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Sensitivity of drug-resistant mutants of hepatitis B virus to poly-IC

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Summary. – The long-term benefits of antiviral treatment are limited by the resistance of hepatitis B virus (HBV). However, the effect of interferon (IFN)α treatment on drug-resistant HBVs is so far unknown. We, therefore, investigated the effects of IFN-α inducer poly-IC on the replication of HBV mutants resistant to drugs such as lamivudine (LAM), adefovir dipivoxil (ADV) and entecavir (ETV) in mice. HBV DNA and HBV DNA intermediate (RI) were employed as markers of the virus replication and 2',5'-oligoadenylate synthase (OAS) mRNA as a marker of IFN-α/β induction. Poly-IC inhibited wtHBV replication and increased levels of OAS mRNA. Compared to the wt virus, the capacity of virus replication was reduced in most LAMr and ETVr mutants except those with mutations rtM(204V+L180M+V173L), and was similary in the ADVr mutants except rt(A121V+N236T). The virus replication was reduced after poly-IC treatment with LAMr and ADVr mutants similary to the wt virus. In contrast, ETVr mutants were resistant to the poly-IC treatment. In conclusion, the capacity of HBV replication and the sensitivity to IFN therapy are influenced by drug-resistant mutations. The IFN therapy may effectively inhibit HBV replication in particular in patients with LAMr or ADVr mutations but not in patients with ETVr mutations.

Keywords: hepatitis B virus; mutants; drugs; mouse; interferon; poly-IC

Introduction

Chronic hepatitis B virus (HBV) infection remains a significant global health problem because of its high prevalence and association with serious liver diseases such as hepatitis, liver cirrhosis, liver failure and hepatocellular carcinoma (HCC), especially in Asia. To date, interferon (IFN)α and five nucleos(t)ide analogs, including lamivudine (LAM), adefovir dipivoxil (ADV), entecavir (ETV), telbivudine (LdT) and tenofovir disoproxil fumarate (TDF) have been approved for the treatment of chronic HBV infection. Long-term antiviral treatment delays the clinical progression of liver diseases and prevents hepatic decompensation in patient with advanced liver disease (Liaw *et al.,* 2004). However, the antiviral treatment cannot eliminate the virus and long-term benefits are limited by the emergence of drug-resistant virus. Lamivudine was the first nucleoside analog approved for the treatment of chronic HBV with potent suppression of HBV replication and a good safety profile. However, development of drug-resistant HBV strains has been found in 70% of the patients after five years of LAM therapy (Lai *et al.,* 2003; Zoulim 2004). The rate of emergence of antiviral resistance for ADV and ETV was 29% and 1.2% at five years of treatment, respectively (Hadziyannis *et al.,* 2006; Tenney *et al.,* 2009). The rate of emergence of antiviral resistance for LdT was 5% in hepatitis B envelope antigen (HBeAg)-postive patients and 2.1% in HBeAg-negative patients after two years of treatment, respectively (Liaw *et al.,* 2009). As L-nucleosides, LdT and LAM have a similar resistance mutation profiles. The LAM mutation conferred cross-resistance to other L-nucleosides and reduced the sensitivity to ETV but not

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Abbreviations: ADV = adefovir dipivoxil; ETV = entecavir; HBV = hepatitis B virus; HBeAg = hepatitis B envelope antigen; HCC = hepatocellular carcinoma; IFN = interferon; LAM = lamivudine; LdT = telbivudine; OAS = 2', 5'-oligoadenylate synthase; r = resistant; RI = replication intermediates; rt,RT = reverse transcriptase; TDF = tenofovir disoproxil fumarate

to ADV or TDF. The ADVr mutation rtA181T/V, however, decreased susceptibility to LAM and ADV, while the ADVr mutations rtA181V and or rtN236T have been shown to confer low-level resistance to TDF *in vitro* (Qi *et al.,* 2007). In addition, the LAM mutation rtL180M was closely associated with frequent virological resistance to ADV therapy (Lee *et al.,* 2012).

The rtM204I and rtM204V mutations are commonly considered as the primary resistance mutations to LAM and decrease the sensitivity to LAM by 100-fold *in vitro* (Allen *et al.,* 1998). The rtL180M mutation as a secondary resistance mutation to LAM is predominantly detected in association with rtM204I/V/S. It functions as the main compensatory mutation with the ability to partially restore HBV replication, and enhances the resistance to LAM (Fu and Cheng 1998; Melegari *et al.,* 1998). The rtV173L mutation was constantly found as a third mutation in conjunction with rtM204V+rtL180M and enhanced viral replication *in vitro* (Delaney *et al.,* 2003). The rtM204V/I + L180M mutations were found to be predominant in 60% of the patients resistant to LAM (Westland *et al.,* 2005). The rtM204V/I and rtL180M mutations required one additional mutation including rtT184S/A/T/G, rtS202G/I, rtM205V, or rtI169T, to cause ETV resistance (Tenney *et al.,* 2007).

Due to antiviral resistance, an appropriate rescue therapy should be initiated with the most effective antiviral effect and the minimal risk of inducing multi-drug resistant strains. IFN-α may be an option since it works directly by inhibiting the synthesis of viral DNA and by activating antiviral enzymes. There were, however, few studies that suggested that IFN-α may provide a novel therapeutic option for management of patients carrying the LAMr virus, and the rate of sustained response with pegylated IFN-α appears to be lower than that in antiviral treatment-naive patients (Sun *et al.,* 2011; Ratnam *et al.,* 2012). How IFN-α inhibits the replication of HBV drug-resistant mutant is not known. Therefore, in this study we constructed different drug-resistant (LAMr, ADV and ETVr) HBV mutants and used the established mouse model of HBV replication to assess HBV DNA replication in liver after the treatment with the IFN inducer poly-IC.

Material and Methods

Plasmid constructs. Wild type pHBV4.1 is an HBV replication competent plasmid that contains 1.3 copies of the HBV genome (subtype ayw). The LAMr mutants: rtM204I, rt(M204I+L180M), rt(M204V+L180M), rt(M204V+L180M+V173L); ADVr mutants: rtA181T, rtA181V, rtN236T, rt(A181V+N236T); ETVr mutants: rt(M204I+L180M+T184L), rt(M204V+L180M+S202G), rt(M204V+ L180M+M250V) were derived from wild type pHBV4.1 by site-directed mutagenesis (Table 1). All clones were confirmed by direct sequencing.

Transfection of mice with plasmids and application of poly-IC. Male Balb/c mice at specific pathogen-free (SPF) level, weighing 18–20 g, were provided by Huaxi Laboratory Animal Center of Sichuan University. To establish the mouse model, mice were injected via tail vein with 10 μg plasmid DNA in PBS to a volume equivalent to 8% of the total body weight of each animal within 5–8 sec. Twenty-four hours after hydrodynamic injection of the HBV plasmids, mice were injected intraperitoneally with 200 μg Poly-IC in 200 μl PBS. The injection was repeated three times, every 24 hr. The control group received only 200 μl PBS. Mice were sacrificed on the third day, 4–6 hr after the final injection of poly-IC. Liver tissue was collected, frozen in liquid nitrogen and stored at -70°C until further study; serum was collected and stored at -20°C until further study.

Southern blot assay of HBV DNA RI. Frozen liver tissue was mechanically ground in liquid nitrogen and HBV DNA RIs were isolated as described previously (Tang *et al.,* 2005). These HBV DNA RIs were diluted in 30 μl of TE buffer, separated by 1% agarose gel electrophoresis, and DNA was blotted to a positively charged nylon membrane (Amersham, USA). The membranes were probed with digoxigenin-labeled HBV DNA to determine the HBV sequence. Membrane hybridization was detected by using DIG Luminescent Detection Kit (Roche Applied Science) and X-ray film.

RT-PCR assay of OAS. The levels of intrahepatic 2', 5'-oligoadenylate synthase (OAS) mRNA transcripts were analyzed by RT-PCR. Total RNA isolated from the mouse liver was used as the template for reverse transcriptase (RT) (TAKARA, China) first stand reaction with an Oligo dT primer.

OAS specific primers: sense: CTTTGATGTCCTGGGTCATGT, anti-sense: GCTCCGTGAAGCAGGTAGAG. β-actin specific primers: sense: CGTTGACATCCGTAAAGACC, anti-sense: AACAGTCCGCCTAGAAGCAC. The PCR amplification was performed under the following conditions: after an initial denaturation for 5 min at 94°C, samples were subjected to 35 cycles of amplification (94°C 15 sec, 63°C 15 sec, 72°C 45 sec), followed by a final extension for 5 min at 72°C. Reaction products were isolated by 2% agarose gel electrophoresis. The expected bands were 281 bp for β-action and 123 bp for OAS.

Table 1. HBV drug-resistance mutants in the RT region

Drug resistance mutations	Resistance mutations	
LAMr	rtL180M	CTG→ATG
	rtM204I	ATG→ATT
	rtM204V	ATG→GTG
	rtV173L	GTG→TTG
ADVr	rtA181T	TGG→TGA
	rtA181V	GCT→GTT
	rtN236T	AAC→ACC
ETVr	rtT184L	ACT→CTT
	rtS202G	AGT→GGT
	rtM250V	ATG→ATG

Real-time PCR assay of HBV DNA. 100 μl of mouse serum predigested with DNaseI was used for the detection of HBV DNA by quantitative real-time PCR using a diagnostic kit for quantification of HBV DNA (Da An Gene, Guangzhou, China).

Results

Replication of HBV mutants in mice

In order to determine the effects of drug-resistant mutations on HBV replication, mice were divided into different groups and each group was given 10 μg of wt or drug-resistant HBV plasmids DNA, respectively. The replication activity of different HBV mutants was analyzed by southern blotting. As shown in Fig. 1, compared to the wt group, the levels of HBV DNA RI were obviously decreased in the LAMr and ETVr mutants groups except the rt(M204I+L180M+173L) mutant group. In the ADVr mutant groups except the rtA(181V+N236T) mutant group, the level of HBV RI were similar to those in the wt group. These results suggested that the position of mutation in the RT region and the number of mutations play an important role in regulating HBV replication.

The effect of poly-IC on replication of wtHBV and its drug-resistant mutants in mice

Poly-IC as a synthetic analog of double-stranded RNA was originally synthesized as a potent inducer of type I IFN. To investigate the effects of poly-IC on HBV replication, we treated the wt or drug-resistant HBV-transfected mice with 200 μg poly-IC in 200 μl PBS. The control groups received only 200 μl PBS.

The effect of poly-IC on wtHBV replication in mice liver

As shown in Fig. 2, the level of HBV DNA RI was obviously decreased in wt group after the poly-IC treatment. OAS, a known marker of IFN-α/β induction, was detected by RT-PCR and was found in all of mice injected with poly-IC or PBS. However the level of OAS mRNA increased in the poly-IC-treated group compared with PBS group, suggesting that the injection of poly-IC induced IFN pathway. Therefore, IFN can inhibit HBV replication in HBV-transfected mice.

The effect of poly-IC on drug-resistant mutants in mice

As shown in Fig. 3, after poly-IC treatment, HBV DNA RI was significantly decreased in the mice transfected with the wt and the LAMr and ADVr mutant plasmids. Compared with the corresponding control groups, the levels of HBV DNA RI in the LAMr mutant groups were decreased to 41.3%, 30.3%, 24.6% and 43.8% in the mice transfected with rtM204I, rt(M204I+L180M), rt(M204V+L180M) and rt(M204V+L180M+V173L), respectively. The levels of HBV DNA RI in the ADVr mutant groups were decreased to 29.1%, 37.1%, 44.0% and 53.9% in the mice transfected with rtA181T, rtA181V, rtN236T and rt(A181V+N236T), respectively. There were no obvious changes in the ETVr mutant groups.

The changes of HBV DNA levels in serum after the poly-IC treatment are shown in Fig. 4. Compared with the PBS-treated groups, the level of HBV DNA was 1.6 log lower in the wt group; 0.77log, 0.78log, 0.79 log and 1.62 log lower in the rtM204I, rt(M204I+L180M), rt(M204V+L180M), rt(M204V+L180M+V173L) mutant groups, respectively; 1.48 log, 1.41 log, 1.04 log and 1.05 log lower in the rtA181T, rtA181V, rtN236T, and rt(A181V + N236T) mutant groups, respectively; 0.3 log lower in the rt(M204V+L180M+T184L) mutant group, 0.03 log higher in the rt(M204V+L180M+S202G) mutant group and 0.29 log higher in the rt(M204V+L180M+M250V) mutant group, respectively.

In summary, most of the drug-resistant mutations tested were sensitive to poly-IC treatment. Compared with the wt HBV, the LAMr and ADVr mutants showed similar sensitivity to the poly-IC treatment while the ETVr mutants showed resistance to poly-IC treatment.

Discussion

Antiviral therapy is critical for the treatment of patients with chronic HBV infection. High replication rates of HBV and high error rates of viral polymerase allow the virus to adapt rapidly to selection pressures, and resistance to nucleoside analogs is inevitable (Deng and Tang, 2011). Long-term benefit is thus limited by the emergence of drug resistance. IFN-α-based therapy is typically administered for a definite period of time and can induce a sustained suppression of viral replication after the treatment suspension. A few studies have shown that in patients with YMDD-mutated virus after previous lamivudine treatment, the response to IFN-α treatment in combination with lamivudine was mixed (Suzuki *et al.,* 2002; Danalioglu *et al.,* 2004). Therefore, understanding the effects of IFN-α on the inhibition of drug-resistant HBV mutants *in vivo* is important and could provide some suggestions on the management of HBV drug resistance in clinical treatment.

First, we observed that mutations in RT region changed the biological characteristics of the mutant virus. The position and number of the mutations affected the replication capacity of the virus. Most LAM- and ETV-resistant mutants have decreased ability of replication compared with

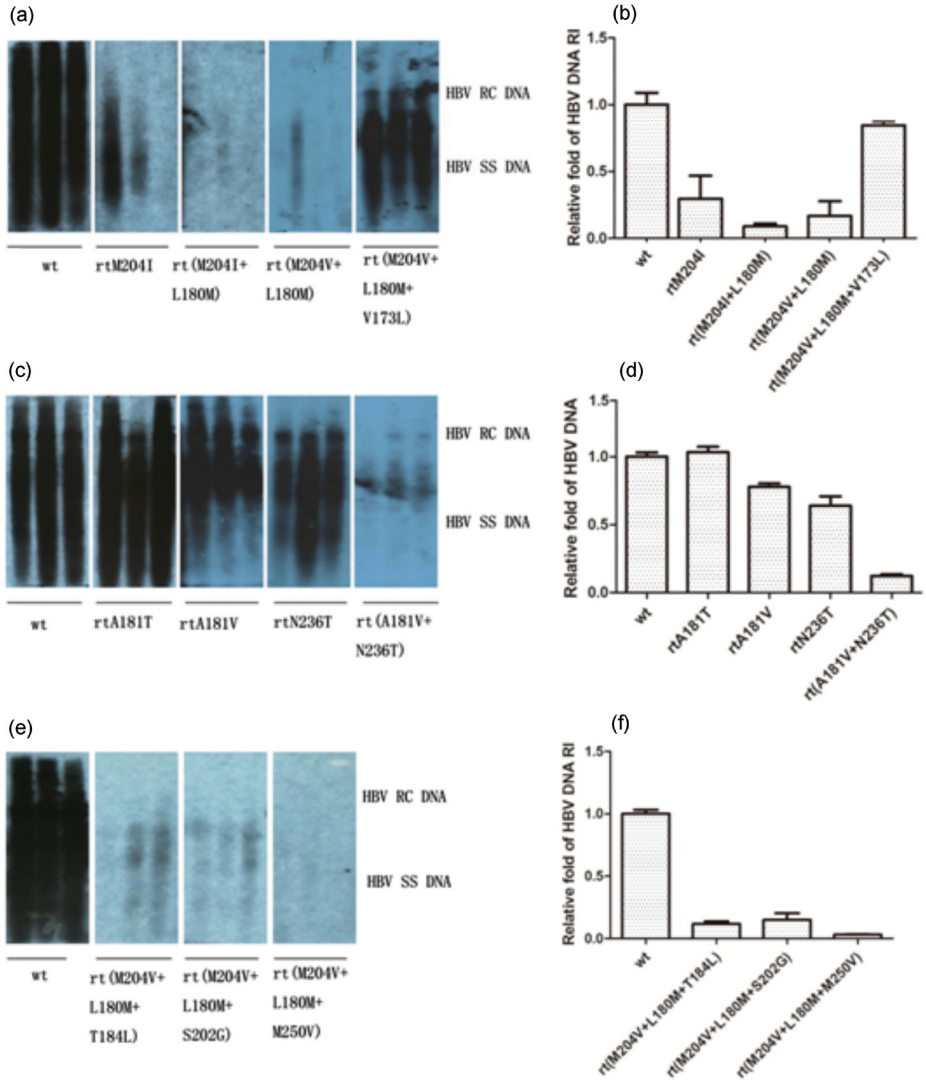

Fig. 1
The effect of drug-resistance mutations in the RT region on HBV replication
HBV DNA RI (rcDNA and ssDNA) levels in mice transfected with wtHBV and LAMr (a, b), ADVr (c, d) and ETVr (E, F) plasmids as assayed by Southern blot hybridization.

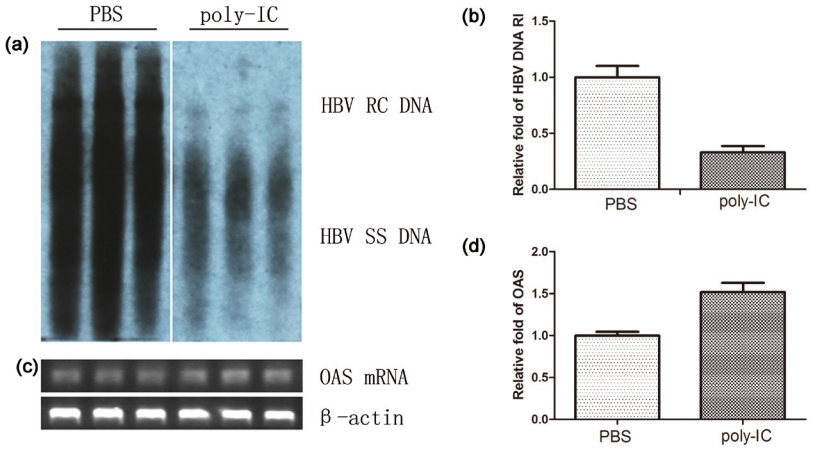

Fig. 2
The effect of poly-IC on wtHBV replication in mice
HBV DNA RI levels in mice treated with poly-IC and PBS, respectively, as assayed by Southern blot hybridization (a, b). OAS levels in mice treated with poly-IC and PBS, respectively, as assayed by RT-PCR (c, d).

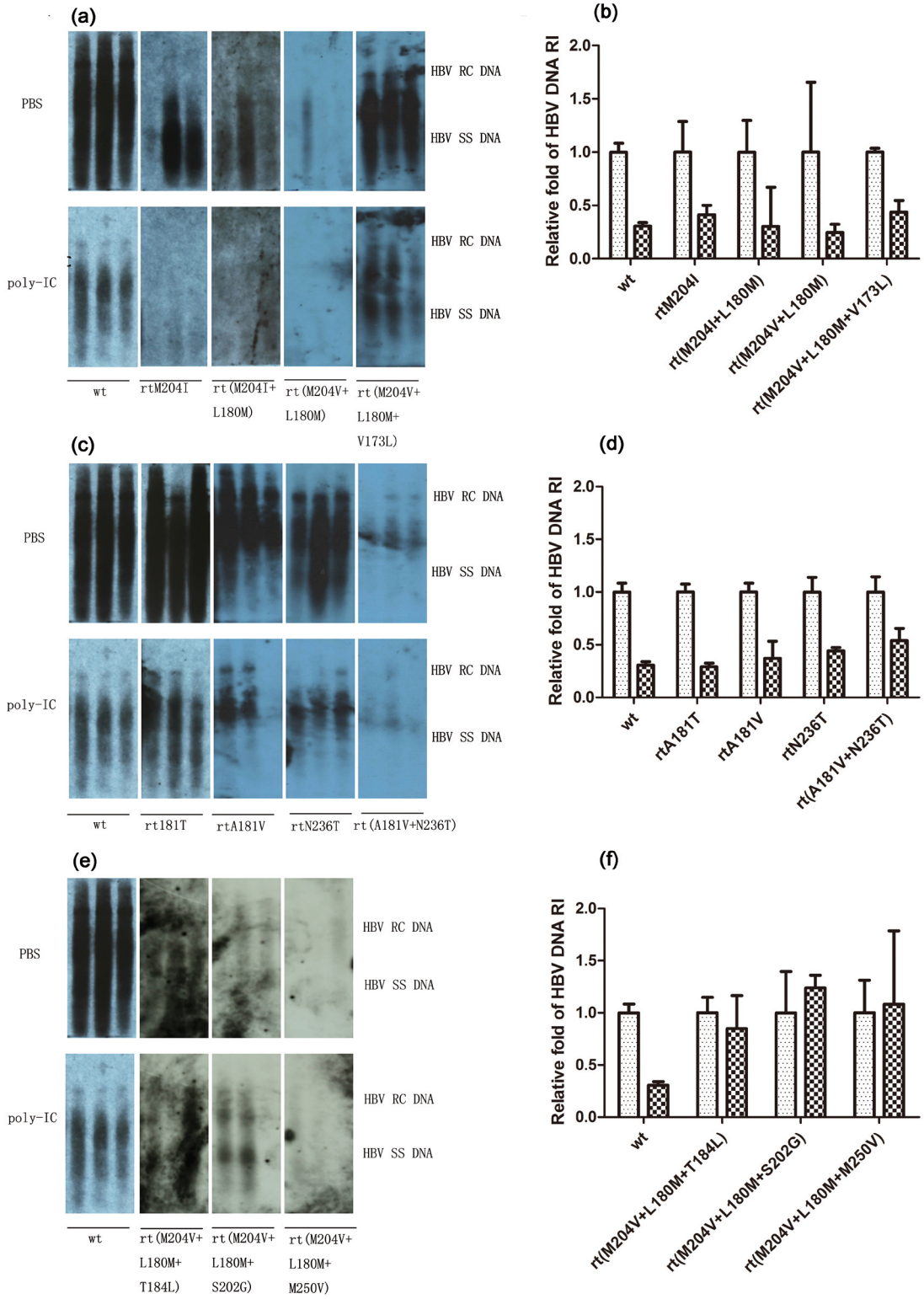

Fig. 3
Effect of mutations in the RT region on the inhibitory effect of poly-IC on HBV replication
HBV DNA RI levels in mice transfected with wt HBV and LAMr (a, b), ADVr (c, d) and ETVr (e, f) plasmids and treated with poly-IC and PBS, respectively. Southern blot hybridization was employed.

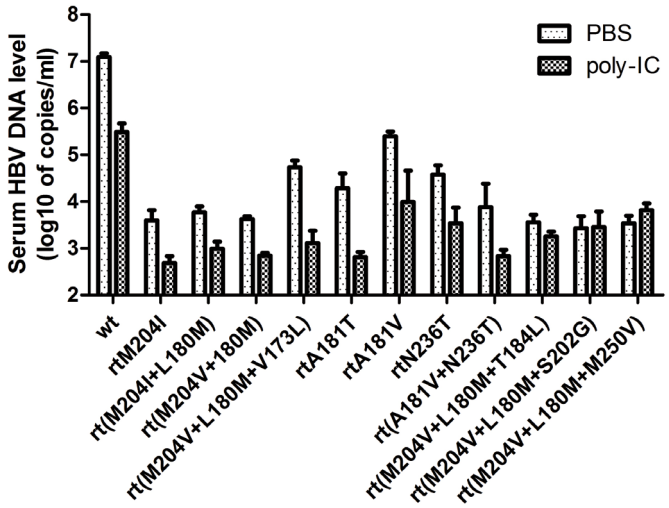

Fig. 4
The effect of poly-IC on the replication of wtHBV and drug-resistant HBV mutants in mice
HBV DNA levels in mice transfected with wtHBV and drug-resistant HBV mutant plasmids and treated with poly-IC and PBS, respectively. Real-time PCR was employed.

the wtHBV as in previous studies (Fu and Cheng, 1998; Melegari *et al.,* 1998; Baldick *et al.,* 2008; Walsh *et al.,* 2010). The compensatory mutations in the RT region were considered to restore the impaired replication capacity of the virus compared with primary mutations (Warner *et al.,* 2007; Ji *et al.,* 2012) such as the rtV173 mutation. In our study, however, the rtL180M mutation as a compensatory mutation to rtM204I/V did not significantly affect the viral replication of rtM204I/V, which was previously reported to enhance mutant virus replication on the ninth day after transfection *in vitro* (Fu and Cheng, 1998; Melegari *et al.,* 1998). The different results may be due to the different HBV replication models and testing time after transfection. Unlike the LAM- and ETV-resistant mutants, the ability of the ADVr mutants to replicate was similar to the wtHBV. This suggested that the position of the mutations in the RT region affects the ability of replication in the mouse liver. A changed ability of replication was found in the rtA181 mutant. The mutation in rtA181T is a truncation, while in rtA181V a substitution. Truncation was found to impair the secretion of HBV surface antigen and to increase and prolong the viral replication in the mouse liver, a lower level of HBV DNA in serum was observed compared with the wild-type and rt181V groups (Warner and Locarnini, 2008; Dai *et al.,* 2012).

Second, the poly-IC treatment inhibited viral replication in the LAMr and ADVr groups, but not in the ETVr groups. Therefore, these results indicate that the mutations in the HBV RT region may reduce the inhibitory effect of IFN-α on HBV replication. Furthermore, the IFN regulatory element mutant in HBV genome reduced the inhibitory effect of IFN-α on HBV replication in our mouse model of HBV replication (Motta *et al.,* 2010; Liu *et al.,* 2012). During IFN-α treatment, multiple mutations occurred in the HBV genome, including the viral polymerase, precore, core and the core promoter regions (Chen *et al.,* 2003). Though IFN has multiple sites of action in the viral life cycle and is independent of the RT activity, the mutation of the HBV genome may directly influence the IFN-mediated inhibition on viral DNA synthesis.

However, as shown in previous studies, LAM-resistant HBV patients with the rtM204V mutation had the highest risk of developing ETV resistance compared with those with the rtM204I mutation (Lee *et al.,* 2013). In addition, the rtL229F mutation effectively restored the replication capacity of the rtM204I strain compared with the rtL229W/M/V (Ji *et al.,* 2012). The replication capacities of LAMr (rtM204V+L180M) HBV with various rtM250 substitutions also displayed different replication levels (Baldick *et al.,* 2008). Different substitutions in the same position of HBV chain had different impact on the replication capacity and the drug resistance risk of the virus. Therefore, further studies to understand how other mutations respond to the IFN-α treatment are important.

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