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BRIEF REPORT

Development and evaluation of a semi-nested PCR technique for amplification and determination of different surface gene variation patterns in patients with chronic HBV infection: development of an amplification method for HBV S gene

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ABSTRACT

Aim: Development of an amplification method for further investigation of HBV S gene variation patterns.
Background: Pre-S/S variants in patients with chronic HBV infection may contribute to the progression of liver damage and Hepatocellular carcinoma (HCC).
Methods: This study was performed on ten patients with chronic HBV infection. Viral DNA was extracted from patient's plasma, primer design was performed, and a semi-nested PCR method was set up to amplify the pre-S/S region of HBV genome. Subsequently, sequencing was performed to analyze the variants of this region.
Results: In the current study, the semi-nested PCR method was successfully set up, and types of variation in the studied samples were investigated.
Conclusion: Pre-S/S variants should be routinely determined in HBV carriers to help identify individuals who may be at a high risk of less favorable liver disease progression. This study showed that the technique could accurately amplify the pre-S/S region, and the product can be successfully used for variation detection by direct sequencing.

Keywords: Hepatitis B virus, PreS/S variations, Semi nested PCR.

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Introduction

Hepatitis B virus (HBV) infection is a global public health problem, and viral hepatitis is the seventh principal reason of death in the world (1). HBV is a partially double-stranded circular DNA virus and a member of the Hepadnaviridae family that causes a range of liver diseases. This virus is classified into eight genotypes (A to I) and a putative genotype (J), four major subtypes, and several subgenotypes (2, 3). The virus genome has four open reading frames (ORF) that each encode proteins, including polymerase (Pol), core (preC/C), X proteins, and envelope (preS/S), which contain small, middle, and large hepatitis B surface antigen proteins (4, 5). Chronic HBV infection (CHB) is a significant cause of viral hepatitis which can induce liver complications such as cirrhosis, fibrosis,

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and hepatocellular carcinoma (HCC). Patients who develop chronic liver diseases after infection with HBV are at increased risk of developing cirrhosis and primary HCC. This progression is determined by the genetic characteristics of the host, environmental and viral factors such as genotype, virus load, and specific mutations (6). HBV has a spontaneous error rate because of the reverse transcriptase function of viral polymerase enzyme, which may lead to variations in genomic DNA after each replication cycle.

Furthermore, the clinical significance of pre-S/S variants has become recognized in patients with chronic HBV infection. These pre-S/S variants may contribute to the progression of liver damage and HCC (7, 8). Therefore, launching effective techniques to identify these genetic variations can be helpful for molecular epidemiology based surveys.

Methods

This baseline study enrolled 10 HBV-positive patients who presented to Taleghani Hospital, Tehran, Iran, between August 2018 and September 2020. Blood samples were collected in EDTA tubes. The samples were centrifuged at 3,000 rpm for 15 minutes at 4 °C to separate serum and plasma from whole blood. The viral DNA was extracted from the resultant plasma samples, which were stored at -80 °C, using a viral DNA extraction kit (QIAGEN, Germany). Because the virus genome is circular (9), part of the pre-S/S region is at the beginning ,and part of it is at the end of the virus genome. To analyze the pre-S/S region, we designed two sets of primers to cover it, including SRMH: F: 5-AACHAGACAYTATTTACACACTC-3; and R1: 5-TGGAATYAGAGGACANACG -3 for the first run, and F2: same as the first run (semi-nested PCR), R2: 5-GATAWCCAGGACAAGTYGGAG -3 for the second run, to amplification of the left part of the pre-S/S region as large as 935 bp. For the right area of the pre-S/S YIR: F1: 5-CHAACCTCCAATCACTCACCAAYC-3 and R: 5-TGCRTCAGCAAACACTTGGC-3 for the first run, and F2: 5-CTCCRAYTTGTCCTGGYTATCG-3, R: same as the first run (semi-nested PCR), for the second run (Figure 1). After that, we launched a nested PCR (polymerase chain reaction) method with appropriate protocols and performed the amplification for the samples (Table 1). HBV genome sequences were determined by direct Sanger sequencing after PCR amplification, then analysis and assembly of sequencing data were performed to determine variants.

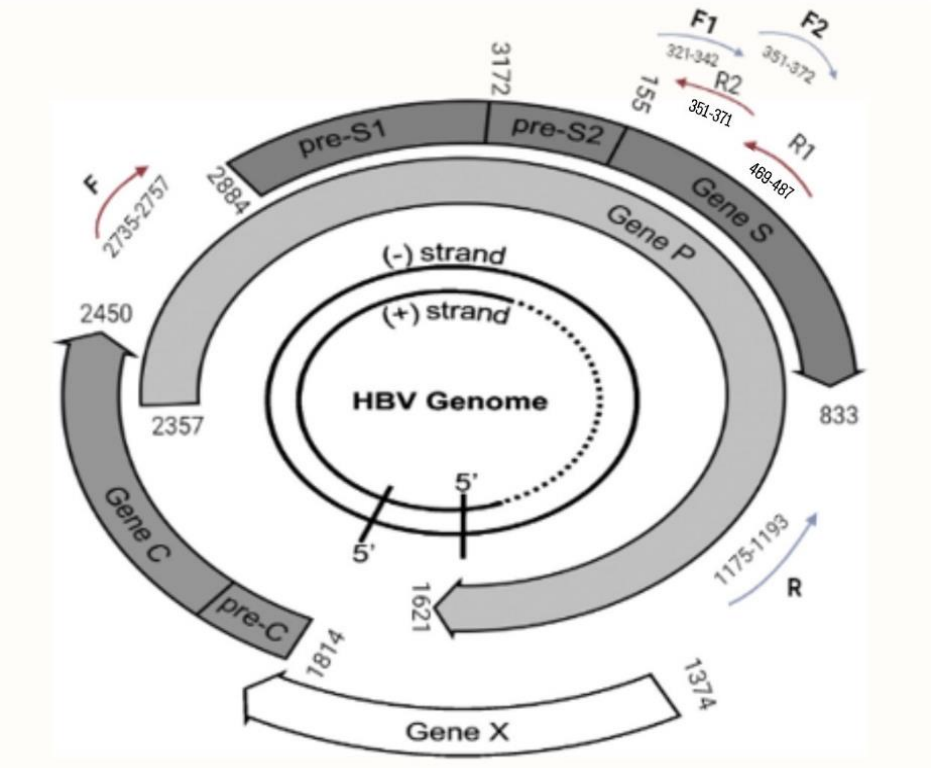

Figure 1. Locations of primers that designed to cover preS/S region on the HBV genome.

Table 1. Semi Nested PCR protocol (*Modifiable, depending on the sample virus load).

Semi nested PCR protocol	Initialization		Denaturation		Annealing		Extension		Final Elongation	
SRMH: First run	95 ℃	5 min	94 ℃	30 s	58 ℃ 35 C	30 s	72 ℃	60 s	72 ℃	7 min
SRMH: Second run	95 ℃	3 min	94 ℃	40 s	66 ℃ 25 C*	40 s	72 ℃	45 s	72 ℃	10 min
YIR: first run	95 ℃	5 min	94 ℃	30 s	60 ℃ 30 C	35 s	72 ℃	60 s	72 ℃	10 min
YIR: second run	95 ℃	3 min	94 ℃	40 s	65 ℃ 35 C*	40 s	72 ℃	45 s	72 ℃	10 min

All sequencing was performed bi-directionally by Applied Biosystems 3130xl genetic analyzer (Foster City, California, USA). Analysis and assembly of sequencing data were performed with the BioEdit software package.

Results

This study aimed to set up an appropriate technique and implement it on clinical samples of patients with chronic hepatitis B. For this purpose, ten patients were evaluated by designed specific primers, and the proper temperature and time protocol for amplification of the desired products containing the preS/S region was set up. Proper product bands of each target were obtained, and these products could be utilized to determine the sequence variations of different isolates, including deletion and substitution (Figures 2 and 3).

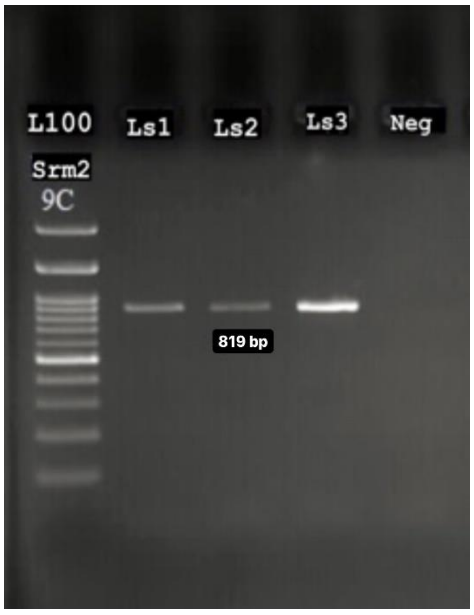

Figure 2. HBV pres/s amplification products electrophoresed on a 1.5% agarose gel L: Ladder 100-1000 bp, Ls: Left area of the pre s/s region, N: Negative control.

Figure 3. HBV pres/s amplification products electrophoresed on a 1.5% agarose gel L: Ladder 100-1000 bp, Rs: Right area of the pre s/s region, N: Negative control.

Discussion

The designed primer sets and the method can be used for different HBV genotypes; however, they should be studied on a larger group of clinical samples. HBV genotypes, subtypes, and subgenotypes determination are useful in epidemiological surveys. In addition, molecular studies have shown that mutations in the hepatitis B virus preS/S region might be related to the progression of liver disease, something that is not noticeable in the clinic and requires appropriate laboratory techniques, including PCR and direct sequencing, and proper sequence analysis (8, 10-12). Deletion in the pre-S2 area is one factor that increases the risk of developing hepatocellular carcinoma (13-15). Pre-S/S variants should be routinely determined in HBV carriers to help identify individuals who may be at a high risk of less favorable liver disease

progression. The technique used in this field can lead to accurate analysis of the pre-S/S region for variants detection and characterization.

Conclusion

Pre-S/S variants should be routinely determined in HBV carriers to help identify individuals who may be at a high risk of less favorable liver disease progression and this study showed that the technique can accurate amplify the pre-S/S region and the product can be successfully used for variation detection by direct sequencing.

Conflict of interests

The authors declare that they have no competing interests.

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