Abstract

Estimates of occult hepatitis B virus (HBV) infection prevalence varies among different studies depending on the prevalence of HBV infection in the study population and on the sensitivity of the assay used to detect HBV DNA. We investigated the prevalence of occult HBV infection in cirrhotic patients undergoing liver transplantation in a Brazilian referral center. Frozen liver samples from 68 adults were analyzed using a nested polymerase chain reaction assay for HBV DNA. The specificity of the amplified HBV sequences was confirmed by direct sequencing of the amplicons. The patient population comprised 49 (72.1%) males and 19 (27.9%) females with a median age of 53 years (range=18-67 years). Occult HBV infection was diagnosed in three (4.4%) patients. The etiologies of the underlying chronic liver disease in these cases were alcohol abuse, HBV infection, and cryptogenic cirrhosis. Two of the patients with cryptic HBV infection also presented hepatocellular carcinoma. Markers of previous HBV infection were available in two patients with occult HBV infection and were negative in both. In conclusion, using a sensitive nested polymerase chain reaction assay to detect HBV DNA in frozen liver tissue, we found a low prevalence of occult HBV infection in cirrhotic patients undergoing liver transplant, probably due to the low prevalence of HBV infection in our population.

Key words: Occult hepatitis B virus infection; Hepatitis B virus; HBV DNA; Liver transplantation

Introduction

Hepatitis B virus (HBV) infection is a major global health problem, with an estimated 350 million people chronically infected with the virus (1). HBV carriers are traditionally identified by detection of the viral surface antigen (HBsAg) in their blood. However, the development of sensitive nucleic acid detection techniques allows identification of patients with occult HBV infection (OBI) – HBsAg-negative subjects with detectable HBV DNA in the serum or liver tissue.

Although OBI occurs worldwide, its distribution may reflect the general prevalence of HBV in various geographic regions and different populations (2). Emerging evidence of the potentially clinical importance of OBI is responsible for the growing interest in this condition. In fact, OBI may have clinical impacts on the possibility of transmission of the infection, the risk of reactivation, the contribution to liver disease progression, and the development of hepatocellular carcinoma (HCC) (3-7). OBI may also be related to cryptogenic chronic liver disease (8,9).

The aim of this study was to investigate the prevalence of OBI in HBsAg-negative cirrhotic patients undergoing liver transplantation in a Brazilian referral center and to describe OBI-related factors with an emphasis on the etiology of the underlying chronic liver disease, the association with HCC, and the profile of serum markers of prior HBV infection.

Patients and Methods

A total of 68 HBsAg-negative adult cirrhotic patients who underwent liver transplantation at the Hospital das
DNA Mini Kit (Qiagen, Germany). The extracted DNA was stored at –70°C until HBV DNA analyses.

HBV DNA analyses
HBV DNA was extracted from 25 mg of frozen liver tissue using the QIAamp® DNA Mini Kit (Qiagen, Germany). The extracted DNA was stored at –70°C until analysis by nested polymerase chain reaction (PCR). The nested PCR was performed with the MiniCycler™ (MJ Research, Inc., USA) using primers with the sequences of the HBV preS-S, precore-core, Pol and X regions as previously described (10). The AmpliTaq Gold PCR Master Mix (Applied Biosystems, USA) was used for first and second reactions under the following amplification conditions: 10 min at 95°C, followed by 35 cycles consisting of 95°C for 30 s, 55°C for 45 s, and 72°C for 1 min. OBI was diagnosed when HBV DNA was detected in at least two different regions of the HBV genome in duplicate assays. Negative and positive controls were included in all PCR reactions. The specificity of the amplified HBV sequences was confirmed by sequencing of the amplicons.

Nucleotide sequences of the amplified PCR products were determined by the Sanger sequencing technology (11) using specific primers as described elsewhere (10). The cycle sequencing reaction was performed with the BigDye Terminator Version 3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems). The sequencing products were read bidirectionally on an ABI3130 instrument (Applied Biosystems), and the chromatogram was read using the Sequencher version 7.1 software (GeneCodes Comp., USA).

Results
The patient population comprised 49 (72.1%) males and 19 (27.9%) females, and had a median age of 53 years (range, 18-67 years). The etiology of the underlying chronic liver disease was attributed to alcohol in 23 (33.8%) patients, hepatitis C virus (HCV) in 17 (25%), autoimmune liver disorders in 10 (14.7%), hemochromatosis in 2 (2.9%), and was cryptogenic in 16 (23.5%). HCC was diagnosed in 20 (29.4%) of these patients, and the tumor was diagnosed in 2 of them only after liver transplantation. Results of hepatitis B core antibody (anti-HBc) tests were available for 61 patients (10 positive, 16.3%); results of hepatitis B surface antibody (anti-HBs) tests were available for 64 patients (20 positive; 31.3%). Five patients were positive for both markers, and 34 were negative for both markers. The patient demographic and clinical characteristics, distribution of markers of previous HBV infection, and etiologies of the underlying chronic liver disease are shown in Table 1.

OBI was diagnosed in 3 (4.4%) patients in whom the etiologies of the underlying chronic liver disease were alcohol abuse, HCV infection, and cryptogenic cirrhosis. Two of these individuals also presented with HCC; and in 1 patient, the tumor was only diagnosed in the explanted liver. Markers of previous HBV infection were available in 2 of the OBI patients and were negative in both (Figure 1). The HBV genome was identified exclusively in nontumor tissue. The preS-S region was amplified in all HBV-positive samples, whereas the precore-core region was absent in all of them.

Discussion
Using the generally accepted criterion of HBV DNA detection of at least two out of four HBV genomic regions to define OBI (12), and searching for HBV DNA in frozen liver tissue, the prevalence of this condition in our study population was relatively low. Other authors found a higher prevalence of OBI in patients with chronic liver disease, particularly in patients with chronic HCV infection (range = 33-57%) (2,13-16). Of note, a recent study conducted in the United States found that 50% of patients of Caucasian origin undergoing liver transplantation for HCV-related cirrhosis were OBI-positive (15). OBI has been less extensively investigated in patients with HCV-negative chronic liver disease, but its prevalence has been reported to range between 20 and 30% in subjects with cryptogenic liver disease (16). The underlying chronic liver diseases in the 3 OBI cases observed in this study — alcohol abuse, HCV infection, and cryptogenic cirrhosis — correspond to the most frequent reasons for liver transplantation in our setting.

OBI prevalence varies among different studies, which has been attributed to the different rates of HBV infection in the various populations, differences in the assays employed for HBV DNA detection, and the biological specimens (serum or liver tissue) used to detect HBV DNA (6,17,18). Furthermore, in some investigations, only anti-HBc positive patients were included.

In our series, we found only 1 case of OBI among the 17 patients with HCV-related cirrhosis. A study of 50 patients...
with HCV-related liver cirrhosis with or without HCC who underwent liver transplantation in São Paulo, Brazil, and in which HBV DNA from serum and paraffin-embedded liver tissue was investigated using real time PCR, found only 1 case of OBI (19). Those authors ascribed this result to the low prevalence of HBV infection in the State of São Paulo.

In two other investigations conducted in the State of São Paulo, OBI was diagnosed by nested PCR in sera of 12 of 50 (24%) (20) and 15 of 106 (14%) anti-HBc positive patients with chronic HCV infection (21). Differences in techniques, and the fact that all patients enrolled in these last two studies had markers of previous HBV infection, probably explain the differences in OBI prevalence in these investigations, our study, and that of Alencar et al. (19).

In OBI, DNA is detected more frequently in liver tissue than in serum and more frequently in frozen than in paraffin-embedded liver samples (17). As we investigated HBV DNA in frozen liver tissue, we expected a higher prevalence of OBI. The relatively low observed prevalence may be explained by the overall low rate of HBV infection in the state of Minas Gerais (22). It is known that HBV DNA levels are low in cryptic HBV infection (17); thus, in order to increase the likelihood of detecting HBV DNA, we employed a nested PCR assay that is more sensitive, but less specific. To confirm the specificity of our results, we performed direct sequencing analysis of the PCR products. Although we used a very sensitive PCR assay, the processing of the liver samples may have caused some breakage of the viral DNA with subsequent reduction in its detection and contributed to the low observed prevalence of OBI.

Regarding the relationship between OBI and HCC, a recent meta-analysis, which included 16 publications, demonstrated that cryptic HBV infection is an important risk factor for HCC development in patients with chronic liver disease (23). The available evidence suggests that OBI can contribute to HCC development by the same mechanisms described in overt HBV infection (6,18). Two of our three patients with OBI also had HCC. The small number of patients with OBI in this study prevented the investigation of any possible association with the occurrence of HCC.

The 2 OBI patients in our study for whom the results of anti-HBc and anti-HBs were available presented no markers of prior HBV infection.
with the presence of markers of previous HBV infection (especially anti-HBc and anti-HBs), but more than 20% of occult-infected individuals are negative for all HBV serum markers (13). Thus, it is possible to distinguish seropositive from seronegative OBI. During the course of HBV infection, some patients may undergo HBsAg seroclearance, either quickly after the resolution of acute hepatitis or after years or decades of overt chronic HBV infection, and become HBsAg negative, but anti-HBc and/or anti-HBs positive, giving rise to seropositive OBI. On the other hand, in seronegative OBI, the anti-HBV antibodies may have been lost gradually or the patients may have remained HBV-negative since the infection began (primary OBI) (18).

The mechanisms implicated in suppression of viral activity until the development of occult HBV are still unclear; nevertheless, the host’s immune status (24,25) and epigenetic mechanisms (26) seem to be involved. Mutations in the surface gene and its regulatory regions may cause a strong reduction of HBsAg expression. However, in the great majority of OBI individuals, these mutations are not detected in HBV isolates, and these variant viruses can be found in isolates from patients with overt HBV infections. Thus, they seem to be responsible for only a minority of cases of OBI (27).

Although the clinical implications of OBI in the context of liver transplantation have not been fully elucidated, growing evidence suggests that it may play a role in different clinical situations. Firstly, livers from OBI donors carry a significant risk of HBV transmission with subsequent hepatitis B in the newly infected recipients. This form of HBV acquisition is a well-known and frequent cause of hepatitis B in liver transplantation recipients without previous HBV infection and forms the basis for the general agreement in using anti-HBV prophylaxis in HBsAg-negative transplanted patients who receive livers from anti-HBc positive donors (6,18,28,29). Secondly, the development of immunosuppression due to the therapy after liver transplantation may disturb the host-virus balance in occult-infected patients prior to liver transplantation, leading to reactivation of virus replication and development of a typical hepatitis B infection, which often has a severe or even a fulminant course (18,28). Thirdly, some preliminary evidence points toward a possible role of HBV in the progression of the liver damage in post-transplant liver disease in HCV-positive patients (18). Fourthly, OBI is assumed to be an important risk factor for HCC development (6,18,28) as discussed above.

In conclusion, the prevalence of OBI was relatively low in cirrhotic patients who underwent liver transplantation in this referral center in the state of Minas Gerais, Brazil. A larger number of patients with OBI is required for analysis of the association between this entity and the occurrence of HCC in our setting. Studies on cryptic HBV infection should continue in order to clarify the various aspects of this disorder, especially its clinical relevance.

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