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ORIGINAL ARTICLE

Establishment of drug-resistant HBV small-animal models by hydrodynamic injection

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Abstract In antiviral therapy of hepatitis B virus (HBV) infection, drug resistance remains a huge obstacle to the long-term effectiveness of nucleoside/tide analogs (NAs). Primary resistance mutation (rtM204V) contributes to lamivudine (LAM)-resistance, and compensatory mutations (rtL180M and rtV173L) restore viral fitness and increase replication efficiency. The evaluation of new anti-viral agents against drug-resistant HBV is limited by the lack of available small-animal models. We established LAM-resistance HBV replication mice models based on clinical LAM-resistant HBV mutants. Double (rtM204V+rtL180M) or triple (rtM204V+rtL180M+rtV173L) lamivudine-resistant mutations were introduced into HBV expression vector, followed by hydrodynamic injection into tail vein of NOD/SCID mice. Viremia was detected on days 5, 9, 13 and 17 and liver HBV DNA was detected on day 17 after injection. The serum and liver HBV DNA levels in LAM-resistant model carrying triple mutations are the highest among the models. Two NAs, LAM and entecavir (ETV), were used to test the availability of the models. LAM and ETV inhibited viral replication on wild-type model. LAM was no longer effective on LAM-resistant models, but ETV retains a strong activity. Therefore, these models can be used to evaluate anti-viral agents against lamivudine-resistance, affording new opportunities to establish other drug-resistant HBV small-animal models.

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1. Introduction

Hepatitis B virus (HBV) is the prototype member of the hepadnavirus family, which is a group of the smallest DNA-containing enveloped animal viruses known. HBV infection is the leading cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC)[1–4]. It is estimated that approximately 350 million people worldwide are chronic HBV carriers and 15%–40% of them eventually develop HBV-related cirrhosis or HCC[5–8]. Around one million deaths per year are due to HBV-related liver pathologies[9,10]. There are two classes of drugs available for the treatment of chronic HBV infection: first is the immune modulator interferon-α (standard or pegylated (PEG)-IFN-α), second are nucleoside or nucleotide analogs (NAs), which act as the HBV polymerase inhibitors, such as lamivudine (LAM), entecavir (ETV), tenofovir disoproxil fumarate (TDF), etc. IFN-α monotherapy has been the standard of treatment for chronic hepatitis B since the middle 1990s[11], but it is poorly tolerated and effective, only effective in 20%–40% patients. With the introduction of LAM, several oral HBV polymerase inhibitors were approved and accounted for the vast majority of therapies for chronic hepatitis B due to higher tolerance and more convenient administration, which reduced the occurrence of HCC and liver cirrhosis[12]. However, drug resistance resulting from HBV polymerase mutations with the NAs treatment is a huge obstacle to successful anti-viral therapy. Clinically, along with long-term LAM treatment, the emergence of lamivudine-resistant HBV was discovered in approximately 24% of patients after 1 year of therapy and in 70% after 5 years of therapy, so it was no longer considered a first-line agent in treatment of chronic HBV infection[13]. Therefore, it is important for designing and exploring new anti-viral agents to understand the mechanism of HBV drug resistance and avoid it.

To date, there are several animal models being described previously for studying the mechanism of HBV infection and exploring new anti-viral agents, but the evaluation of new anti-viral agents against drug-resistant HBV is limited by the lack of available small-animal models. Here we describe recently developed drug-resistant HBV mice models that alleviate many experimental constraints. Adapting the hydrodynamic-based procedure, we established convenient and replication-competent drug-resistant HBV NOD/SCID mice models based on clinic LAM-resistant mutants and evaluated the availability of these models in the assessment of anti-viral drugs[14].

2. Materials and methods

2.1. Reagents and animals

Female NOD/SCID mice at 6–9 weeks of age were purchased from Vital River Laboratory Animal Technology Co., Ltd., Beijing, China. Animals were bred and cared under specific pathogen-free conditions in the Experimental Animal Center of the Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences. Our studies on mice were carried out in strict accordance with the recommendations in the Code of Ethics of the World Medical Association.

Lamivudine (LAM) and entecavir (ETV) tablets were purchased from GlaxoSmithKline Pharmaceuticals (Suzhou) Co., Ltd. and Sino-American Shanghai Squibb Pharmaceuticals Co., Ltd., respectively.

2.2. Construction of lamivudine-resistant mutations

pTmcs-HBV1.3 and the Sleeping Beauty transposase expression plasmid (pCMV-SB) were generously provided by F.V. Chisari's laboratory. pTmcs-HBV1.3 is an HBV replication-competent plasmid, which encodes a wild-type, terminally redundant (1.3-unit-length) HBV genome (Genbank accession number V01460). The supergenomic DNA was flanked by the inverted repeat (IR) recognition sequences of the Sleeping Beauty transposase. pTmcs-HBV1.3-3TCR and pTmcs-HBV1.3-3TCR-V173L are two different lamivudine-resistant mutants carrying rtL180M–rtM204V double mutations and rtL180M–rtM204V–rtV173L triple mutations, respectively. For their generations, rtL180M, rtM204V and rtV173L mutations were introduced into pTmcs-HBV1.3 by site-mutagenesis with the appropriate primers using QuickChange® Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) as per the manufacturer's recommendations. The primer pairs of 5′–TTGGCTTTCAGTTATGTGGATGATGTGGTATTG–3′ and 5′–CAATACCACATCATCCACATAACTGAAAGCCAA–3′ were for rtM204V mutation, 5′–GTGGGCCTCAGCCCGTTTCTCATG-GCTCAGTTTACTAGTGCC–3′ and 5′–GGCACTAGTAAACTGA-GCCATGAGAAACGGGCTGAGGCCCAC–3′ were for rtL180M mutation, 5′–AAAATTCCTATGGGATTGGGCCTCAGCCCG-TTT–3′ and 5′–AAACGGGCTGAGGCCCAATCCCATAGGAA-TTTT–3′ were for rtV173L mutation. All constructs were sequenced to confirm that no additional mutations had been introduced.

2.3. Hydrodynamic injection of plasmid

The method of hydrodynamic injection of plasmids has been previously described[15]. In brief, a total of 13.5 μg of pTmcs-HBV1.3, pTmcs-HBV1.3-3TCR or pTmcs-HBV1.3-3TCR-V173L and 4.5 μg of pCMV-SB were co-injected into the tail vein of 6- to 9-week-old NOD/SCID mice in a volume of saline equivalent to 8% of the mouse body weight (*e.g.*, 1.6 mL for mouse of 20 g). The total volume was delivered within 5–8 s. The mouse serum samples were taken from the retro-orbital vessels on day 5 after injection to measure the HBV surface antigen (HBsAg) level by using the Diagnostic Kit for Hepatitis B Virus Surface Antigen (ELISA) (Shanghai Kehua Bio-Engineering) according to the manufacturer's instructions. HBsAg/Cut-off value in serum serves as an estimate of transfection efficiency in these experiments.

2.4. Evaluation of the availability of mouse models

The HBsAg-positive mice were randomly divided into three groups with 10 mice each on day 5 after hydrodynamic injection. Briefly, LAM (20 mg/kg) and ETV (0.1 mg/kg) were given twice per day by oral gavage administration in a medication course lasting 12 days, and the equivalent volume of saline was used as a control. To assess the efficacies of LAM and ETV against HBV replication, the mouse serum samples were collected every four days after drug administration. On the last day, the mice were sacrificed and the liver tissues were harvested and stored at −70 °C until assayed.

2.5. Quantitation of HBV DNA in mouse sera

The mouse serum samples were collected on days 0, 4, 8 and 12 after drug administration, and the method of purifying encapsidated viral DNA has been previously described[15]. Briefly, 50 μL of isolated serum was first digested with DNase I to eliminate

residual plasmid DNA (20 units of DNase I in $10 \times$ DNase I Buffer; incubated at 37 °C for 12–16 h). The encapsidated viral DNA was then released by proteinase K digestion (20 mmol/L EDTA/1% SDS/400 units proteinase K; incubated at 37 °C for 12 h), followed by extraction with 500 μL of Tris-saturated-phenol and precipitated with 1 mL cold ethanol. After centrifugation at 12,000 rpm for 10 min, precipitates were again resuspended in 50 μL of double-distilled H_2O and stored at −20 °C. Serum HBV DNA levels were quantified in 20 μL reaction system by a real-time polymerase chain reaction (PCR, see below).

2.6. *Quantitation of HBV DNA and input DNA in mouse livers*

Total genomic DNA was purified from 15 mg of liver tissues using the DNeasy® mini kit (QIAGEN) as per the manufacturer's recommendations. Briefly, the purified DNA was eluted with 50 μL of double-distilled H_2O, then its concentration was measured with NanoDrop 2000 (Thermo Scientific) and stored at −20 °C. HBV DNA and input vector copy numbers were measured by real-time PCR in a 20 μL reaction mixture containing 50 ng of liver genomic DNA (see below). An excess >2-fold of HBV over input plasmid was interpreted as evidence that active replication was ongoing in the liver.

2.7. *Real-time PCR*

HBV DNA and input vector copy numbers were determined by real-time PCR in 20 μL reaction system in Power SYBR® Green PCR Master Mix (Applied Biosystems), using an Applied Biosystems 7500 fast real-time thermocycler (Applied Biosystems). Thermal cycling parameters consisted of 10 min denaturation step at 95 °C, followed by 40 cycles of denaturation (15 s at 95 °C) and annealing/extension (60 s at 60 °C). A series of plasmid pTmcs-HBV1.3 diluted to 10^8, 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 copies per reaction with 0.5 ng/μL human genomic DNA was used as standard. All samples were analyzed in triplicate. The PCR primers used to amplify each respective amplicon were: HBV DNA primers HBV 469-488F (5′–CCCGTTTG-TCCTCTAATTCC–3′) and HBV 588-569R (5′–GTCCGAAGGTT-TG;GTACAGC–3′), input vector pTHBV-5585U20 (5′–CCAG-TCGGGAAACCTGTCGT–3′) and pTHBV-5675L20 (5′–GCAGC-GAGTCAGTGAGCGAG–3′)[16]. pTHBV-5585U20 and pTHBV-5675L20 annealed to sequences within the Sleeping Beauty transposase IR sequences in the plasmid backbone of vector pTmcs.

2.8. *Statistical analysis*

Bar graphs were plotted to show mean ± standard deviation (SD) and statistical analyses were performed. A *P* value of <0.05 in the Student's *t*-test was considered statistically significant.

3. Results

3.1. *Introducing the drug-resistant HBV replicative plasmid into NOD/SCID mice leads to viral gene expression in vivo*

The application of NAs is hampered by the appearance of drug-resistant HBV. Thus, it is essential to understand the mechanism of resistance to HBV polymerase inhibitors in long-term treatment strategy. Yang et al.[15,17] previously described a mouse model supporting HBV gene expression and replication in the liver of NOD/SCID mice through hydrodynamic injection, which introduced an 1.3-fold HBV DNA (pTmcs-HBV1.3) and Sleeping Beauty transposase (pCMV-SB) into the tail vein of mice. Taking advantage of the hydrodynamic-based procedure, we established two LAM-resistant HBV replication mouse models by injecting pTmcs-HBV1.3-3TCR and pTmcs-HBV1.3-3TCR-V173L, respectively, into NOD/SCID mice to transfect hepatocytes *in vivo*. On day 5 after injection, the percentage of HBsAg-positive with injection of pTmcs-HBV1.3, pTmcs-HBV1.3-3TCR and pTmcs-HBV1.3-3TCR-V173L were 95.24%, 100% and 100%, respectively (Fig. 1A) and the viremia were 49.2×10^6, 7.9×10^6, and 5.8×10^6 copies/mL, respectively (Fig. 1B). As show in Fig. 1C, HBV DNA levels were more than 2-fold higher in mouse liver compared to their own input vector level. These results indicated that hydrodynamic injection of the drug-resistant HBV replicative plasmids leads to viral gene expression *in vivo*.

3.2. *Viremia of wild-type and LAM-resistant mouse models*

For a successful experimental animal model of HBV infection, high level and long life-span viremia are important[15]. Fig. 1B shows the viremia of three HBV replication mouse models. On day 5 after injection, the viral replication in wild-type mouse serum is the highest among three models; HBV DNA copy numbers of LAM-resistant viruses carrying double or triple mutations were 16.1% and 11.8% of wild-type virus, respectively. It is interesting that viral replication gradually decreased with time in one of the LAM-resistant models (double mutants) and HBV DNA copy numbers were 3.52×10^4 copies/mL on day 17 after injection of pTmcs-HBV1.3-3TCR. However, in another LAM-resistant model (triple mutants), the level reached the peak on day 9 after injection of pTmcs-HBV1.3-3TCR-V173L and the decrease speed was slower than the wild-type model. The copy numbers are 116.5×10^6 and 65.0×10^6 copies/mL on day 9 and 17 after injection of pTmcs-HBV1.3-3TCR-V173L. Taken together, serum viral level and sustained time were different among the three models, with pTmcs-HBV1.3-3TCR-V173L displaying the superiority.

3.3. *Replication ability of wild-type and LAM-resistant HBV DNA in mouse liver*

To investigate the replication ability of liver HBV DNA in wild-type and LAM-resistant HBV mouse models, the mice were sacrificed and the liver tissues were harvested on day 17 after injection. Total genomic DNA was extracted from liver tissues and HBV DNA levels were measured. As shown in Fig. 1D, compared with wild-type mouse model, viral replication decreases about 10.06-fold in the rtL180M–rtM204V double mutants, but increases about 7.79-fold in the rtL180M–rtM204V–rtV173L triple mutants. The HBV DNA copy numbers were 24.5×10^6 copies/mL in wild-type mouse liver, and the values in LAM-resistant mouse livers carrying double or triple mutations were 2.4×10^6 and 191.3×10^6 copies/mL, respectively (Fig. 1D), suggesting that rtM204V and rtL180M double mutations in LAM-resistant HBV mutants resulted in the decline of replication ability, but the third compensatory rtV173L mutation significantly increased the HBV replication ability *in vivo*.

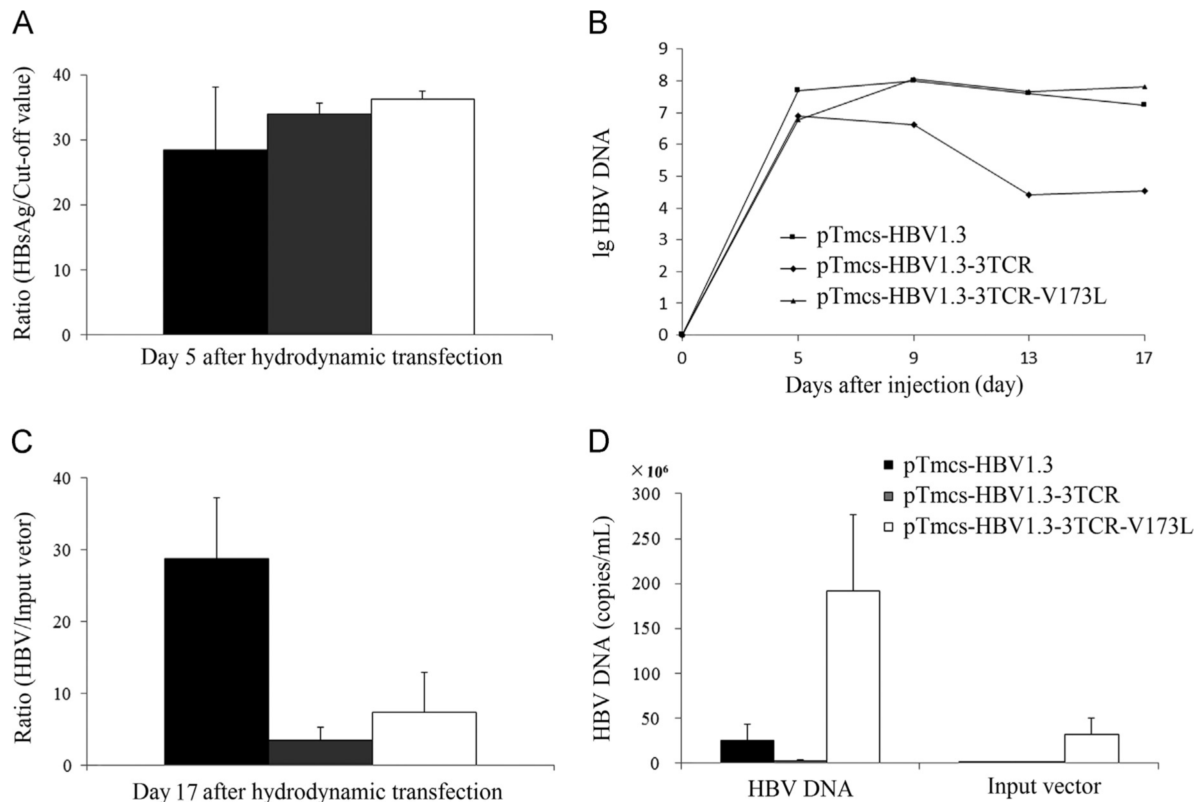

Figure 1 Viremia and viral gene expression in the livers after hydrodynamic transfection with different DNA. (A) The mice sera were isolated on day 5 after injection to measure the HBV surface antigen (HBsAg) level. HBsAg/Cut-off value in serum serves as an estimate of transfection efficiency in these experiments. HBsAg/Cut-off value greater than 1 was HBsAg-positive. HBsAg-positive NOD/SCID mice were qualified for the next experiment. (B) HBV DNA was purified from serum on days 5, 9, 13 and 17 after injection and analyzed for kinetics of viremia by real-time PCR. (C) HBV DNA was purified from the liver on day 17 after injection. Ratios of HBV/Input vectors greater than 2 verified active viral replication in mouse liver. (D) HBV DNA was purified from the liver on day 17 after injection. The copies of Liver HBV DNA and input vector were detected by real-time PCR.

3.4. The availability of these mouse models to assess the anti-viral drugs

To verify whether the HBV replication mouse models can be used for the evaluation of anti-HBV agents, we used two nucleotide analogs, LAM and ETV, to test the availability of these models. Our results showed that both LAM and ETV could decrease HBV DNA level in wild-type mouse serum and liver. In serum, the HBV levels in LAM- and ETV-treated groups significantly decreased by 54.34% and 99.96%, respectively, compared with negative control on day 12 after treatment. In liver, the HBV levels in LAM- and ETV-treated groups significantly decreased by 43.04% and 97.47%, respectively (Figs. 2A and 3A). Furthermore, as shown in Figs. 2B, 2C, 3B and 3C, in two LAM-resistant mouse models, LAM cannot decrease serum and liver HBV level significantly on day 12 after ETV treatment, but ETV retains strong efficacy in the liver with the inhibition rates of 87.18% and 84.22% in double and triple mutants, respectively, compared with negative control. Although ETV decreased the serum HBV level significantly in triple mutants, ETV no longer had its strong efficacy in serum HBV DNA level in double mutants (Fig. 2B), which may be due to the decrease of HBV replication efficiency in double mutants. These results indicated that LAM-resistant HBV mouse models, especially triple mutations, were established successfully and can be used to evaluate the efficacy of anti-viral compounds against drug-resistant HBV infection.

4. Discussion

Hepatitis B virus (HBV) belongs to the Hepadnaviridae family, which includes human HBV, other mammalian viruses such as the woodchuck hepatitis virus (WHV), the ground squirrel hepatitis virus (GSHV), the artic squirrel hepatitis virus (ASHV) and the woolly monkey hepatitis virus (WMHV), and avian viruses such as duck hepatitis B virus (DHBV), the heron hepatitis virus (HHV) and snow goose hepatitis virus (SGHV)[18–23]. There are several animal models such as DHBV, WHV, HBV-infected chimpanzees and HBV transgenic mice, offering opportunities for *in vivo* studies of the mechanism of genome replication, viral persistence and disease pathogenesis[21,22,24,25], and they have assisted the discovery of new antiviral drugs. Yang and his colleagues[15] established a more ideal and convenient mouse model by introducing the HBV DNA to the livers of immunocompetent mice based on the hydrodynamic transfection method.

However, the evaluation of new anti-viral agents against drug-resistant HBV is limited by the lack of available small-animal models. Along with long-term NA treatment, the emergence of drug-resistant HBV was a huge problem. Clinically, the LAM-resistant viral mutations are rtL180M–rtM204V in HBV polymerase. Moreover, rtV173L was invariably found as a third compensatory mutation in conjunction with rtM204V and rtL180M in one of three patients who failed in lamivudine therapy[26], and it was not observed as a single change in chronic hepatitis B patients[27].

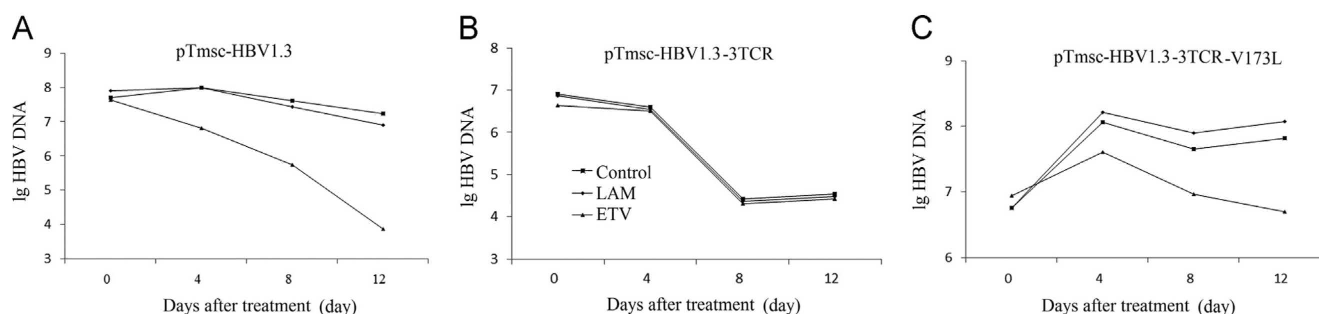

Figure 2 The effect of two NAs on serum HBV DNA on days 0, 4, 8 and 12 after drug treatment in three different mouse models. (A) In wild-type HBV (pTmcs-HBV1.3) transfection mouse serum, the HBV levels in LAM and ETV-treated groups were significantly decreased by 54.34% and 99.96%, respectively, compared with negative control on day 12 after treatment. (B) In double mutant HBV (pTmcs-HBV1.3-3TCR) transfection mouse serum, the HBV levels declined rapidly. Low viral titers concealed the efficacy of ETV. (C) In triple mutant HBV (pTmcs-HBV1.3-3TCR-V173L) transfection mouse serum, the viral replication sustained high level; LAM did not decrease serum HBV level; ETV displayed strong efficacy, the inhibition rate was 92.30% on day 12 after ETV treatment.

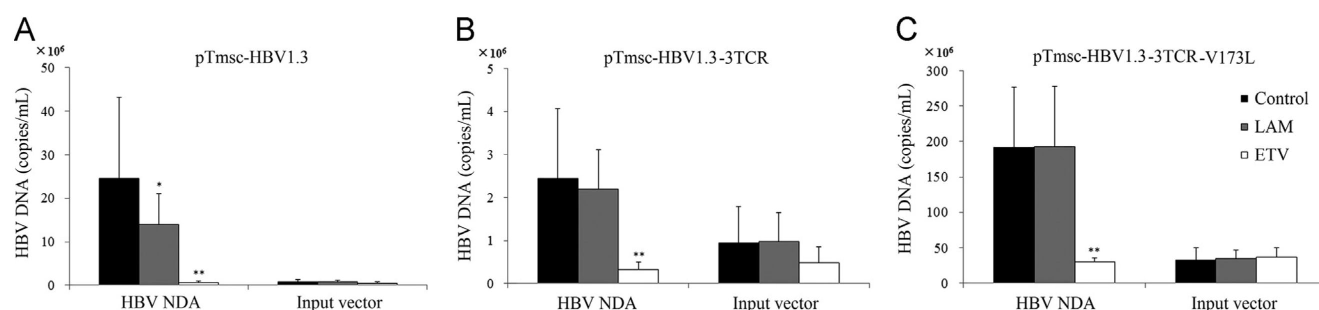

Figure 3 The effect of two NAs on liver HBV DNA on day 12 after treatment in three different mouse models. (A) In wild-type HBV (pTmcs-HBV1.3) transfection mouse liver, the HBV levels in LAM and ETV-treated groups were significantly decreased by 43.04% and 97.47%, respectively, compared with negative control on day 12 after treatment. (B) In double-mutant HBV (pTmcs-HBV1.3-3TCR) transfection mouse liver, LAM did not decrease HBV level significantly; ETV retained efficacy in the liver, the inhibition rate is 87.18%. (C) In triple mutant HBV (pTmcs-HBV1.3-3TCR-V173L) transfection mouse liver, LAM also did not decrease the HBV level; ETV displayed strong efficacy, the inhibition rate is 84.22%. $^{*}P<0.05$, $^{**}P<0.01$ *vs.* wild-type HBV (pTmcs-HBV1.3) transfection mouse.

Thus, taking advantage of the hydrodynamic injection and clinical LAM-resistant data, we successfully established the LAM-resistant mouse models by introducing HBV DNA carrying double or triple LAM-resistant mutations into the tail vein of NOD/SCID mice, which is lack of functional T/B cells and beneficial to viral persistent replication.

Two NAs targeting viral polymerase, LAM and ETV, are used for the HBV treatment. In long-term monotherapy, they are associated with the emergence of drug-resistant mutations in the RT domain of the polymerase[28,29]. Viral drug-resistant sites are different between LAM and ETV. When treating with LAM, drug resistance arises rapidly and amino acid substitutions compromise rtM204V/I, rtL180M and rtV173L, etc. When treating with ETV, drug resistance arises less rapidly, amino acid substitutions compromise rtS202I, rtT184G and rtI169T, etc. They do not share cross-resistance. Moreover, ETV can be used for the treatment of LAM-resistant HBV patients in clinic.

In our experiment, rtL180M–rtM204V double mutations reduced HBV replication capacity *in vivo* compared with wild-type HBV. In serum, the decreased values were 95.86%, 99.94% and 99.79% on days 5, 9 and 17 after injection, respectively. In the liver, the decreased value was 90.96% on day 17 after injection. These results are consistent with Delaney's observation[26]. In rtL180M–rtM204V double mutations HBV mouse model, low viral titer partially concealed the efficacy of ETV in serum, although ETV displayed efficacy in the liver (Figs. 2B and 3B).

As illustrated in Fig. 4, rtM204V/I are primary resistance mutations which mapped in conserved YMDD motif within C domain of the viral RT and facilitate ongoing viral DNA synthesis in the presence of LAM. The compensatory mutations include rtL180M and rtV173L within the B domain that restore viral fitness. In Delaney's observation, the rtL180M–rtM204V double mutations can reduce HBV replication *in vitro* compared with wild-type HBV, but the addition of rtV173L mutation to LAM-resistant HBV can increase HBV replication. As illustrated in Fig. 1B and 1D, the replication of rtL180M–rtM204V–rtV173L triple mutant increased compared to wild-type HBV construct and double mutant in NOD/SCID mice.

We evaluated the availability of the mouse models through the application of nucleotide analogs. LAM and ETV are all effective in wild-type models (Figs. 2A and 3A), but the models carrying the double or triple mutations were highly resistant to LAM (Figs. 2B and 3B). Sherman and colleagues[30] found that ETV was less potent in LAM-resistant patients. Despite an increased dose of 1 mg/kg, only 19% and 40% of these patients achieved undetectable HBV DNA after one and two years, respectively. For the level of serum HBV DNA, we observed the same results in LAM-resistant models carrying double mutations. However, ETV remains potent on the model carrying triple mutations, which

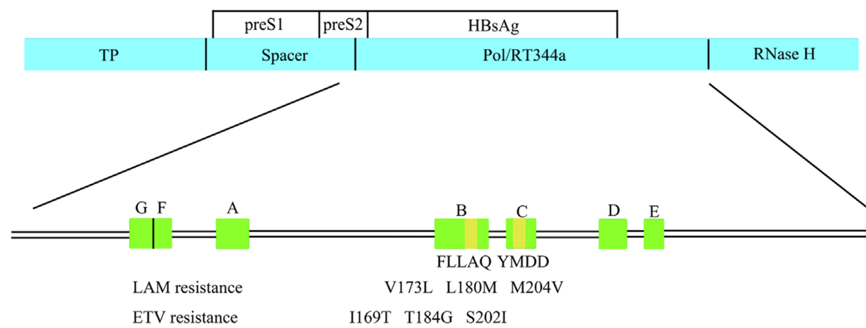

Figure 4 HBV polymerase gene and drug-resistant mutation[29]. The locations of LAM resistance mutations in HBV polymerase (Pol) open reading frame (ORF) are shown. HBV Pol comprises four regions: terminal protein (TP), spacer, reverse transcription region (Pol/RT) for viral HBV replication and RNaseH region. RT region is divided into 7 domains (A–G). The conserved motif of tyrosine, methionine, aspartate and aspartate (YMDD) locates in C domain. HBsAg ORF overlaps RT domain. LAM resistance arises rapidly; amino acid substitutions include rtM204V/I, rtL180M and rtV173L, etc. ETV resistance arises less rapidly; amino acid substitutions include rtS202I, rtT184G, rtI169T, etc.

may be due to the enhancement of the viral replication caused by the third compensatory mutation rtV173L. We also observed that ETV significantly decreased the level of HBV DNA in LAM-resistant (double and triple mutants) mouse liver compared to the saline control (Fig. 3B and 3C).

Based on our data, we confirmed that the wild-type and LAM-resistant HBV replication mouse models were established successfully in our laboratory. These models can afford new opportunities for further understanding of the molecular mechanism of genome replication, viral persistence and drug resistance. More importantly, they may play an important role in discovering new agents against HBV mutants and developing a new mouse model of other drug resistance.

Acknowledgments

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