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Biological impacts of "hot-spot" mutations of hepatitis B virus X proteins are genotype B and C differentiated

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Abstract

AIM: To investigate the biological impacts of "hot-spot" mutations on genotype B and C HBV X proteins (HBx).

METHODS: Five types of "hot-spot" mutations of genotype B or C HBV X genes, which sequentially lead to the amino acid substitutions of HBx as I127T, F132Y, K130M+V131I, I127T+K130M+V131I, or K130M+V131I+F132Y, respectively, were generated by means of site-directed mutagenesis. To evaluate the anti-proliferative effects, HBx or related mutants' expression vectors were transfected separately to the Chang cells by lipofectamine, and the cells were cultured in hygromycin selective medium for 14 d, drug-resistant colonies were fixed with cold methanol, stained with Giemsa dyes and scored (increase of the colonies indicated the reduction of the anti-proliferation activity, and vice versa). Different types of HBx expression vectors were co-transfected separately with the reporter plasmid pCMVβ to Chang cells, which were lysed 48 h post-transfection and the intra-cellular β-galactosidase activities were monitored (increase of the β-galactosidase activities indicated the reduction of the transactivation activity, and vice versa). All data obtained were calculated by paired-samples *t*-test.

RESULTS: As compared to standard genotype B HBx, mutants of I127T and I127T+K130M+V131I showed higher transactivation and anti-proliferative activities, while the mutants of F132Y, K130M+V131I, and K130M+V131I+F132Y showed lower activities. As compared to standard genotype C HBx, I127T mutant showed higher transactivation activity, while the other four types of mutants showed no differences. With regard to anti-proliferative activity, compared to standard genotype C HBx, F132Y and K130M+V131I mutants showed lower activities, and K130M+V131I+F132Y mutant, on the other hand, showed higher activity, while the mutants of I127T and I127T+K130M+V131I showed no differences.

CONCLUSION: "Hot-spot" mutations affect the anti-proliferation and transactivation activities of genotype B and/or C HBx, and the biological impacts of most "hot-spot" mutations on HBx are genotype B and C differentiated.

Key words: Hepatitis B virus; Genotype; X gene; Mutation

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INTRODUCTION

HBV belongs to the hepadnaviridae family, which has a double-stranded DNA genome with a single-stranded gap. The genome of HBV encodes four kinds of proteins including the envelope protein (S/Pre-S), the core protein (C/pre-C), the polymerase (P), and the X protein (HBx). HBx is a multi-functional transcriptional transactivator[1] and has been shown to be crucial in the pathogenesis of HBV-related diseases[2].

Based on an intergroup divergence of 8% or more in the complete or partial nucleotide sequences, HBV can be classified presently into eight genotypes[3,4], termed A-H, in which B and C genotypes are prevalent in East Asia including China[5]. Recent studies[6,7] revealed that the infection of genotype B or C HBV brought about quite different clinical manifestations and liver histological changes, yet the exact mechanisms involved remain largely obscure. Considering the important role HBx plays in the pathogenesis of HBV, it is reasonable to speculate that the clinical and histological differences of the infection of genotype B and C HBV may be partially related with the functional differentiations between these two genotypes of HBx due to the genetic variability.

Genetic variability[8] of HBx includes both of the genotype-specific variations and the mutations emerged during chronic infection. We had shown in our previous study[9] that there were 16 amino acid genotype-specific

variations between genotype B and C HBx, and the biological functions including transactivation activities and anti-proliferation capacities were different between these two HBx genotypes. It was also demonstrated that during chronic infection, genotype B and C HBx shared the same "hot-spot" mutations in the amino acid positions of 127, 130, 131, and 132[10], we wondered whether these "hot-spot" mutations effected HBx, and more importantly, whether the influences of the same type of mutation on HBx showed genotype B or C specificity. To address these issues, we constructed HBx mutants of each genotype (B or C) with the same "hot-spot" mutations, and comparatively analyzed their differences in biological functions.

MATERIALS AND METHODS

Plasmids

pcDNA3.1-XB and pcDNA3.1-XC were recombinant pcDNA3.1/Hygro(-) (Invitrogen, USA) vectors harboring either genotype B or genotype C HBV X gene with conserved sequences (the amino acid alignment of these two genotypes of HBx is shown in Figure 1) and could express HBx in transfected Chang cells, as detected by immunohistochemistry.

Generation of genotype B or C HBV X mutants with "hot-spot" mutations

pcDNA3.1-XB or pcDNA3.1-XC was used as the template and the mutagenesis was carried out using GeneTailor site-directed mutagenesis system (Invitrogen, USA) following the manufacturer's instructions. In brief, 200 ng of pcDNA3.1-XB or pcDNA3.1-XC DNA was methylated by 8 U methylase in a total volume of 32 μL, and 2 μL of methylated DNA was used as the template for PCR using paired primers (reverse primer R was used together with one of the forward primers F1-F5 to generate nucleotide mutation, sequentially resulting in amino acid substitution) (Table 1). After amplification, 5 μL of PCR product was used to transform *E. coli* $DH_{5\alpha}$-T1, and transformants were screened by growth on bacterial culture plates with ampicillin for selection. All mutants were sequenced to confirm the correctness of the induced mutations.

Cell culture and transfection

The human liver cell line, CCL13 (Chang cells) was cultured in Dulbecco's modified Eagle minimal essential medium with penicillin (100 U/mL) and streptomycin (100 μg/mL), supplemented with 10% fetal calf serum. Recombinant plasmid DNA was extracted and purified with Qiagen maxiprep kits, and used to transfect Chang cells in 60-mm plates or six-well plates by lipofectamine 2000. Duplicate plates/wells were used for all samples. Empty vector pcDNA3.1/Hygro(-) was used as a mock transfection control.

Colony formation assay

Chang cells (3×10^5) in six-well plates were transfected with 2.5 μg of HBx constructs or empty vector. Forty-eight hours after transfection, the cells were subcultured at a ratio of 1∶3, and cultured in 200 μg/mL hygromycin (Roche, Germany) selective medium for 14 d. Drug-resistant colonies were fixed with cold methanol, stained with Giemsa, and then scored.

β-galactosidase test

Two micrograms of HBx constructs and 2.0 μg reporter pCMVβ plasmids (Promega, USA) were co-transfected into Chang cells in 60-mm plates (5×10^5 cells per plate). Cells were harvested and lysed at 48 h post-transfection. After protein normalization by the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA), equal amounts of protein (30 ng) were used for β-galactosidase assay following the manufacturer's instructions.

Statistical analysis was performed using a paired *t*-test. $P<0.05$ was taken as significant.

RESULTS

Generation of genotype B or C HBx mutants

Mutations at HBV nucleotides 1 753, 1 762, 1 764, and 1 768 resulted in amino acid substitution of HBx at positions 127, 130, 131, and 132, respectively. In this study, nucleotide mutations were generated in each genotype of HBx with five types of combinations as $1\ 753^{T\rightarrow C}$, $1\ 762^{A\rightarrow T}+1\ 764^{G\rightarrow A}$, $1\ 753^{T\rightarrow C}+1\ 762^{A\rightarrow T}+1\ 764^{G\rightarrow A}$, $1\ 762^{A\rightarrow T}+1\ 764^{G\rightarrow A}$

Table 1 Sequences and related applications of the primers used for site-directed mutagenesis and nomenclature of HBx mutants

Primers	Sequences (5'→3')	Applications		Nomenclature of the HBx mutants
		Nucleotide mutation[2]	Amino acid substitution[3]	
R	CTCCTCCCCCAACTCTTCCCACTCAGTAAAC			
F1	GAAGAGTTGGGGGAGGAGAC[1] TAGGTTAAAGGTC	$1\ 753^{T\rightarrow C}$	I127T	XB/XC-127
F2	GAAGAGTTGGGGGAGGAGACTAGGTTA ATGATCTTTGTACTAGG	$1\ 753^{T\rightarrow C}+1\ 762^{A\rightarrow T}+1\ 764^{G\rightarrow A}$	I127T+K130M+V131I	XB/XC-127-130-131
F3	GAAGAGTTGGGGGAGGAGATTAGGTTA ATGATCTTTGTACTAGG	$1\ 762^{A\rightarrow T}+1\ 764^{G\rightarrow A}$	K130M+V131I	XB/XC-130-131
F4	GAAGAGTTGGGGGAGGAGATTAGGTTA ATGATCTATGTACTAGGAGGC	$1\ 762^{A\rightarrow T}+1\ 764^{G\rightarrow A}+1\ 768^{T\rightarrow A}$	K130M+V131I+F132Y	XB/XC-130-131-132
F5	GAAGAGTTGGGGGAGGAGATTAGGTTA AAGGTCTATGTACTAGGAGGC	$1\ 768^{T\rightarrow A}$	F132Y	XB/XC-132

[1]Mutated nucleotides are shown as underlined; [2]1 753, 1 762, 1 764, 1 768 indicate the positions of mutated nucleotides in HBV genome; [3]127, 130, 131, 132 are positions of substituted amino acids in HBx.

+1 $768^{T\to A}$ and 1 $768^{T\to A}$, which sequentially resulted in amino acid substitutions as I127T, K130M+V131I, I127T+K130M+V131I, K130M+V131I+F132Y, and F132Y, respectively (Table 1 and Figure 1). The X mutants were named according to their genotype belongings (B or C) and the position of the substituted amino acids, for example, XB-127 for I127T mutant of genotype B HBx, while XC-127 for I127T mutant of genotype C HBx.

```
          5         15        25        35        45        55
XB  MAARLCCQLD PARDVLCLRP VGAESRGRPL PGPLGALPPA SPPVVPTDHG AHLSLRGLPV
XC  ....V.....  .........  .........V S..F.P..SP .SSA..A...  ..........
          65        75        85        95        105       115
XB  CAFSSAGPCA LRFTSARRME TTVNAHRNLP KVLHKRTLGL SAMSTTDLEA YFKDCVFTEW
XC  ..........  ..........  ......QV..  ..........  ..........  .....L.KD.
          125       135       145
XB  EELGEDIRLKVFVLGGCRHK LVCSPAPCNF FTSA
XC  ..........  ..........  ..........  ....
```

Figure 1 There were 16 amino acid variations between genotype B and C HBx, which were located at amino acid positions 5, 30, 31, 34, 36, 39, 40, 42, 43, 44, 47, 87, 88, 116, 118, and 119, respectively. Four boxed amino acids, located at the positions of 127, 130, 131, 132, respectively, were to be substituted by site-directed mutagenesis.

Anti-proliferation activities of genotype B or C HBx mutants

To study the anti-proliferation activities of HBx mutants, we performed colony formation assay[11], in which the increasing anti-proliferation activities should be paralleled with the reduction of cell colonies, and vice versa. Chang cells were transfected separately with parental vector pcDNA3.1/Hygro(-) or recombinant vectors harboring different types of HBx; cells were subcultured in hygromycin selective medium 48 h post-transfection, and the drug-resistant colonies formed 14 d later were stained with Giemsa and scored. The results showed that, as compared with XB (standard genotype B HBx), expression of mutants XB-127 and XB-127-130-131 caused the reduction in the number of colonies, and expression of mutants XB-132, XB-130-131, or XB-130-131-132 caused the elevation (Figure 2). As for genotype C HBx, expression of mutant XC-130-131-132 caused the sharp reduction of colonies to zero, and mutants of XC-132 and XC-130-131, on the other hand, caused the elevation in the number of colonies as compared with XC (standard genotype C HBx), while XC-127 and XC-127-130-131 showed no differences from XC (Figure 3). It indicated that effects of three types of "hot spot" mutations, i.e., I127T, I127T+K130M+V131I, and K130M+V131I+F132Y, on anti-proliferation of HBx were

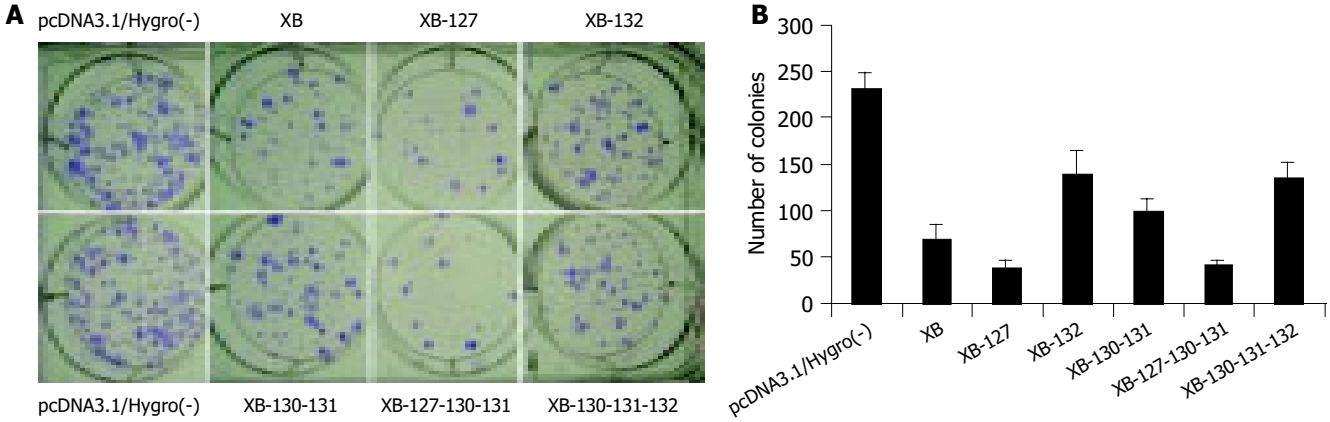

Figure 2 Chang cells were transfected by genotype B X gene expression vectors and screened by hygromycin, the drug-resistant colonies formed 14 d post-transfection were fixed by cold methanol, stained with Giemsa (**A**) and counted (**B**). The results are shown as mean±SD of six separate experiments.

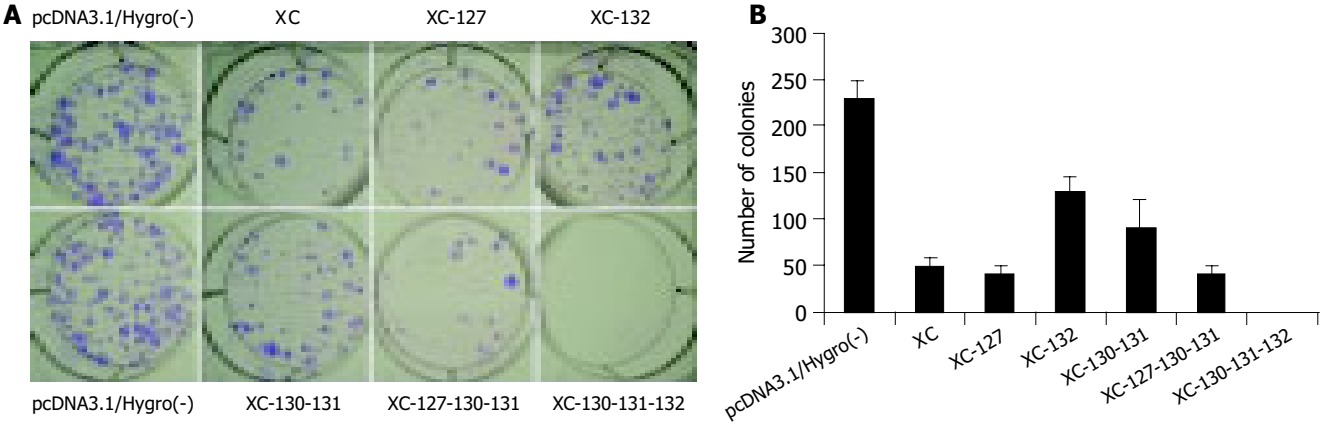

Figure 3 Chang cells were transfected by genotype C X gene expression vectors and screened by hygromycin, the drug-resistant colonies formed 14 d post-transfection were fixed by cold methanol, stained with Giemsa (**A**) and scored (**B**). The results are shown as mean±SD of six separate experiments.

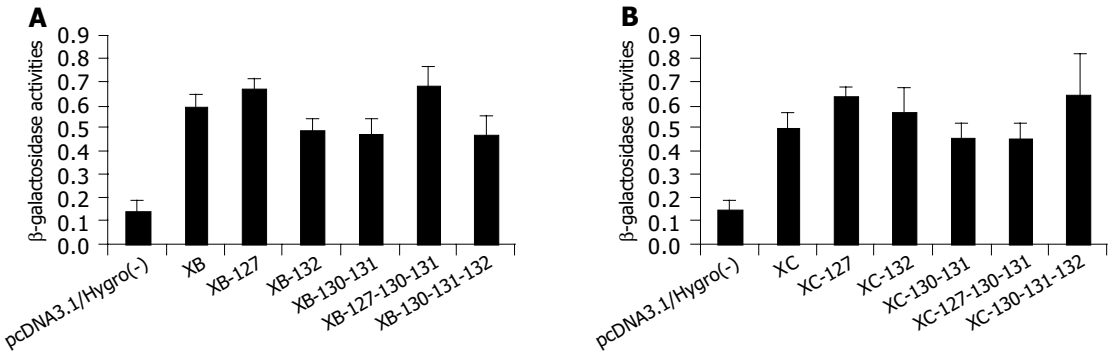

Figure 4 Chang cells were co-transfected by pCMVβ together with the X gene or related mutants with genotype B (**A**) or genotype C (**B**). Forty-eight hours post-transfection, the cells were lysed and the intracellular β-galactosidase activities were monitored after protein normalization. The results are shown as mean±SD of six separate experiments.

genotype B and C differentiated. In addition, it was also demonstrated that all HBx caused dramatic reduction of colonies as compared with the empty vector pcDNA3.1/Hygro(-), irrespective of genotypes (B or C) or the types (the standard or the mutants), indicating the retaining of anti-proliferation activities of all HBx.

Transactivation activities of genotype B or C HBx mutants

It was reported previously that HBx could transactivate the CMV immediate early promoter[12], so plasmid pCMVβ containing the CMV immediate early promoter and expressing β-galactosidase was used as the indicator to evaluate the transactivation activities of HBx mutants. In our study, pCMVβ was co-transfected with an HBx construct to Chang cells, which were lysed 48 h post transfection, and intra-cellular β-galactosidase activities were monitored. The results showed that, as for genotype B HBx, expression of mutants XB-127 and XB-127-130-131 induced increase in β-galactosidase activities as compared with XB, and three other mutants, i.e., XB-132, XB-130-131, and XB-130-131-132 induced decreased activities (Figure 4A and Table 2), as for genotype C HBx, XC-127 induced the increase in β-galactosidase activities as compared with XC, while the other four types of mutants showed no differences (Figure 4B and Table 2). The results revealed that effects of four types of "hot spot" mutations, i.e., I127T+K130M+V131I, K130M+V131I, K130M+V131I+F132Y, and F132Y, on transactivation of HBx were genotype B and C differentiated. In addition, the results also showed that expression of HBx, irrespective of genotypes (B or C) or the mutant types, induced the increase in intra-cellular β-galactosidase activities as compared with the empty vector pcDNA3.1/Hygro(-), indicating the retaining of transactivation activities of all HBx.

Table 2 Intracellular β-galactosidase activities of transfected Chang cells by pCMVβ and X gene expression vectors

DNA transfected	β-galactosidase activities[1]	*P*[2]	*P*[3]
pcDNA3.1/Hygro(–)	0.147±0.039	-	-
XB	0.593±0.051	<0.01	-
XB-127	0.666±0.054	<0.01	<0.05
XB-132	0.492±0.052	<0.01	<0.01
XB-130-131	0.477±0.065	<0.01	<0.01
XB-127-130-131	0.680±0.090	<0.01	<0.05
XB-130-131-132	0.472±0.081	<0.01	<0.05
XC	0.505±0.064	<0.01	-
XC-127	0.641±0.044	<0.01	<0.01
XC-132	0.571±0.104	<0.01	>0.05
XC-130-131	0.459±0.065	<0.01	>0.05
XB-127-130-131	0.457±0.067	<0.01	>0.05
XB-130-131-132	0.646±0.177	<0.01	>0.05

[1]All data are expressed as mean±SD of six separate experiments; [2]Paired-samples *t*-test between pcDNA3.1/Hygro(–) and all HBx; [3]Paired-samples *t*-test between genotype B(XB)/genotype C(XC) and the relative mutants.

DISCUSSION

There are increasing evidences that the clinical manifestations, the long-term prognosis, the liver histological changes, and the response to treatment of interferon and nucleosidic inhibitors may differ depending on which genotype of HBV the patient has been infected with[13]. In Taiwan, where the genotypes of HBV were predominantly B and C, genotype C is associated with more severe liver disease and genotype B is associated with the development of hepatocellular carcinoma (HCC) in young non-cirrhotic patients[6]. In contrast, genotype B has a relatively good prognosis in Japan and China and is rarely associated with the development of HCC[7]. However, the impact of genotypes on HBV pathogenesis remains largely obscure.

HBx is a promiscuous activator[1] and considered to be of importance in the pathogenesis of HBV, it can indirectly activate miscellaneous viral and cellular genes[14], induce or block apoptosis of the infected cells[15,16], and induce HCC in certain lines of transgenic mice[17,18]. We demonstrated previously that there were 16 amino acid differences between standard genotype B and C HBx, and the activities including transactivation and anti-proliferation were different between these two genotypes of HBx. These results indicated that the B, C genotype-specific structural and functional differentiations of HBx may partially contribute to the different clinical outcomes and histological changes of the infection with genotype B or C HBV.

In addition to the genotype-specific variations, both of B and C genotypes of HBx also showed the same gene mutations within their coding regions (nt1 376-1 840), i.e., nt1 $753^{T\to C}$, nt1 $762^{A\to T}$, nt1 $764^{G\to A}$, and nt1 $768^{T\to A}$, which accordingly led to the amino acid variations as I127T, K130M, V131I, and F132Y. These "hot-spot" mutations

Table 3 Summary of the biological impact of "hot-spot" mutations on genotype B and C HBx

HBx mutants	Genotype B[1]		Genotype C[2]	
	Transactivation	Antiproliferation	Transactivation	Antiproliferation
I127T	↑[3]	↑	↑	-[5]
I127T+K130M+V131I	↑	↑	-	-
K130M+V131I	↓[4]	↓	-	↓
K130M+V131I+F132Y	↓	↓	-	↑
F132Y	↓	↓	-	↓

[1]Changes of biological functions were compared with XB; [2]Changes of biological functions were compared with XC. [3, 4,] and [5] accordingly indicate increased, decreased and equal activities of HBx mutants as compared with XB/XC.

emerged due to the error-prone reverse transcriptase activity of HBV polymerase[19] and the long-term virus-host interaction, and occurred mainly during chronic infection including different clinical stages of chronic hepatitis[20] and HBV-associated HCC as well[21]. In fact, these "hot-spot" mutations appeared predominantly in five types: I127T, F132Y, K130M+V131I, I127T+K130M+V131I, or K130M+V131I+F132Y. Our results demonstrated that these mutations affected the anti-proliferation and transactivation activities of genotype B and/or C HBx, and more importantly, the impacts of most types of the "hot-spot" mutations on HBx were genotype B and C differentiated, i.e., as concerning the anti-proliferation activities, impacts of HBx mutants of I127T, I127T+K130M+V131I, and K130M+V131I+F132Y showed genotype B and C differentiated, and regarding the transactivation activities, impacts of mutants of I127T+K130M+V131I, K130M+V131I, K130M+V131I +F132Y, and F132Y showed genotype B and C differentiated (Table 3).

It was also revealed that all HBx mutants, irrespective of genotypes or mutant types, retained their biological functions. It may be therefore concluded that the effects of the mutations on HBx was relatively mild and was in strong contrast to the distal COOH-terminal deletion mutants isolated from the integrated chromosomes of HCC tissues, which completely abrogated the transactivation or anti-proliferation activities[22,23]. In addition, it was previously reported that the transactivation activities of HBx are often linked to anti-proliferation function[24]. Our results strikingly showed that the linkage of these two activities only occurred in genotype B HBx, in which the increasing activation activities of HBx mutants were coupled with the enhanced anti-proliferation activities, and vice versa, while it seemed that the activities of anti-proliferation and transactivation were completely separated in genotype C HBx (Table 3). Therefore, it was concluded that association of transactivation with anti-proliferation activities was a common feature of HBx with genotype B rather than genotype C.

There is no indisputable evidence showing that the transactivation activity of HBx contributes to liver disease. However, reduction of the anti-proliferation effect may endow a growth advantage on cells containing these particular HBx mutants, while increase of anti-proliferation effects will lead to cell disruption and hence accelerate intracellular spread of HBV[25]. So in this point, any functional changes of anti-proliferation are pathogenic.

In summary, "hot-spot" mutations affect the anti-proliferation and transactivation activities of genotype B and/or C HBx, and the biological impacts of most "hot-spot" mutations on HBx are genotype B and C differentiated. Together with our previous findings demonstrating the different biological functions between genotype B and C HBx[9,26], we conclude that genetic variability results in functional differentiations between genotype B and C HBx. Nevertheless, considering the sophisticated mechanism of HBV pathogenesis[27], whether these genotype B and C specific functional differentiations of HBx are responsible for the different clinical outcomes and liver histological changes between two genotypes of HBV, and more importantly, the exact mechanisms involved await further studies.

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