Genetic diversity of hepatitis B virus and mutations associated to hepatocellular carcinoma in patients from Venezuela, with different stages of liver disease.

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Keywords: HBV; HCC; HBV subgenotype F2; BCP mutations.

Abstract. Globally, about 50% of liver cancer originates as a result of long term infection with hepatitis B virus (HBV), and some genotypes and mutations have been associated with an increased severity of infection. The aim of this study was to evaluate the genetic diversity of HBV in patients from Venezuela, with chronic infection, cirrhosis and hepatocellular carcinoma (HCC) and to compare the occurrence of mutations in all patient groups. Samples from patients with different pathologies of the liver, associated with HBV infection, were collected. The HBV S region was analyzed for genotype determination and, when available, the whole genome sequence was examined for mutations analysis. Genotype F was the most common genotype (87%). While the HBV subgenotype F3 was the most frequent genotype in the whole group of samples (44%), the subgenotype F2 predominated in HCC patients (56%). Mutations were more common in HCC and cirrhosis cases (p=0.01). The A1762T mutation was significantly associated with the advanced stage of liver disease (p=0.008). Additionally, mutations were more common in early stages of liver disease in HBV subgenotype F2-infected patients, and a significant association between this subgenotype and the emergence of T1753C, A1762T, A1762T/G1764A (p=0.04) and C1773T (p=0.001) mutations in chronic patients was found, when compared to the HBV subgenotype F3. By comparing F2 with all other HBV subgenotypes, a positive association for the three basal core promoter (BCP) mutants (A1762T, A1762T/G1764A p=0.01, G1764A p=0.04) was found. These results suggest that the HBV subgenotype F2 might be associated to more severe forms of liver disease in comparison with the HBV subgenotype F3.
Diversidad genética del virus de la hepatitis B y mutaciones asociadas con carcinoma hepatocelular en pacientes de Venezuela, con diferentes estados de la enfermedad del hígado.

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Palabras clave: VHB; carcinoma hepatocelular; subgenotipo F2 de VHB; mutaciones del promotor basal de la cápside.

Resumen. Mundialmente, alrededor del 50% del cáncer de hígado se origina como consecuencia de la infección a largo plazo con el virus de la hepatitis B (VHB), y algunos genotipos y mutaciones han sido asociados con severidad incrementada de la infección. El objetivo de este estudio fue evaluar la diversidad genética del VHB en pacientes de Venezuela con infección crónica, cirrosis y carcinoma hepatocelular (CHC) y comparar la ocurrencia de mutaciones en los tres grupos de pacientes. Se reunieron muestras de pacientes con diferentes patologías de la enfermedad del hígado asociada a la infección por VHB. La región S del VHB fue analizada para la determinación del genotipo y cuando estuvo disponible, la secuencia del genoma completo fue examinada para análisis de mutaciones. El genotipo F de VHB fue el más frecuente (87%). Mientras que el F3 fue el subgenotipo más encontrado en el grupo completo de muestras (44%), el F2 fue predominante en pacientes con CHC (56%). Las mutaciones fueron más comunes en casos de pacientes con cirrosis y CHC (p=0,01). La mutación A1762T estuvo asociada significativamente con estado avanzado de la enfermedad del hígado (p=0,008). Adicionalmente, las mutaciones fueron más comunes en estados tempranos de la enfermedad del hígado en pacientes infectados con el subgenotipo F2, encontrándose una asociación significativa entre este subgenotipo y la ocurrencia de las mutaciones T1753C, A1762T, A1762T/G1764A (p=0,04) y C1773T (p=0,001) en pacientes crónicos, en comparación con el subgenotipo F3. Por otro lado, al comparar F2 con los demás subgenotipos de VHB, se encontró una asociación positiva para las tres mutantes del promotor basal de la cápside (PBC) (A1762T, A1762T/G1764A p=0,01, G1764A p=0,04). Estos resultados sugieren que el subgenotipo F2 de VHB puede estar asociado a formas más severas de la enfermedad del hígado en comparación al subgenotipo F3.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary liver cancer and the most severe complication of chronic liver disease (1). According to GLOBOCAN estimates (http://globocan.iarc.fr/) (2), about 782,000 new cases of liver cancer were diagnosed in 2012, being the fifth most common cancer in men (554,000 cases, 7.5% of the total) and the ninth in women (228,000 cases, 3.4%). Liver cancer is the second most common cause of death from cancer worldwide, estimated to be responsible for nearly 746,000 deaths in 2012 (9.1% of the total). The major etiological factors for the developing of HCC are hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, dietary intake of aflatoxin B1 (AFB1) and chronic alcohol abuse (3). Globally, about 50% of liver cancer originates as a result of long term infection with HBV (4), and there is important evidence suggesting an association between the infecting genotype and the generation of specific mutations with the progression of liver disease (5-9).
HBV infection is a global health problem. About 2 billion people, a third of the world population, have been exposed to HBV and at least 380 million are chronic carriers of HBV infection (6% of the world population). Up to ten genotypes (A to J) and several subgenotypes have been reported. In Venezuela the HBV genotype F is the most frequent in the overall population (10, 11), being the HBV subgenotype F3 the most common (12).

Among the major mutations in HBV that have been associated with the development of HCC, those found in the region of the basal core promoter (BCP), which involve a transversion of adenine for thymine at nucleotide 1762 (A1762T) and a transition of guanine to adenine at nucleotide 1764 (G1764A), have been widely studied and associated with an increased severity of infection (13). On the other hand, several studies have associated the emergence of C1653T, T1753C, C1766T, T1768A and G1899A mutations with an increased risk of HCC (7, 9, 14-16).

In Venezuela, the information available on the genetic diversity of HBV in cirrhosis and HCC patients is scarce, and even less is known about the frequency of occurrence of HBV BCP mutants in these patients compared to chronic patients. The aim of this study was to evaluate the genetic diversity of HBV in patients from Venezuela, with chronic infection, cirrhosis and HCC and to compare the occurrence of mutations (X, BCP, Enhancer II and preCore regions) in all patient groups, representing the first study of its kind in our country.

PATIENTS AND METHODS

Patients and blood samples

This study was approved by the Bioethics Committee of the Instituto Venezolano de Investigaciones Científicas (IVIC). A total of 35 blood samples from HBV positive patients with different pathologies of the liver disease were analyzed, after signing an informed consent. The samples were stored at -70°C until use. Additionally, four samples of liver tissue biopsies embedded in paraffin were included in this study. To summarize, the group of samples consisted of nine HCC cases (mean age 44 years, 56% men), 10 from cirrhosis (mean age 55 years, 80% male) and 20 cases of patients with chronic viral infection without severe liver disease (mean age 41 years, 45% men). All samples were positive for HBV DNA. The patients were from the Capital District and the state of Mérida, Venezuela.

PCR and Sequencing

All samples were analyzed by nested PCR of the region S of HBV (10). For the determination of mutations in the X, BCP, preCore and enhancer II regions of HBV, in a total of 30 samples the whole genome of HBV was amplified, using in the first and second PCR round primers previously described (17, 18). For the remaining nine samples smaller fragments of the HBV genome were amplified. All PCR products were purified using QIAquick PCR Purification Kit (Qiagen) and subjected to direct nucleotide sequencing. In all cases, both sense and antisense inner primers were used for sequencing and all sequences were performed by Macrogen Service Center, Seoul, Korea.

Phylogenetic and sequence analysis

Nucleotide sequences were aligned using DNAMan 5.2.2 (Lynnon Bio Soft, Canada). Phylogenetic analyses were performed by the Neighbor Joining method (1000 bootstrap replicates, with genetic distances estimated with Kimura 2 parameter correction). HBV sequences were compared with sequences available in GenBank. To investigate the presence of mutations in the X, BCP, enhancer II and preCore regions of HBV, HBV isolates were analyzed by comparing them with a HBV subgenotype F3 sequence (AY311370). Nucleotide sequence data have been deposited into the GenBank database under the accession numbers KP995082-KP995126.

Statistical Analysis

Statistical differences were evaluated by the Chi-square test with Yates correction, or Fischer Exact test, using Epi Info version 7.1.1.14 (Center
for Disease Control and Prevention, Atlanta, GA, USA). A p value < 0.05 was considered statistically significant.

RESULTS

A fragment of the HBV S region (680 nt) was analyzed on 38 isolates from patients with several pathologies of the liver, and compared to HBV reference sequences available in GenBank (Fig. 1). For one sample, the HBV subgenotype was determined using a smaller fragment of the HBV S region (340 nt) (data not shown). The HBV genotype F was the most frequent genotype, present in 87% of chronic infection, cirrhosis and HCC cases, with a small proportion of cases (13%) due to other HBV genotypes. While the HBV subgenotype F3 was the most common in the whole group of samples (44%), especially in the groups of chronic and cirrhosis patients, the genotype F2 was the most often found in the group of HCC patients (56%), although this tendency did not reach statistical significance (Table I).

![Fig. 1. Phylogenetic analysis of a fragment of 680 nt corresponding to the S gen of 38 isolates of HBV. Reference sequences are designated by their GenBank accession number. In bold isolates names from this study are shown. Numbers at each node correspond to bootstrap values (greater than 50%) obtained with 1000 replicates. The scale bar is in units of nucleotide substitutions per site.](image-url)
TABLE I
HBV GENOTYPES FOUND IN PATIENTS WITH CHRONIC INFECTION, CIRRHOSIS AND HCC

<table>
<thead>
<tr>
<th>Cases</th>
<th>HBV subgenotypes (n)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F1b</td>
<td>F2</td>
</tr>
<tr>
<td>Chronic infection</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>HCC</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>3</td>
<td>12</td>
</tr>
</tbody>
</table>

The frequency of occurrence of mutations in the X gene, BCP, enhancer II and preCore regions of HBV was evaluated in 36 samples (Table II). A1762T, G1764A, the double BCP mutant, T1753C and C1773T mutations were the most frequently found. Mutations were more common in patients with cirrhosis and HCC, for which a positive association was found. Also, A1762T and T1768A mutants were associated with advanced stage of the liver disease.

TABLE II
FREQUENCY OF OCCURRENCE OF MUTATIONS IN THE X, BCP, ENHANCER II AND PRECORE REGIONS OF HBV

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Clinical stage HCC+Cirrhosis (%)</th>
<th>Chronic (%)</th>
<th>p</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1613A</td>
<td>3/16 (19)</td>
<td>0/20 (0)</td>
<td>0.08</td>
<td>3/36 (8)</td>
</tr>
<tr>
<td>C1653T</td>
<td>4/16 (25)</td>
<td>2/20 (10)</td>
<td>0.37</td>
<td>6/36 (17)</td>
</tr>
<tr>
<td>A1703C</td>
<td>5/16 (31)</td>
<td>1/20 (5)</td>
<td>0.07</td>
<td>6/36 (17)</td>
</tr>
<tr>
<td>G1719T</td>
<td>1/16 (6)</td>
<td>0/20 (0)</td>
<td>0.44</td>
<td>1/36 (3)</td>
</tr>
<tr>
<td>A1726C</td>
<td>2/16 (13)</td>
<td>2/20 (10)</td>
<td>1</td>
<td>4/36 (11)</td>
</tr>
<tr>
<td>G1727A</td>
<td>3/16 (19)</td>
<td>0/20 (0)</td>
<td>0.08</td>
<td>3/36 (8)</td>
</tr>
<tr>
<td>T1753C</td>
<td>7/16 (44)</td>
<td>5/20 (25)</td>
<td>0.41</td>
<td>12/36 (33)</td>
</tr>
<tr>
<td>G1757A</td>
<td>2/16 (13)</td>
<td>0/20 (0)</td>
<td>0.19</td>
<td>2/36 (6)</td>
</tr>
<tr>
<td>A1762T</td>
<td>12/16 (75)</td>
<td>5/20 (25)</td>
<td>0.008</td>
<td>17/36 (47)</td>
</tr>
<tr>
<td>G1764A</td>
<td>10/16 (63)</td>
<td>6/20 (30)</td>
<td>0.11</td>
<td>16/36 (44)</td>
</tr>
<tr>
<td>A1762/G1764A</td>
<td>10/16 (63)</td>
<td>5/20 (25)</td>
<td>0.05</td>
<td>15/36 (42)</td>
</tr>
<tr>
<td>C1766T</td>
<td>2/16 (13)</td>
<td>0/20 (0)</td>
<td>0.19</td>
<td>2/36 (6)</td>
</tr>
<tr>
<td>T1768A</td>
<td>4/16 (25)</td>
<td>0/20 (0)</td>
<td>0.03</td>
<td>4/36 (11)</td>
</tr>
<tr>
<td>C1773T</td>
<td>8/16 (50)</td>
<td>8/20 (40)</td>
<td>0.79</td>
<td>16/36 (44)</td>
</tr>
<tr>
<td>C1799G</td>
<td>1/16 (6)</td>
<td>0/20 (0)</td>
<td>0.44</td>
<td>1/36 (3)</td>
</tr>
<tr>
<td>G1896A</td>
<td>2/16 (13)</td>
<td>0/20 (0)</td>
<td>0.19</td>
<td>2/36 (6)</td>
</tr>
<tr>
<td>G1899A</td>
<td>1/16 (6)</td>
<td>2/20 (10)</td>
<td>1</td>
<td>3/36 (8)</td>
</tr>
<tr>
<td>Any mutation</td>
<td>16/16 (100)</td>
<td>12/20 (60)</td>
<td>0.01</td>
<td>28/36 (78)</td>
</tr>
</tbody>
</table>
Some mutations, especially BCP mutants, were more common in early stages of liver disease in HBV subgenotype F2 infected patients (Table III). Even more, a significant association between the HBV subgenotype F2 and the emergence of T1753C, A1762T, A1762T/G1764A and C1773T mutations in chronic patients, in relation to HBV subgenotype F3, was found. Also, by comparing F2 with all other HBV subgenotypes a positive association for the three BCP mutants (A1762T, A1762T/G1764A and G1764A) was found.

**DISCUSSION**

In this study, the HBV genotype F was found to be highly prevalent in Venezuelan patients with chronic infection, cirrhosis and HCC associated to HBV infection, with a small proportion of cases due to other genotypes. These results are similar to those previously reported (11, 19), about the prevalence of the HBV genotype F in Venezuela. Regarding HBV subgenotypes, F3 was the most common (44%), followed by F2 (31%). This distribution of HBV subgenotypes, with predominance of F3 was similar to that observed in the overall population of Venezuela (20), in Amerindians from Venezuela and the overall population of Colombia (10). It should be noted that in patients with HCC, the HBV subgenotype F2 was the predominant, whereas the HBV subgenotype F3 was more frequent in cirrhotic and chronic patients. Several studies have established relationships between infection with several HBV genotypes and the development of HCC. For the HBV genotype F, three studies suggest a relationship with more severe forms of liver disease. The first one, made in Spain, found that death related to liver disease was more frequent with the HBV genotype F than with HBV genotypes C or D (21). The second study, conducted in Alaska native patients, found a significant association between HBV genotype F

### TABLE III

<table>
<thead>
<tr>
<th>HBV subgenotype</th>
<th>Clinical stage</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T1753C</td>
</tr>
<tr>
<td><strong>F2 (n=11)</strong></td>
<td>Chronic (%)</td>
<td>4/6 (67)</td>
</tr>
<tr>
<td></td>
<td>Cirrhosis+HCC (%)</td>
<td>4/5 (80)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>8/11 (73)</td>
</tr>
<tr>
<td><strong>F3 (n=16)</strong></td>
<td>Chronic (%)</td>
<td>1/10 (10)</td>
</tr>
<tr>
<td></td>
<td>Cirrhosis (%)</td>
<td>2/6 (33)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>3/16 (19)</td>
</tr>
<tr>
<td><strong>Other (n=9)</strong></td>
<td>Chronic (%)</td>
<td>0/4 (0)</td>
</tr>
<tr>
<td></td>
<td>Cirrhosis (%)</td>
<td>1/5 (20)</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>1/9 (11)</td>
</tr>
</tbody>
</table>

*HCC cases not considered

\(^{a}\)p=0.04 by comparing chronic patients with HBV subgenotype F2 vs F3 for A1762T and A1762T/G1764A

\(^{b}\)p=0.01 by comparing chronic patients with F2 vs all other HBV subgenotypes for A1762T and A1762T/G1764A

\(^{c}\)p=0.04 by comparing chronic patients with F2 vs all other HBV subgenotypes for G1764A

\(^{d}\)p=0.001 by comparing chronic patients with HBV subgenotype F2 vs F3 for C1773T
and the development of HCC in young patients (6),
being the HBV subgenotype F1b, which circulates
in Alaska (22). The third, a more recent study,
made in Colombia, found that HBV genotype F,
specifically the subgenotype F3, was prevalent in
samples of patients with severe forms of hepatic
disease (HCC + Cirrhosis) (23). On the other hand,
Venezuelan patients with HCC had a higher average
age than that reported in the study of Alaska (6).
Additionally, the average age in HCC patients
infected with HBV subgenotype F2, was lower than
that observed for the group of HCC associated to
HBV subgenotype F3 (data not shown). These data
seem to suggest a more rapid progression to HCC by
HBV subgenotype F2 than by F3, which would be
more associated with the development of cirrhosis.
Thus, the results of association of HBV genotype
F with HCC seem to be variable in the region. One
possible explanation is that the severity of infection
by HBV genotype F might be influenced by the HBV
subgenotype infecting. In addition, the occurrence of
mutations, which are an important factor associated
with HCC, may be distinct between different HBV
subgenotypes.

In the past decade, attention has focused on HBV
variant strains that contribute to the clinical severity
of liver disease, especially HCC (24). Thus, a number of
mutations pattern of HBV, such as the mutant
preCore at nucleotide 1896 or the double mutant in
the region of the BCP, have been widely studied in
relation with clinical severity (5, 7). Several studies
have shown the relationship between the occurrence
of HBV mutants, the infection with certain HBV
subgenotypes and development of HCC (9, 16,
25-28). In the present investigation, the mutations
more frequently found were A1762T, G1764A and
the double BCP mutant. Others fairly common
mutations were T1753C and C1773T. Mutations,
especially BCP mutants, were present mostly in
patients with HCC and cirrhosis associated to HBV
subgenotype F2 and F3 infection. Although the
number of HCC cases gathered was low, mutations
were observed from early stages of liver disease in
patients infected with the HBV subgenotype F2,
finding an association between the occurrence of
T1753C, A1762T, A1762T/G1764A and C1773T
mutations, the HBV subgenotype F2 infection and
the development of HCC. Again, these results suggest
a possible increased tendency of HBV subgenotype
F2 to the development of cancer and in any case
subgenotype differences on the progression of liver
disease. These subgenotype differences have been
observed in other studies developed in our region.
A recent study, made in Argentina, found a bias of
mutations among genotypes (A2, D, F1b and F4).
Mutations in the BCP were more frequently found
in subgenotype F1b than in A2, D and F4. Genotype
F showed a lower seroconversion rate of hepatitis B
“e” antigen (HBeAg). These findings suggest that
intrinsic biological features of each genotype may
lead to a longer HBeAg positive stage and therefore
to different implications in the progression of the
infection (29).

In conclusion, these results represent the first
study that shows an association between the infection
with the HBV subgenotype F2 and the generation
of mutations related to more severe forms of liver
disease, so further studies are required to establish a
definitive association between infection with HBV
subgenotype F2 and the development of HCC. As
expected, the HBV subgenotype F3 was the most
frequent, while F2 was more common in HCC
patients. The occurrence of mutations was more
common from early stages of liver disease in HBV
subgenotype F2 infected patients.

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