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Hepatitis B virus PreS2 mutants in liver cancer patients

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ABSTRACT

Liver cancer, or Hepatocellular Carcinoma (HCC), is one of the most common cancers in the world. Chronic infection with the hepatitis B virus (HBV) is one of the most critical factors that cause it. During chronic infection with HBV, variants of the virus are created. They may have deletion mutations in the PreS2 region. These variants may play a role in the occurrence of HCC. This study aims to determine the presence of these mutants in patients with liver cancer in China. For this purpose, virus DNA was extracted from the serum of 10 patients with HCC. After amplifying the PreS region and determining the sequence of this region from the genome, the presence of PreS2 mutants in these patients was investigated compared to the database. The results showed that a point mutation was observed at the level of the start codon of PreS2 in two samples. In three of the isolates, several amino acids were deleted at the end of the PreS2 region. In PreS2 deletion mutants, the T-cell and B-cell epitopes on the PreS2 region product are generally deleted. As a result, conditions are created where the virus can escape from the immune system. These mutant PreS2 proteins accumulate in the endoplasmic reticulum (ER) network and cause ER stress. In this way, in addition to creating unstable conditions in the cell genome, the proliferation of hepatocytes is stimulated indirectly. As a result, there is a possibility that the cells will progress toward becoming cancerous.

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Introduction

The Hepatitis B virus (HBV) causes many acute and chronic liver diseases. In most cases, acute HBV infections are usually self-limiting. While chronic infection usually remains throughout life (1-3). Clinical manifestations of chronic HBV infection include chronic hepatitis, chronic carrier, cirrhosis, and hepatocellular carcinoma (HCC) (4). Hepatocellular Carcinoma (HCC) is one of the most common cancers in the world, especially in Africa and East Asia (5). It is also the third cause of cancer death among men and the seventh cause of cancer death among women, causing the death of one million people annually. It happens all over the world. Epidemiological studies show that chronic infection with HBV is one of the leading causes of HCC (6). Hepatitis B virus (HBV) is a small and enveloped virus that belongs to the Hepadnaviridae family. The genome of this virus is 2.3 Kb long and consists of an open reading frame (ORF) that encodes the Core X protein, polymerase, and surface proteins of the virus (7-9).

The surface proteins of the virus include Small (S), Middle (M), and Large (L) proteins, all of which are the product of a single ORF (9, 10). M protein is the product of PreS2 and S region. The PreS2 region encodes 55 amino acids of the amino terminus of protein M. The amino end of the PreS2 region contains epitopes recognized by B cells and T cells (11). Antibodies induced by peptides in this region have protective properties. In several countries, HBs particles containing Pre-S2 are transferred to Chinese hamster egg cells. Thus HBs vaccines containing Pre-S2 are produced and used (12, 13).

There are several hypotheses related to HBV carcinogenesis. Among the most important of them, we can mention the transactivation role of protein X and the deletion variants of PreS2 (14). There are two types of these options. The first category refers to the variants that cannot make M protein due to the mutation at the start codon level (11, 15). The second category includes the variants that have a deletion mutation in half of the amino end of the PreS2 region. As a result, the M protein in this group is produced shorter than usual. Both types of these variants may occur during chronic HBV infection (12). The evidence from various studies in other parts of the world shows that deletion mutations in this region of the genome may play a role in the occurrence of HCC (14, 16). This study aims to determine the possible presence of deletion mutants of the hepatitis B virus isolated from patients with HCC in China.

Materials and Methods

A study was conducted on ten serum samples of HCC patients who were chronically infected with HBV. These patients were referred to the Hospital of Wannan Medical College, Wuhu, Anhui 241000, China. None of the patients were infected with hepatitis D, C or HIV.

Using the QIAamp DNA Blood Mini Kit (QIAGEN, CA), viral DNA was extracted from 200 microliters of serum, according to the kit's instructions. Finally, the extracted DNA was separated from the silica membrane by a 50ul separation buffer. Then the Pre-S region of the hepatitis B virus was amplified by the Semi-Nested PCR method

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and using Taq DNA Polymerase Kit (QIAGEN, CA). The reaction of both PCR stages was carried out on 100 ul of the reaction mixture according to the kit's instructions. 5ul DNA was used in the first step reaction, and 1ul DNA was used in the second step. The characteristics of the designed primers for Semi-Nested PCR method are mentioned in Table 1.

The products of the second stage of PCR, which were 834 bases long, were transferred on 1% agarose gel, and after staining with ethidium bromide, they were observed by a transilluminator. Then, the second stage PCR product sequence was determined directly and bilaterally using ABI sequencer model 3130 XL and PS1 and PS3 primers. The obtained sequences were modified by Chromas software. The above sequences are with the sequences available in GenBank, which are available on the NCBI website and in the Blast section. To determine the genotype, a comparison was made. The sequence of nucleotides and related amino acids were compared with the sequences available in GenBank, including the sequences related to viruses isolated from China and the sequence related to genotype D with the number ABO33559 (17). The comparison was made by BioEdit software models 7.0.5.3.

Results

By comparing the entire Pre-S sequence with the sequences available in GenBank, it was determined that all ten isolated viruses are in the D genotype group. Then by comparing the sequence of the Pre-S2 region of viruses isolated from patients and the sequences related to genotype D available in GenBank, the following results were obtained:

In the nucleotide sequence of the Pre-S2 region in the virus isolated from three patients, there were deletion mutations with lengths of 18, 9, and 24 bases (Figure 1), which caused the deletion of 3, 6, and 8 amino acids, respectively (Figure 2). Also, a point mutation was found at the start codon level of the Pre-S2 region of the virus isolated from two patients (Figure 1). This type of mutation at the amino acid level has led to the change of methionine to inulin (M1I). In one of the samples (005), both types of mutations mentioned together (i.e., start codon mutation and deletion) have occurred.

Discussion

In this study, the PreS2 region related to 10 HBV viruses isolated from HCC patients was compared with the wild-type virus found in GeneBank. We found three patterns of PreS2 mutants in this number of samples. The first group has undergone a mutation in the start codon, which has led to the change of methionine to inulin (M1I) at the amino acid level. The second category, in addition to the mutation in the start codon, also has a deletion mutation. The third category of variants only has deletion-type mutations in this region. Deletion-type mutations are all located at the 5' end of the PreS2 region. In patterns 1 and 2, due to mutation in the start codon, there is a disturbance in the M protein translation stage. As a result, these mutants lack M protein. Deletion-type mutations in models 2 and 3 affect only the amino end of the PreS2 region.

In a case report, a virus variant was isolated from a patient with HCC, which was present in the Deletion region of PreS2 with a length of 34 nucleotides (1).

In another study, out of 40 patients with HCC, the patients had deletion variants of the PreS2 region (18).

Also, a study illustrated the whole genome of viruses isolated from 16 patients with HCC caused by HBV. In this study, 50% of HCC patients were infected with PreS deletion variants (2).

Also, 19 patients in Damascus with HCC were examined for the existence of PreS2 variants, and 84% of them were infected with these variants (19). In Bangladesh, considering the type of genotype, PreS region mutations were investigated. This research showed that the abundance of deletion mutants of the PreS region depends on the type of genotype on the one hand (more was obser-

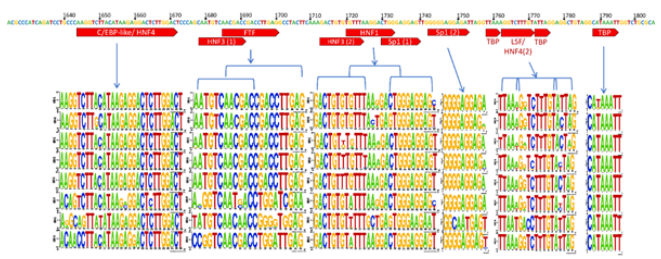

Figure 1. Nucleotide sequence of PreS2 region related to viruses isolated from HCC patients in comparison with D genotype available in GenBank.

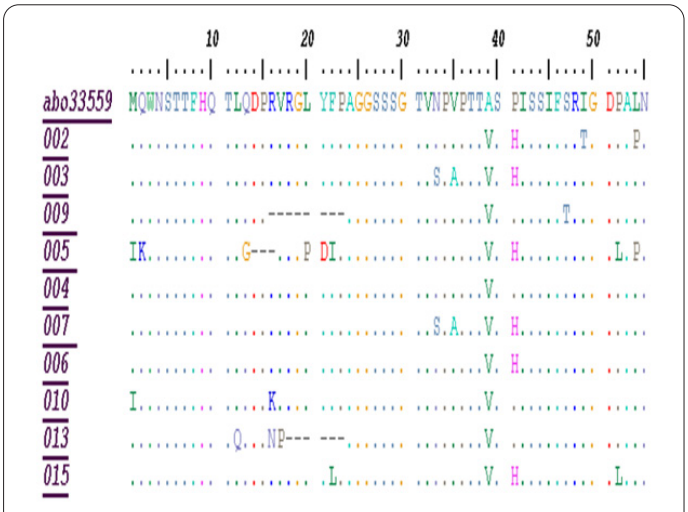

Figure 2. The amino acid sequence of the PreS2 region adapted from the nucleotide sequence.

Table 1. The characteristics of the designed primers for Semi-Nested PCR method.

Primer		Sequence	Nucleotide
PS1	Sense	5'TCAGAATTCTCACCATATTCTTGGGAACAA3'	2817-2839
PS2	Antisense	5'CACTAGTAAACTGAGCCA3'	668-687
PS3	Antisense	5'AGTAAGCTTAGAAGATGAGGCATAGCAGC3'	415-434

ved in genotype C) and, on the other hand, the degree of liver disease progression (20). In addition, according to a recent study in Indonesia, the frequency of PreS2 deletion mutants in patients with advanced stages of liver diseases, especially HCC, is higher than in the control group (1). These studies and other reports in this field have shown that infection with this variant has a close relationship with active HBV infections, especially HCC. Of course, most of the mentioned studies were done on C and B genotypes (21). On the contrary, the viruses we studied are in the D genotype group. Such studies have been done less on genotype D.

The PreS2 region overlaps with the Spacer region of the polymerase gene, which has no known role in the function of the polymerase enzyme (22). For this reason, variants of the virus that have a deletion mutation in this region are no different from the wild virus in terms of DNA replication and virion secretion (23). The studies in this field show that in the amino end half of this genome region, epitopes are recognized by B cells and T cells (11). In chronic infection with HBV for a long time due to the pressure of the immune system, these mutants appear gradually (24). These conditions are a selective advantage for these variants to escape from the host's immune system and prevail among the viral population (25). On the other hand, a defect in the production of PreS2 causes an imbalance in the production of surface proteins (26). This imbalance is associated with increased production and accumulation of PreS1 inside the cell and endoplasmic reticulum, which is highly toxic for the cell and gives the liver cell an appearance similar to Ground Glass Hepatocyte (27).

A similar study in the same field showed that it is possible that the spatial shape of HBS mutant proteins that have undergone deletion mutations in the amino terminus of the PreS2 region has changed, leading to their accumulation in the endoplasmic reticulum and subsequently to ER stress (14).

ER stress ultimately leads to DNA oxidative damage and genome instability on the one hand (28-31). On the other hand, activating several signal transduction pathways will lead to cell proliferation (26). Therefore, it can be concluded that PreS2 deletion mutants may play a long-term role in HBV-related HCC pathogenesis. Of course, more *in vivo* and *in vitro* studies are needed to clarify this relationship further.

In general, in PreS2 deletion mutants, T cell and B cell epitopes on the product of the PreS2 region are deleted. As a result, conditions are created where the virus can escape from the immune system. These mutant PreS2 proteins accumulate in the endoplasmic reticulum (ER) network and cause ER stress. In this way, in addition to creating unstable conditions in the cell genome, the proliferation of hepatocytes is stimulated indirectly. As a result, there is a possibility that the cells will progress toward becoming cancerous.

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