudine resistance. Can the KorR86Q and AorE591T mutants also prevent Lamivudine from appropriately configuring into the nucleotides binding site of the reverse transcriptase? It needs more research.

A 4-year clinical research showed that YMDD mutants only could explain the 75% of Lamivudine resistances. Polymerase gene mutations were observed in 82.5% of virological breakthroughs but also in 75% of the non-responders<sup>[32]</sup>. So the mutations outside the YMDD motif in the P protein can independently cause DHBV resistant to Lamivudine is not very strange.

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