Functional significance of hepatitis B virus subgenotype Cs genomic markers

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KEY MESSAGES

1. The G1613A mutation suppresses hepatitis B e antigen (HBeAg) secretion and increases the viral load.
2. The T2170G mutation decreases the extracellular concentration of HBeAg, although it has a milder effect when compared with G1613A.
3. Co-mutations of G1899A and T2170G/T2441C counteract the effect of G1613A and inhibit hepatitis B virus (HBV) DNA synthesis.
4. RFX1 plays a significant role in transactivating HBV core promoter activity with the G1613A mutation.
5. Mutations at the nt. 1613, 1899, 2170, and 2441 of the HBV genome could affect subcellular localisation of the HBV core protein.

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Introduction

Chronic hepatitis B virus (HBV) infection increases the risk of developing primary hepatocellular carcinoma (HCC); about one million people die from HBV-related diseases annually. Development of new strategies for the diagnosis and treatment of chronic HBV infection is important. In a case-control study to identify markers in the HBV genome that can be associated with HCC development, the complete HBV genomes from 200 HCC patients and controls were sequenced and genotyped; several hot-spot mutations in the HBV genome were identified as markers in each genotype.[1] In this study, four genomic markers of HBV subgenotype Cs were selected to evaluate the impact of these markers on viral DNA replication and protein synthesis.

Methods

This study was conducted from January 2010 to December 2011. Four genomic markers on HBV subgenotype Cs were selected (Table). Replication-competent HBV subgenotype Cs' clones of the wild-type HBV genome were originally extracted from the serum of an HBV-positive individual. A 1.3 x HBV genome of the Cs subgenotype was successfully constructed in the pUC18 vector. Other replication-competent HBV clones with either single or combinational mutations were subsequently constructed by site-directed mutagenesis.

The clones were then transfected into Huh7 hepatoma cells to enable viral replication. HBV surface antigen and HBV e antigen (HBeAg) were detected both in culture media and in lysate by enzyme-linked immunosorbent assay. The HBV DNA was then detected by quantitative real-time polymerase chain reaction and Southern blot analysis. To explore the impact of the mutations on HBV core antigen (HBcAg), the subcellular localisation of the HBcAg in HBV mutants was observed by using confocal microscopy.

The wild-type and G1613A mutated core promoters were cloned. The effect of the G1613A mutation on core promoter activity was evaluated by dual-luciferase reporter assay. The effect of the mutation on the binding of endogenous cellular proteins was assessed by the electromobility shift assay (EMSA).

Continuous variables were compared using *t* test or Mann-Whitney *U* test as appropriate. Categorical variables were compared using Chi-squared test or Fisher's exact test as appropriate. All statistical tests were two sided, and a P value of <0.05 were considered statistically significant.

Results

Using confocal microscopy, the localisation pattern of HBV core protein and virions in HBV-transfected Huh-7 cells was observed. Four distinct patterns of localisation were identified. Each pattern represented a stage of the life cycle during HBV replication. Although all patterns could be observed in liver cells transfected with wild-type HBV and HBV mutants, the distribution of phenotypes in some mutants was

quite different from the others.

essential for pregenomic RNA encapsidation and viral DNA replication.[6] However, the exact role of phosphorylation of this serine remains elusive.

In this study, the N90K and S181P mutations on the core protein affected the pattern of core protein localisation in hepatocytes. It was hypothesised that T2170G (N90K) mutation may disrupt the structure of the core protein and hence modify the structure of the capsid. This may affect core capsid formation and therefore the subsequent viral replication process. Moreover, the T2441C (S181P) mutation may alter the interaction between the HBV pregenomic RNA and the core protein during RNA encapsidation, and therefore affect the process of DNA replication.

In this study, the G1613A mutation led to suppression of HBeAg secretion. Emergence of the HBeAg-negative/-reduced mutant during chronic infection led to more active disease on liver histology, especially when the mutant became predominant with a high viral load in serum. The G1613A mutation, which triggered suppression of HBeAg secretion and enhancement of viral DNA production, may contribute to a more aggressive stage of liver disease.

Two main protein complexes were found to be associated with the NRE sequence of the core promoter and showed differential binding affinity towards the wild-type and mutant NRE sequences. This suggested a possible role of the G1613A mutation on regulating core promoter activity, and hence modulating viral replication and protein secretion. RFX1 was identified as binding to the G1613A mutant with higher affinity than the wild-type sequence and possessed the transactivating effect to enhance core promoter activity in liver cells. The G1613A-mutated NRE sequence shared higher homology with the consensus RFX1 binding sequence than the wild-type sequence, indicating that the higher affinity of the mutant NRE to the protein led to transactivation of the core promoter activity.

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