Hepatitis G Virus Infection in Patients with Hepatocellular Carcinoma in Recife, Brazil

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The evidence of a higher incidence of hepatitis G virus (HGV) infection among patients with hepatocellular carcinoma (HCC) and the relatively high prevalence of patients with primary liver carcinoma without apparent risk factors in our country motivated the present study, the objective of which was to determine the frequency of HGV-ribonucleic acid (RNA) in a series of patients with HCC. The diagnosis of HCC was established based on $\alpha$-fetoprotein levels (>400 ng/ml), a compatible image and/or biopsy of the hepatic nodules. Markers of hepatitis B virus (HBV) (HBsAg and anti-HBc), hepatitis C virus (HCV) (anti-HCV) and HGV (HGV-RNA) were investigated using MEIA and RT-PCR (reverse transcriptase polymerase chain reaction). There were 32 patients evaluated, including 20 males (63%), with a mean age of 58 years. Twenty-eight (88%) patients were cirrhotic (Child–Pugh: A = 8 patients, B = 14, and C = 6) and 50% reported alcohol consumption. Serological hepatitis markers were detected in 26 (81%) patients, including HBV in 19 (59%), HCV in 12 (38%) and HGV in 9 (28%). Only one (3%) patient was positive for HGV alone. The prevalence of HGV in blood donors from the same region is 10%. The findings suggest that, despite the frequent detection of HGV markers in patients with HCC, isolated infection with this agent does not seem to be a relevant factor in the etiology of this carcinoma.

Key words: hepatitis G virus — hepatocellular carcinoma — cirrhosis — hepatitis B virus — hepatitis C virus

INTRODUCTION

Hepatocellular carcinoma (HCC) is currently one of the most frequent neoplasms worldwide, with an estimated incidence of 300 000—1 000 000 new cases/year and accounts for about 4% of all malignant tumors (1,2). High prevalence rates are observed in the Asian and African continents, reaching peaks of 100 HCC cases per 100 000 inhabitants (2). Recently, an increase in the number of HCC cases resulting from hepatitis C virus (HCV) infection has been observed and in the next decade HCC related to HCV infection will reach its incidence peak (1,2).

Hepatitis B virus (HBV) and HCV are the main agents involved in hepatocarcinogenesis, with their relevance varying according to regional characteristics (2–4). In China, chronic HBV infection is present in 80% of HCC patients, whereas in Japan, Spain and Italy HCV accounts for most cases (3–5).

In Brazil, clinical and epidemiological data regarding HCC are scarce and there are no reports analyzing in detail regional characteristics and variations. A national survey conducted in 1997 showed that HBV and HCV are the main responsible agents despite the high percentage (42%) of cases of undetermined etiology (6).

In the mid 1990s, Linnen et al. (7) and Simons et al. (8) almost simultaneously described the hepatitis G virus (HGV). The epidemiology of HGV is not well defined, but studies on blood donors have shown variations in infection
rates ranging from 1 to 2% in the USA to 19% in South Africa (9–11). A recent meta-analysis involving 13,610 blood donors from different countries identified 649 (4.8%) cases of viremia (HGV-RNA (ribonucleic acid)), with no differences between different ethnic groups (11). In Brazil, studies on blood donors have reported a HGV prevalence of about 10% (12–14).

HGV is mainly transmitted through the parenteral route, with polytransfused patients and intravenous drug users being considered high-risk groups (9–11). However, the high prevalence rate of HGV among blood donors from undeveloped countries suggests that other routes, especially vertical and sexual transmission, play a relevant role in the dissemination of the virus (10).

The involvement of HGV in the development of HCC has been investigated over the last few years and some studies deserve special attention. Lightfoot et al. (15) compared the frequency of HGV-RNA between 167 black South-African patients with HCC and the same number of controls and found no significant differences. In contrast, Tagger et al. (16) studying 170 patients with HCC and 306 controls, observed a 7.3 times higher relative risk of detecting HGV in patients with liver cancer. Similar data were reported by Yuan et al. (5) who studied 144 patients with HCC and 252 controls and observed a 5.4 times higher risk of infection with HGV among patients with HCC, irrespective of concomitant infection with HBV or HCV. In the Brazilian literature no data concerning the prevalence of the HGV in patients with HCC were found.

The evidence of a higher incidence of HGV infection among patients with HCC and the relatively high prevalence of patients with primary liver carcinoma without apparent risk factors in our country motivated the present study, whose objective was to determine the frequency of HGV-RNA in a series of patients with HCC.

PATIENTS AND METHODS

Patients

Thirty-two patients with HCC evaluated between June 1996 and July 1999 who fulfilled the criteria for the diagnosis of HCC were studied. The patients were submitted to anamnesis and clinical examination, and were invited to participate in the study after the diagnosis of HCC had been established. The study was approved by the local Ethics Committee.

Diagnosis of Hepatocellular Carcinoma

The diagnosis of HCC was established based on an increase in α-fetoprotein (AFP) and liver injury detected by imaging methods, and/or histopathological analysis irrespective of AFP titers. Serum AFP levels were determined using commercial kits (AxSym, Abbott, Chicago, IL, USA) according to the manufacturer’s instructions. Levels lower than 10 ng/ml were considered to be normal, whereas values higher than 400 ng/ml were classified as specific (17,18). In cases with normal or intermediate serum levels, the diagnosis was confirmed by biopsy of the hepatic lesion and/or lipiodol arteriography.

LABORATORY ASSESSMENT

The following biochemical parameters were measured: alanine aminotransferase (ALT, normal: <53 IU/l), aspartate aminotransferase (AST, normal: <40 IU/l), alkaline phosphatase (normal: <92 IU/l), bilirubin (normal: <1.2 mg/dl), albumin (normal: >3.2 g/dl) and prothrombin time (normal: up to 3 s of control).

HBV surface antigen (HBsAg), anti-core antibody (anti-HBc) and anti-HCV antibody were assayed by MEIA (AxSym, Abbott, Chicago, IL, USA). Patients with positive HBsAg and anti-HBc were considered as having active infection by the HBV and those with isolated anti-HBc as having previous contact with this virus. Patients with anti-HCV were considered infected by the HCV independently of the presence of HCV-RNA.

HGV-RNA was detected by the reverse transcriptase-polymerase chain reaction (RT-PCR) as follows.

EXTRACTION AND AMPLIFICATION OF HGV-RNA

Total RNA was extracted from 250 μl of serum with Trizol reagent (Gibco/BRL—Invitrogen®, CA, USA) according to the manufacturer’s recommendations. The pellet was resuspended in 100 μl sterile ultrapure water (Milli-Q®) and stored in a freezer at −70°C. After extraction, 10 μl of the RNA extracted from samples and controls was converted into cDNA by incubation with avian myeloblastosis virus (AMV) RT (Gibco/BRL) for 30 min at 42°C and amplified by nested PCR. Reverse transcription was carried out in the same mixture as the first amplification together with the other components. Two distinct regions were used for amplification of the HGV genome, with the selection of two primer pairs for amplification of the NS3 region and two primer pairs for amplification of the 5′NTR region.

Amplification of the NS3 Region

The reaction mixture of the first amplification contained 1X PCR buffer (Gibco/BRL), 1.5 mM MgCl₂, 1 mM dNTP, 16 U RNAsin (Gibco/BRL), 2.5 U Taq polymerase (Gibco/BRL), 64 U AMV RT (Gibco/BRL) and 100 μl of each primer. For the first amplification, primers designed from the GBV-C-s1 and GBV-C-a1 sequences at positions 4257 (5′GGN ACN AGG TCN CCR TCY TTC ATG AT 3′) and 4470 (5′ACN ACN AGG TCN CCR TCY TTC ATG AT 3′) were used as described by Linnen et al. (7). Amplification was carried out in a total volume of 100 μl in a Perkin-Elmer Minicycler thermocycler (Wellesley, MA, USA) under the following conditions: denaturation at 95°C
for 5 min, followed by 25 cycles at 95°C for 30 s, 55°C for 60 s and 72°C for 60 s. Prolonged incubation at 72°C for 7 min was added to guarantee the amplification of all not yet amplified products. After the first amplification, 10 μL of the amplified product was added to the second amplification pool containing 1X PCR buffer (Gibco/BRL), 1.5 mM MgCl₂, 1 mM dNTP, 2.5 U Taq polymerase (Gibco/BRL), and 100 μL of each primer. For the second amplification, the 4269 sense (5’TAT CCC TTT TAT GGG CAT GG 3’) and 4435 anti-sense primers (5’YTC RTT GAT GAT GGA ACT GTC GTC 3’) were used in a total volume of 100 μL. Amplification was carried out in a Perkin Elmer Minicycler under the following conditions: denaturation at 95°C for 4 min, followed by 35 cycles at 95°C for 30 s, 55°C for 60 s and 72°C for 60 s, and a final extension step at 72°C for 7 min. The PCR products were separated by electrophoresis on 1.8% agarose gel containing 0.5 μg/ml ethidium bromide and visualized under UV light using a 100-bp molecular marker as standard, generating fragments of 167 bp.

**AMPLIFICATION OF THE 5’NTR REGION**

The primer sequences were derived from HGV isolates PNF2161, R10291 and GBV-C (7,19). The first and second amplifications were carried out in the same reaction mixture and at the same temperatures and cycle durations as employed in the amplification of the NS3 region using primers 108 (5’ AGG TGG ATG GGT GAT 3’) and 531 (5’TGC CAC CCG CCC TCA CCC GAA 3’) for the first amplification and primers 134 (5’TGG TAG TGC GTA AAT CCC GGT 3’) and 476 (5’GGR GCT GGG TGG CCY CAT GCW T 3’) for the second amplification. The PCR products were separated by electrophoresis on 1.8% agarose gel containing 0.5 μg/mL ethidium bromide and visualized under UV light using a 100-bp molecular marker as standard, generating fragments of 343 bp.

**STATISTICAL ANALYSIS**

The means of continuous variables were compared by the Student t-test. The χ² test or Fisher’s exact test was used for comparison of the frequency of qualitative variables. A 95% confidence interval was adopted and differences were considered to be significant when P < 0.05.

**RESULTS**

The mean age of the 32 patients was 58 ± 12 years (range, 24–90 years). Twenty (62%) patients were males, with a male:female ratio of 1.6:1. Twenty-one (66%) patients reported previous surgery and 12 (38%) had a history of blood transfusion (Table 1). Habitual alcohol consumption (>80 g/day) was observed in 16 (50%) patients, all of them males. No intravenous drug use was reported.

Among the 32 patients evaluated, 28 (88%) presented clinical-laboratory or histological abnormalities suggestive of hepatic cirrhosis. According to the Child–Pugh classification cirrhosis was classified as class A in 8 (25%) patients, as class B in 14 (44%), and as class C in 6 (19%). Hepatic cirrhosis was observed in all patients who were considered to be habitual alcoholics.

Serum AST and ALT levels were elevated in 28 (88%) and 22 (68%) patients, respectively, with AST levels being higher than ALT levels. Serum alkaline phosphatase levels were normal in only one of them (3%). Serum albumin levels were reduced in 23 (72%) patients and AFP presented a wide variation, being lower than 10 ng/ml in five (16%), whereas 16 (50%) patients showed titers higher than 400 ng/ml and 11 (34%) presented intermediate levels. The mean laboratory data are shown in Table 2.

Viral markers were detected in 26 (81%) of the 32 patients, with 25 (78%) being positive for HBV and/or HCV (Fig. 1). HGV-RNA was detected in nine (28%) of the 32 patients, but was detected alone in only one case (3%).

**Table 1. Clinical and demographic characteristics of the 32 patients with hepatocellular carcinoma, according to the presence or absence of HGV-RNA**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total (n = 32)</th>
<th>HGV positive (n = 9)</th>
<th>HGV negative (n = 23)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male:female)</td>
<td>1.6 : 1</td>
<td>3.5 : 1</td>
<td>1.3 : 1</td>
<td>0.42</td>
</tr>
<tr>
<td>Age (years)</td>
<td>58 ± 12</td>
<td>58 ± 17</td>
<td>58 ± 13</td>
<td>1.00</td>
</tr>
<tr>
<td>Surgical history</td>
<td>21 (66%)</td>
<td>4 (44%)</td>
<td>17 (74%)</td>
<td>0.21</td>
</tr>
<tr>
<td>Blood transfusion</td>
<td>12 (38%)</td>
<td>2 (22%)</td>
<td>10 (43%)</td>
<td>0.42</td>
</tr>
<tr>
<td>Alcoholism</td>
<td>16 (50%)</td>
<td>5 (56%)</td>
<td>11 (48%)</td>
<td>1.00</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>28 (88%)</td>
<td>8 (89%)</td>
<td>20 (87%)</td>
<td>1.00</td>
</tr>
</tbody>
</table>

HGV, hepatitis G virus; RNA, ribonucleic acid.

**Table 2. Laboratory characteristics of the 32 patients with hepatocellular carcinoma, according to the presence or absence of HGV-RNA**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total (n = 32)</th>
<th>HGV positive (n = 9)</th>
<th>HGV negative (n = 23)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (IU/ml)</td>
<td>167 ± 161</td>
<td>240 ± 255</td>
<td>138 ± 97</td>
<td>0.27</td>
</tr>
<tr>
<td>ALT (IU/ml)</td>
<td>76 ± 74</td>
<td>73 ± 62</td>
<td>77 ± 80</td>
<td>0.87</td>
</tr>
<tr>
<td>Bilirubin (mg/dl)</td>
<td>1.8 ± 1.7</td>
<td>1.46 ± 0.83</td>
<td>1.96 ± 1.91</td>
<td>0.45</td>
</tr>
<tr>
<td>Direct bilirubin (mg/dl)</td>
<td>1.1 ± 1.5</td>
<td>0.80 ± 0.60</td>
<td>1.24 ± 1.67</td>
<td>0.45</td>
</tr>
<tr>
<td>AP (IU/ml)</td>
<td>348 ± 269</td>
<td>421 ± 379</td>
<td>322 ± 225</td>
<td>0.36</td>
</tr>
<tr>
<td>Prothrombin time (s)</td>
<td>1.9 ± 1.6</td>
<td>1.2 ± 1.3</td>
<td>2.2 ± 1.6</td>
<td>0.11</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>2.9 ± 0.6</td>
<td>3.0 ± 0.6</td>
<td>2.9 ± 0.6</td>
<td>0.59</td>
</tr>
<tr>
<td>AFP (ng/dl)</td>
<td>565 ± 498</td>
<td>746 ± 466</td>
<td>494 ± 503</td>
<td>0.20</td>
</tr>
</tbody>
</table>

AST, aspartate aminotransferase; ALT, alanine aminotransferase; AP, alkaline phosphatase; AFP, alpha-fetoprotein.
Among the six patients without viral markers, two reported habitual alcohol consumption. The other four (13%) patients did not present any of the studied risk factors for the development of HCC (Fig. 1).

The mean age of the nine patients with HGV-RNA was 58 ± 17 years (range, 39–90 years); seven (78%) of them were males, with a male:female ratio of 3.5:1. Four (44%) patients reported previous surgery and two (22%) had a history of blood transfusion (Table 1). Habitual alcohol consumption was reported by five (56%) patients and cirrhosis was observed in eight (89%) of the nine patients.

In contrast, the mean age of the 23 HGV RNA-negative patients was 58 ± 13 years (range, 24–84 years); 13 (57%) of them were males, with a male:female ratio of 1.3:1. Seventeen (74%) patients reported previous surgery and 10 (43%) had a history of blood transfusion. Habitual alcohol consumption was observed in 11 (48%) patients and hepatic cirrhosis in 20 (87%). Comparison of the two groups (HGV-RNA positive and negative) revealed no significant difference in gender, age, history of surgery or blood transfusion, alcohol consumption, or signs of hepatic cirrhosis (Table 1). As can be seen in Table 2, there were also no significant differences in the laboratory data between the two groups.

**DISCUSSION**

We evaluated the frequency of HGV in patients with HCC in order to verify a possible role of this viral infection in the etiology of HCC in our country. The mean age of the 32 studied patients (58 years) was similar to that reported in other studies and the gender distribution (Table 1) was close to that observed in countries with a low or intermediate prevalence of HCC (2,3,5,6). Habitual alcohol consumption was observed in 50% of the patients studied, a frequency slightly higher than the 37% reported among patients with HCC from the same country (11). Cirrhosis was detected in 88% of the 32 patients with HCC.

The mean AFP level was 565 ng/dl; however, it should be noted that this mean value was possibly underestimated due to fixation of the upper AFP limit at 1050 ng/ml by the equipment used (Table 2). In addition, the percentage of patients with normal AFP (16%) was low compared to the frequency of about 30% reported in the literature (17,18,20). This finding might be due to the small number of patients with a biopsy for confirmation of the diagnosis of HCC and indicates the need for an increased AFP level as an inclusion criterion in the present study.

No markers for HBV, HCV or HGV, or evidence of alcohol consumption were observed in four cases, corresponding to 13% of patients without apparent risk factors, a rate lower than the 42% of patients with undetermined etiology reported in a Brazilian survey (6). Markers for HBV and HCV and HGV-RNA were detected in 81% of the 32 patients with HCC, with 78% presenting positive serology for HBV or HCV (Fig. 1). It should be noted that positive serology for HBV was not only defined as the presence of HBSAg but also as the presence of anti-HBc. In fact, recent studies have emphasized that, despite the lower risk of HCC in cases with serological ‘cure’, i.e. detection of anti-HBs, compared to patients with persistent viremia, the chance of the occurrence of liver tumors seems to be higher among patients with a history of HBV infection than in the general population (21,22).

HGV was detected in nine (28%) patients, but was present alone in only one case (3%). This frequency of HGV is similar to that detected in China where the virus was identified among 27% of patients with HCC and higher than that reported in Italy, the USA and Japan where the frequency ranged from 4 to 9% (5,16, 23,24). In contrast, the frequency of HGV alone among patients with HCC was similar to that reported in Italy (2.9%) and lower than that observed in China (9%) (5,16).

The observation of concomitant infection with HBV and/or HCV in eight (89%) of the nine patients infected with HGV suggests that the agents might have shared the same transmission routes, i.e. co-infection or superinfection. The possibility of superinfection with HGV in cases of chronic liver disease caused by HBV or HCV seems to be relevant because these patients frequently require medical care, with a consequent higher risk of parenteral contamination. However, because HBV markers were detected in seven (88%) of eight HGV-infected patients, one may speculate that contamination occurred through the sexual or vertical route and not through the hematogenic route. As a consequence, in the present study no correlation was observed between cases with HGV infection and a history of surgery or blood transfusion (P = 0.42), or reports of intravenous drug use. However, the lack of a correlation might be due to the small number of patients studied. Nevertheless, the detection of concomitant infection with the three viruses, including HCV, in a small portion of patients (25%) raises the possibility of parenteral transmission.

Another interesting finding of the present study was that patients with concomitant infections could have longer disease duration because they presented higher AST levels,
although without statistical difference (Table 2). The HGV co-infected also presented higher alkaline phosphatase and AFP levels, suggesting that these patients could have a larger size of the hepatic tumors. In principle, longer disease duration may result in a higher risk of contamination with HGV or, alternatively, the association between infections may potentiate the oncogenic effect of viral agents.

Indeed, a recent review article confirmed a higher risk of HCC in patients with HBV/HCV co-infection compared to monoinfected subjects (21). Additionally, Yang et al. (25), in Taiwan, showed that HBV/HGV co-infection increases the risk of the development of HCC, although a higher risk was not observed for HCV/HGV co-infection.

In summary, in the present study most patients with HCC had hepatic cirrhosis (88%), which was related to the presence of HBV (62%), HCV (38%) and HGV (28%). However, isolated HGV virus infection was only detected in one of the seven patients who presented no markers for HBV or HCV, suggesting that this agent is of low relevance in the etiology of HCC. Further studies are necessary to better understand the true hepatocarcinogenic potential of HGV alone or in combination with HBV or HCV infection.

**Conflict of interest statement**

None declared.

**References**