Hepatology International (2020) 14:944–946
https://doi.org/10.1007/s12072-020-10116-z

EDITORIAL

HBx mutations emerged during antiviral therapy: a new face of a multifaceted HBV protein?

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Received: 24 August 2020 / Accepted: 23 November 2020 / Published online: 3 December 2020

The hepatitis B virus (HBV) is a major human pathogen. The HBV genome encodes four overlapping open-reading frames (ORFs) including the S-ORF, C-ORF, P-ORF, and X-ORF. Among them, polymerase which encoded by P-ORF is the target of all approved nucleos(t)ide analogues (NAs) for antiviral therapy. NAs are generally efficient and well tolerated. However, resistance to non-first line antiviral agents is a major issue affecting long-term therapy [1]. HBx encoded by X-ORF is a nonstructural protein that serves multiple functions during the various stages of chronic HBV infection (CHB) through interaction with a number of host proteins.

In this issue of *Hepatology International*, Lin et al. identified HBx mutants that emerged in patients experiencing lamivudine (LAM) resistance or entecavir (ETV) suboptimal response by sequence analysis and characterized their roles in the HBV replication cycle. Co-transfection of these HBx-mutant plasmids and HBV replication-competent clone into cell lines result in increased nuclear-to-cytoplasmic ratios of core antigen and HBV-DNA, as well as the level of nuclear covalently, closed circular DNA (cccDNA). These results demonstrate that HBx mutants can emerge during LAM or ETV therapy and compensate for the replication suppression of NAs by increased cccDNA. This is an innovative study in which the researchers extend the resistant-associated mutations from polymerase to the X gene, and explore the effect of these mutations on HBV replication in vitro [2].

Mutations provide possibilities for the virus to survive a complex environment. Mutations often arise spontaneously in viral quasispecies followed by some of them were subsequently selected as predominant strains for their adaption in changing conditions. We have known that HBV resistance to NAs is achieved through polymerase mutations. Antiviral agents always target important virological mechanisms; as a result, resistance mutation often led to decreased replication fitness. There are many documented examples of the fitness costs that are associated with NA resistance in HBV antiviral therapy. In most cases, the cost can be ameliorated by the acquisition of compensatory mutations. Hughes et al. classified the common resistance-compensatory mechanisms into four categories [3]. Two of these compensating mechanisms that often appear in antiviral resistance are compensation by an intragenic mutation and compensation by an intergenic mutation. According to the above definitions, regular HBV initial primary mutation and compensatory mutation introduced by Lok et al. belong to compensation by an intragenic mutation, in which primary mutations in the reverse transcription (RT) region of polymerase cause an amino acid substitution that results in reduced susceptibility to an antiviral agent while secondary RT mutations cause amino acid substitutions that restore functional defects in viral polymerase activity (i.e., replication fitness) associated with primary drug resistance. For example, typically rtM204V/I is a primary lamivudine resistance mutation which causes a greater than a 100-fold decrease in susceptibility to LAM in phenotypic assays. rtL180M is the most common compensatory mutation which restores the replication fitness of HBV polymerase that harbors the rtM204V/I mutation [4, 5]. The mutations reported by Lin et al. are involved above intergenic compensation between polymerase and HBx. The results not only enrich our knowledge on the resistance-compensation mechanism of HBV but also provide more thoughts on the role of HBx mutations.

The development of NA resistance can be divided into three phases during HBV antiviral therapy. In the first phase, the wild-type dominates quasispecies at the initiation of antiviral therapy. Along with the application of NAs, a rapid decline was observed in virus load; in the second phase, accompanied by the rapid elimination of wild-type virus,

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resistant strains were selected and replicated at a low level due to their replication fitness cost. In the third phase, the replication capacity of drug-resistant strains is improved by the intragenic (Fig. 1a) or intergenic (Fig. 1b) compensation. It must be pointed out that not all phases are necessary for the development of NA resistance. Some drug-resistant strains can establish a low-level virus replication without phase three [6]. Some resistant strains are both capable of resistance and high-level replication and became dominant strains rapidly under specific NA treatment [7]. In such cases, phase two is skipped. It is undeniable that in the practical case of NA resistance, intragenic and intergenic compensation may occur simultaneously. However, there is no reported study of both compensation mechanisms at the same time.

HBV intergenic compensation mechanism for NA resistance mutations is gradually recognized in these years. There are other non-polymerase resistance-compensation mutations reported, in which naturally occurred core protein mutations were found to compensate for the reduced replication fitness of a lamivudine-resistant HBV isolate [8]. As mentioned in this study, these mutations do not necessarily occur just during resistance or suboptimal response. Moreover, the selection relationship of these mutations with specific NAs is uncertain, i.e., no hot spots were found in patients treated with the same NAs. Although it is not excluded that these HBx mutations were selected in patients with NA resistance or suboptimal response, there is a possibility that they were discovered randomly. By understanding the different pathways of resistance and intragenic compensation of the polymerase (RT region), clinicians have obtained the theoretical guidance for the NA combination therapy. These intergenic findings are inadequate as evidence to optimize antiviral treatment at the present.

Although this is an innovative study of HBx mutations in the development of NA resistance, there is already a mountain of researches on HBx mutations and its complex functions. On the one hand, HBx interacts with cccDNA and basal transcriptional machinery and activates transcription in the nucleus; on the other hand, HBx stimulates signal transduction pathways to benefit virus replication, including factors that affect cell survival, metabolism, proliferation, and transcription pathways in the cytoplasm [9]. A plethora of evidence suggests that mutated HBx plays important role

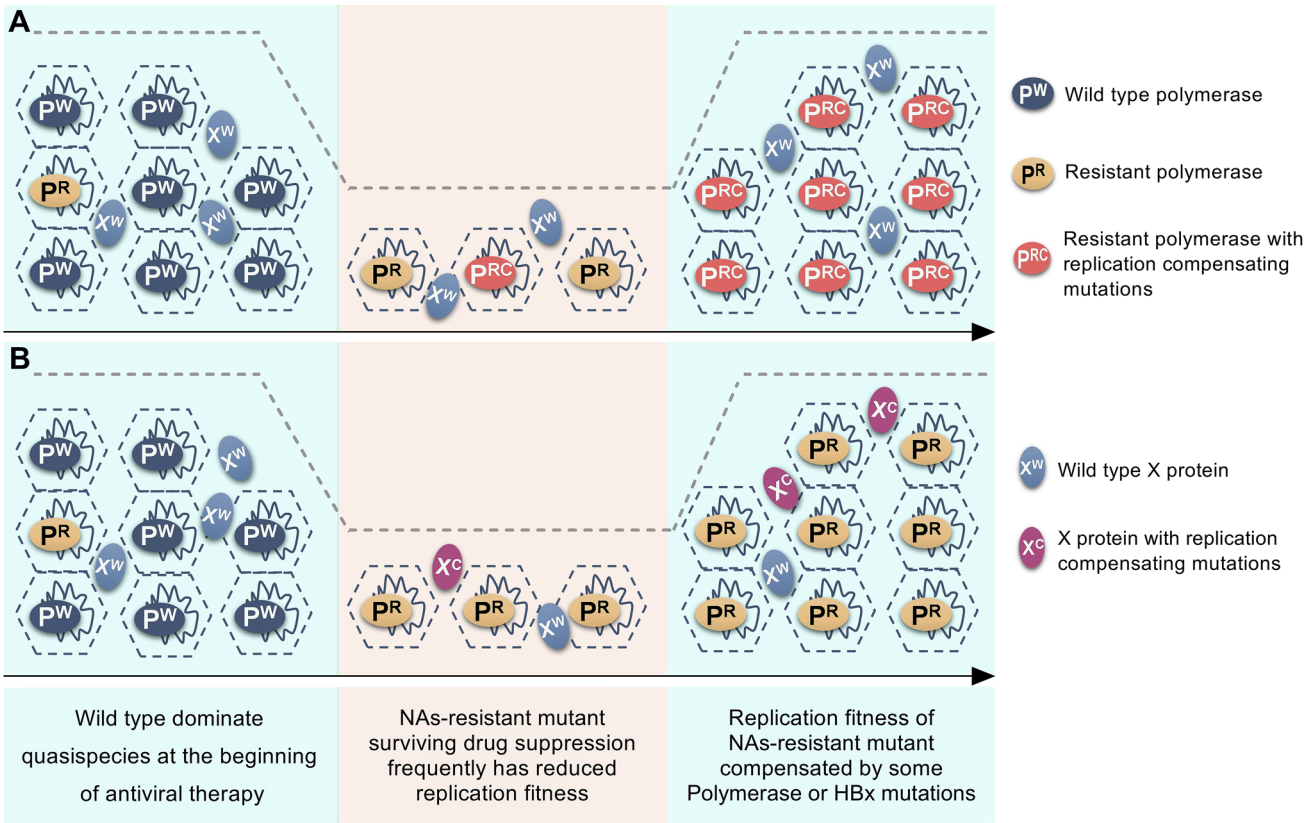

Fig. 1 Intragenic and intergenic compensation mechanism on the replication fitness of HBV NA resistance. **a** The model of widely accepted polymerase intragenic-resistant compensation for the reduced replication fitness of a NA resistance by secondary polymerase mutations; **b** the model of intergenic compensation proposed by Lin et al., in which HBV replication was promoted by HBx mutation-mediated effect on transactivation and cccDNA

in inducing hepatocellular carcinoma (HCC), behaving as a prominent oncogenic driver for HBV-related HCC. If the mutations found in this study were confirmed to be associated with the cell cycle and HBV-related HCC, theoretically, the same effect may occur in any case where the same mutation is found. All these results suggest that we need to consider the multifaceted consequences of mutant HBx on viral replication and host cellular physiology.

Except for the main results above, the authors gave a contradictory statement in the paper. On the one hand, 15 HBx amino acid residues with relatively higher mutation frequencies were presented in the result section. On the other hand, the authors acknowledged that they failed to propose hot spots HBx mutation in the discussion section. We noted that the inconsistency is caused using different reference sequences. 15 HBx mutations with higher frequencies are the result referring to HBV genotype B sequence from GenBank. There are many genotype C samples in Lin's study. Therefore, 15 HBx "mutations" are genotype-specific amino acid residues rather than mutations. For example, the L30V is described as a high-frequency mutation in the paper, what is shown in alignment is that the amino acid residue of genotype B is L, while genotype C is V at the 30th position of HBx. The other so-called higher frequency mutations are the same. As the HBx mutations associate with resistance in a specific patient, the sequence of HBx gene obtained before antiviral treatments (ETV and LAM) was used as reference and compared with the post-treatment sequences to identify amino acid substitutions. In vitro experiments were subsequently proceeded using these naturally isolated clones, which is the conventional method for identifying new emerging mutations in quasispecies. This indicates that the concept of genotype is very important in the identification of mutations. It is necessary to select the appropriate reference when identifying mutations.

In summary, NAs have achieved great success in HBV suppression currently, while HBV cccDNA still exists in hepatocyte of most treated patients. In this regard, it should be noted that the synthesis and function of X protein in hepatocyte of treated patients still need to be determined. The data in this report increase current knowledge on compensatory HBx role in NA-resistant/suboptimal patients. Due to the close relationship of X protein with HBV replication and hepatocellular carcinoma, more detailed studies need to be undertaken to dissect and clarify this issue.

Funding National Science and Technology Major Project (2018ZX10301202 to JH), Local Innovative and Research Teams Project of Guangdong Pearl River Talents Program (2017BT01S131 to JH).

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