Occult Hepatitis B Infection and HBV Replicative Activity in Patients with Cryptogenic Cause of Hepatocellular Carcinoma

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We aimed to investigate the incidence of occult hepatitis B infection (OBI) in patients with “cryptogenic” hepatocellular carcinoma (HCC) and to study the HBV replicative activity in these patients. Tumorous and adjacent nontumorous liver tissues were obtained from 33 cryptogenic HCC patients and 28 HCC patients with identifiable causes (13 with chronic hepatitis B [CHB], six with chronic hepatitis C, and nine alcohol-related). OBI was identified by nested polymerase chain reaction (PCR). Intrahepatic HBV DNA, covalently closed circular DNA (cccDNA), and pregenomic RNA (pgRNA) were quantified by real-time PCR and reverse-transcription PCR (RT-PCR), respectively. OBI was identified in 24 (73%) cryptogenic HCC patients, one (17%) HCC patient with HCV, and five (56%) patients with alcohol-related HCC. In HCC patients with OBI, HBV DNA were detected in ≥2 HBV genomic regions more often in nontumorous tissues than in tumorous tissues (90% versus 57%, respectively; P = 0.007). Cryptogenic HCC patients with OBI had lower intrahepatic total HBV DNA levels than HCC patients with CHB (median: 0.010 versus 3.19 copies/cell, respectively; P < 0.0001). Only six (26%) cryptogenic HCC patients with OBI had detectable cccDNA (median: <0.0002 copies/cell), which was significantly lower than that of the CHB patients (median: 0.005 copies/cell; P < 0.0001). HBV pgRNA were detectable in 12 (52%) cryptogenic HCC patients with OBI (median: 0.0001 copies/cell), which was significantly lower than that of the CHB patients (median: 2.90 copies/cell; P < 0.001). Conclusion: 73% of patients with apparently unidentifiable causes for HCC were HBV-related. The detection rate was higher in nontumorous tissues than tumorous tissues. The low intrahepatic HBV DNA and pgRNA levels indicated that persistent viral replication and possibly HBV integration are the likely causes of HCC in OBI patients. (HEPATOLOGY 2011;54:829-836)

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undetectable circulating HBsAg. With the advance of sensitive nucleic acid detection techniques, HBsAg-negative subjects with detectable HBV DNA in sera or liver tissues can be identified. Patients who have undetectable HBsAg and yet have detectable HBV DNA in sera or liver tissues are defined as having occult HBV infection (OBI). OBI as a cause of liver disease in HBsAg-negative patients is becoming an important disease entity.\(^4\)\(^-\)\(^7\) Two studies conducted by our research group demonstrated that patients with HBsAg sero-clearance can still develop advanced liver diseases and HCC.\(^8\)\(^,\)\(^9\) There have been a number of early studies that reported the detection of HBV DNA in the liver tissues of HBsAg-negative HCC patients using DNA hybridization techniques.\(^10\)\(^-\)\(^12\) Since then there have been many reports using nucleic acid amplification techniques to detect HBV DNA in either liver tissues or sera in HBsAg-negative HCC patients.\(^13\) The prevalence of OBI in HBsAg-negative, anti-HCV-negative HCC patients varies among different populations, ranging from 16% in the USA,\(^14\) which has a low prevalence of CHB, to 70% in CHB endemic areas like China.\(^15\) OBI is also common in patients with HCC patients with chronic hepatitis C infection or alcoholic liver diseases.\(^16\)\(^-\)\(^19\) However, most of these studies were not based on the currently accepted criteria of the identification of OBI, which are the positive detection of HBV DNA in at least two different HBV genomic regions by nested polymerase chain reaction (PCR).\(^13\) Detailed studies on the detection of HBV DNA in both tumorous and nontumorous tissues from HCC patients are limited.

HBV covalently closed circular DNA (cccDNA) is an important intermediate in the life cycle of HBV, from which the HBV pregenomic RNA (pgRNA) and all HBV messenger RNA (mRNA) transcripts originate. Although the level of HBV replication in OBI patients is expected to be low, little is known about the levels of cccDNA and HBV pgRNA in HCC patients with OBI.

In the present study we aimed to use both the tumorous and adjacent nontumorous tissues from HCC patients with unknown etiology (“cryptogenic” HCC) to investigate the incidence of OBI as well as the levels of intrahepatic HBV DNA, cccDNA, and pgRNA in these patients.

**Patients and Methods**

**Patients.** Tumorous and adjacent nontumorous liver tissues were obtained from 61 Asian HCC patients who underwent surgical resection. Of these, 33 had HCC of unknown causes (cryptogenic HCC), and 28 had known etiologies, which included 13 with CHB, six with chronic hepatitis C, and nine with alcohol-related HCC with chronic alcohol consumption of >60 g per day. All patients had paired tumorous and nontumorous tissues available except that nontumorous tissues were not available in three cryptogenic HCC patients and one HCV patient. These tissues were rapidly frozen in liquid nitrogen and stored at \(-80^\circ\)C after resection. Serum samples were collected from these patients before liver surgery (range: 0-54 days). All patients consented to provide the tissues for investigations described below. This study was approved by the Institutional Review Board, University of Hong Kong.

**Isolation of Intrahepatic HBV DNA and RNA.** Total DNA was extracted from about 30 mg of liver tissues by the QIAamp Allprep Kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. This extraction kit allows simultaneous extraction of DNA, RNA, and protein from the same piece of liver tissue. During RNA isolation, on-column DNase digestion was performed using RNase-free DNase (Qiagen) to get rid of DNA contamination. The concentration of total RNA was estimated spectrophotometrically at 260 nm and the quality of RNA was checked by agarose gel electrophoresis.

**PCR Detection of Intrahepatic HBV DNA.** Intrahepatic HBV DNA was detected using a previously described nested PCR method with primers targeting to the S, polymerase (Pol), precore-core, and X regions of the HBV genome,\(^20\) with slight modification. The primers used are listed in Table 1. The condition for the first and second round PCR was 95°C 10 minutes, followed by 40 cycles of 95°C 30 seconds, 58°C 30 seconds, 72°C 1 minute, and final extension of 72°C for 10 minutes. Appropriate controls were included in each PCR.

**Quantification of Serum and Intrahepatic HBV DNA.** Serum HBV DNA levels were measured using the COBAS TaqMan HBV Monitor Test (Roche Diagnostics, Branchburg, NJ), with a lower limit of detection of 20 IU/mL (100 copies/mL). Intrahepatic total HBV DNA, cccDNA, and human genomic DNA (hgDNA) content in the liver tissues were measured by real-time PCR as described.\(^9\)\(^,\)\(^21\) Briefly, intrahepatic total HBV DNA and cccDNA were measured using primer-probe sets against the S and DR1-DR2 nicked regions, respectively.\(^21\) Plasmid-Safe ATP-dependent DNase (Epicentre, Madison, WI) was used to digest the single-strand region of HBV genome, allowing enrichment of cccDNA for subsequent real-time
PCR detection. The LightCycler DNA control kit was used for the measurement of hgDNA (Roche Applied Science, Mannheim, Germany). All real-time PCRs were performed using the Quantifast Probe real-time PCR kit (Qiagen) in the RotorGene Q PCR system (Qiagen). Using the estimation of 6.667 pg of hgDNA/cell, the amount of HBV DNA in the liver tissues was expressed as copies/cell. Taking into account the tissue size and the real-time PCR system used, we determined that the lower limit of detection of intrahepatic total HBV DNA and cccDNA quantification were $1 \times 10^{-4}$ copies/cell and $2 \times 10^{-4}$ copies/cell, respectively.

**Reverse Transcription and Analysis of HBV-Specific Transcripts.** Quantitative measurement of HBV pgRNA was performed using real-time RT-PCR as previously described with a few modifications.22 The RNA UltraSense One-step qRT-PCR system (Invitrogen, Carlsbad, CA) was used to measure the HBV RNA levels. The specific primers and probes used for RT-PCR for the detection of pgRNA, precore mRNA, and contamination HBV DNA were the same as those described.22 Because of the overlapping nature and the different starting site of the HBV pgRNA and precore mRNA, the pgRNA primers-probes set measured both the pgRNA and precore mRNA simultaneously, and the precore primers-probes set measured only the precore mRNA. The level of pgRNA expression, which reflects the HBV replicative activity, was then calculated as the difference between the quantitative values obtained from pgRNA and precore primers/probes sets. Because both HBV DNA and RNA were extracted from the same piece of liver tissue, normalization of HBV DNA and RNA expression levels to the number of cells can be done conveniently. The lower limit of detection for pgRNA is 10 copies/assay, which is equivalent to $1 \times 10^{-4}$ pgRNA copies/cell when the size of the liver tissues was taken into account.22

**Statistical Analysis.** All statistical analyses were performed using the Statistical Program for Social Sciences (SPSS 18.0 for Windows, Chicago, IL). The Mann-Whitney U test was used to test continuous variables with skewed distribution. Categorical variables were tested by the chi-square test or Fisher's Exact test when appropriate. For statistical calculations, undetectable intrahepatic total HBV DNA, cccDNA, and pgRNA levels were recorded as the lower limit of detection of the respective assays ($1 \times 10^{-4}$ copies/cell, $2 \times 10^{-4}$ copies/cell, and $1 \times 10^{-4}$ pgRNA copies/cell, respectively). Statistical significance was defined as $P < 0.05$.

**Results**

**Identification of Occult HBV Infection in HCC Patients.** Demographic data of the 61 HCC patients are shown in Table 2. Antibody-to-hepatitis B core antigen (anti-HBc) was detectable in 22 out of 33 (67%) cryptogenic HCC patients, four out of six (67%) HCV patients, and five out of nine (56%) CHB patients, four out of six (67%) HCV patients, and five out of nine (56%)
patients with alcohol-related HCC. Antibody-to-HBsAg (anti-HBs) serological data were available for all patients except for three cryptogenic HCC patients. Anti-HBs was detectable in 18 out of 30 (60%) cryptogenic HCC patients, one out of six (17%) HCV patients, and seven out of nine (78%) patients with alcohol-related HCC. Intrahepatic HBsAg immunostaining data were available for 25 cryptogenic HCC patients, three HCV patients, seven alcoholic patients, and 10 CHB patients. All non-CHB patients had negative intrahepatic HBsAg immunostaining. Positive intrahepatic HBsAg immunostaining was detected in six (60%) of 10 CHB patients. Out of 33 cryptogenic HCC patients, seven (21%) had radiological and/or histological evidence of nonalcoholic steatohepatitis (NASH) and 11 (33%) had diabetes mellitus (DM). Nineteen out of 33 (58%) cryptogenic HCC patients had not been diagnosed with NASH or DM. Cirrhosis was identified in the nontumorous tissues in 21 out of 33 (64%) cryptogenic HCC patients, all six HCV patients, all nine patients with alcohol-related HCC and 9 out of 13 (69%) CHB patients.

Detection of HBV DNA in the tumorous and adjacent nontumorous tissues in the study patients was performed using nested PCR, using primers against four regions (S, Pol, precore-core, and X) of the HBV genome. Representative nested PCR results are shown in Fig. 1. For the 13 CHB patients, HBV DNA was detectable in all four HBV regions in all 13 nontumorous tissues. In the tumorous tissues, HBV DNA was detectable in all four HBV regions in 10 cases, in three regions in one case, and two regions in two cases.

Nested PCR was then used to determine the presence of OBI in the cryptogenic HCC patients, HCV patients, and patients with alcohol-related HCC. OBI was defined as the positive detection of HBV DNA in at least two out of four HBV regions. Using this criterion, OBI was detected in 24 out of 33 (73%) cryptogenic HCC patients, one out of six (17%) HCV patients, and five out of nine (56%) alcohol-related HCC patients (Fig. 2). In total, 30 HCC patients (24 cryptogenic, one HCV, and five alcohol-related) were identified to have OBI, in which HBV DNA was detectable in all four regions in six nontumorous tissues and six tumorous tissues, in three regions in 10 nontumorous and three tumorous tissues and in two regions in 10 nontumorous and eight tumorous tissues. Of these 30 OBI patients, 21 (18 cryptogenic and three alcoholic) were anti-HBc-positive and 18 (14 cryptogenic and four alcoholic) were anti-HBs-positive. In total, 3/30 OBI patients (10%) were seronegative for all HBV serological markers. Of the 24 cryptogenic HCC patients with OBI, 14 (58%) had no evidence of NASH or DM.

The distribution of PCR-positive regions in the nontumorous and tumorous tissues of the 30 OBI patients is listed in Table 3. There were more samples with detectable HBV DNA in the X regions than in the other three regions in both the nontumorous and
tumorous tissues: 27/29 (93%) of the nontumorous tissues had detectable HBV DNA in the X region, compared with 18 (62%), 13 (45%), and 19 (66%) of the nontumorous tissues with detectable HBV DNA in the S, precore-core, and Pol regions, respectively ($P = 0.013, < 0.001, and 0.026$, respectively). Similarly, compared with the proportion of tumorous tissues with detectable HBV DNA in the X region (22/30; 73%), the proportions of tumorous tissues with detectable HBV DNA in the S (10/30; 33%), precore-core (14/30; 47%), and Pol (11/30; 37%) regions were significantly lower ($P = 0.006, 0.020$, and 0.011, respectively). Overall, there were more nontumorous tissues with detectable HBV DNA in ≥2 regions than tumorous tissues (26/29 [90%] versus 17/30 [57%], respectively, $P = 0.007$; odd ratios 6.6; 95% confidence interval 1.6-26.8).

Among the 48 non-CHB HCC patients (33 cryptogenic, six HCV, and nine alcoholic HCC patients), OBI was not identified in 18 of them (38%; nine cryptogenic, five HCV, and four alcoholic). Of these 18 HCC patients, four had detectable HBV DNA for one HBV genomic region in both the nontumorous and tumorous tissues, six had detectable HBV DNA for one HBV genomic region in the nontumorous tissues only, three had detectable HBV DNA for one HBV genomic region in the tumorous tissues only, and five (three cryptogenic and two HCV) had undetectable HBV DNA in both the nontumorous and tumorous tissues.

Quantitative Detection of Intrahepatic HBV DNA. Because the detection rate of HBV DNA was significantly higher in the nontumorous tissues than in the tumorous tissues, the levels of intrahepatic total HBV DNA and cccDNA were measured in the nontumorous tissues using quantitative real-time PCR method. The number of patients with detectable intrahepatic total HBV DNA, cccDNA, serum HBV DNA, and pgRNA (see below) is schematically shown in Fig. 2. In the 23 nontumorous tissues obtained from the cryptogenic HCC patients with OBI, 22 (96%) had detectable intrahepatic total HBV DNA (median: 0.010 copies/cell; range: <0.0001-0.36 copies/cell) and six (26%) had detectable cccDNA (median: <0.0002 copies/cell; range: <0.0002-0.12 copies/cell). Intrahepatic total HBV DNA was detectable in all five OBI patients with alcohol-related HCC (median: 0.012 copies/cell; range: <0.0001-0.36 copies/cell).

Table 3. Distribution of HBV Regions Detectable by Nested PCR in the Nontumorous and Tumorous Tissues of the 30 HCC Patients with OBI

<table>
<thead>
<tr>
<th>No. of PCR-Detectable HBV Regions in NT/T</th>
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<td>4/4</td>
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<tr>
<td>Total no. of cases/PCR positive regions</td>
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<td>18</td>
<td>13</td>
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range: 0.0005-0.22 copies/cells) and the HCV patient with OBI (0.0064 copies/cell). cccDNA was not detectable in either the HCV and alcoholic HCC patients. Compared to CHB patients, cryptogenic HCC patients with OBI had significantly lower intrahepatic total HBV DNA (3.19 versus 0.010 copies/cell, respectively; \( P < 0.0001 \)) and cccDNA levels (0.0052 versus <0.0002 copies/cell, respectively; \( P < 0.0001 \)). The quantitative levels of intrahepatic total HBV DNA, cccDNA, and pgRNA (see below) in each patient group are shown in Fig. 3.

Serum HBV DNA was also measured in the CHB patients and HCC patients with OBI. Serum HBV DNA was detectable in 12/13 CHB patients (median: 1.02 \( \times 10^{4} \) copies/mL; range: <100-5.48 \( \times 10^{7} \) copies/mL). All 30 HCC patients with OBI (24 cryptogenic, one HCV, and five alcohol-related) had undetectable serum HBV DNA.

**Intrahepatic HBV pgRNA Expression.** The levels of HBV pgRNA expression, which reflect HBV replicative activity in the nontumorous tissues, were measured (Fig. 3). HBV pgRNA was detectable in 12/23 (52%) cryptogenic HCC patients with OBI (median: 0.0001 pgRNA copies/cell; range: <0.0001-0.027 pgRNA copies/cell). HBV pgRNA was detectable in 12/13 (92%) CHB patients (median: 2.90 pgRNA copies/cell; range: <0.0001-169.66 pgRNA copies/cell) and 3/5 (60%) alcoholic HCC patients with OBI (median: 0.0002 pgRNA copies/cell; range: <0.0001-0.0020 pgRNA copies/cell), and was not detectable in the HCV patient with OBI. Cryptogenic HCC patients with OBI had lower median pgRNA levels (<0.0001 copies/cell) than CHB patients (2.90 copies/cell; \( P < 0.001 \)).

The levels of HBV replicative activity were also expressed in terms of pgRNA level / cccDNA copies. In the CHB patients, each cccDNA molecule generated a median value of 746 copies of pgRNA (range: 0.0080-2,870). The median levels of HBV replicative activity in the cryptogenic HCC patients with OBI was only 0.22 pgRNA/cccDNA (range: 0.025-9.47 pgRNA/cccDNA).

**Discussion**

Although the causal relationship between CHB infection and HCC is well known, the association between OBI and HCC is less well defined. There have been a number of studies on the detection of HBV DNA in HBsAg-negative HCC patients.\(^{10-14,20,23}\) However, detailed studies on the detection of OBI in both tumorous and nontumorous tissues as well as intrahepatic HBV DNA and pgRNA levels in patients with cryptogenic HCC are limited.

In the present study we used the generally accepted criterion of the detectability of HBV DNA in at least two out of four HBV genomic regions to define OBI in patients with a cryptogenic cause of HCC.\(^{13}\) In our control group of HCC patients with CHB, all four HBV genomic regions, namely S, Pol, precore-core, and X, were detectable in the nontumorous tissues in all 13 patients by this method. When this method was employed to measure HBV DNA in cryptogenic HCC patients, 73% of the cryptogenic HCC patients were identified to have OBI. This high figure is likely related to the high prevalence of CHB in Hong Kong. Nevertheless, it is possible that this figure was an underestimation. Our nested PCR results revealed that only five HCC patients (three cryptogenic and two HCV) had undetectable HBV DNA in both the nontumorous and tumorous tissues. There remains the possibility that, due to extremely low HBV DNA levels, HBV DNA is only detectable in one HBV region but undetectable in three other regions in some OBI patients, who would have been excluded by the currently accepted criterion for OBI detection. Thus, taking patients with detectable HBV DNA in any one region in either the nontumorous or tumorous tissues as potentially having OBI, the percentage of patients with OBI would be considerably higher.
This study, as well as other studies on OBI in different areas, suggested a causal role of OBI in HCC development. Although NASH and DM are possible risk factors for cirrhosis/HCC, 58% of the cryptogenic HCC patients with OBI had no evidence of NASH or DM in the present study. Thus, even without the added risk of NASH or DM, OBI alone is a likely risk factor for HCC in our study patients. Possible mechanisms of OBI-induced hepatocarcinogenesis include HBV-induced liver injury such as necroinflammation and fibrosis, potential transforming activities of the hepatitis B X protein and truncated hepatitis B middle surface protein, and chromosomal instability resulting from integration of HBV DNA into host chromosome.

We found that there was a significant difference in the detection rate of HBV DNA between the tumorous and nontumorous tissues in OBI subjects. Only a few studies have addressed the difference in HBV replication level between tumorous and nontumorous tissues. In a previous study we measured HBV DNA levels in tumorous and nontumorous tissues of HCC patients with CHB and found that HBV replication was lower in the tumorous tissues than in the corresponding nontumorous tissues. Although the present study did not examine this particular aspect, the number of tumorous tissues with detectable HBV DNA in ≥2 HBV regions was significantly lower than that of the nontumorous tissues. Both the present study and our previous study led to a speculation that tumorous tissues may not have the optimal environment for HBV replication. Although further studies are needed to elucidate this speculation, this finding may shed some light on some of the difficulties encountered in the identification of OBI in HCC patients using liver HBV DNA detection. Based on the criterion for OBI identification by nested PCR method, 13 (43%) out of the 30 OBI patients identified would have been missed if only tumorous tissues were used, whereas only three (10%) of OBI patients would have been missed if only nontumorous tissues were used. Moreover, if only quantitative detection of serum HBV DNA were used for the identification of OBI patients in this study, all OBI patients would have been misclassified as HBV-negative. Taken together, this implies the importance of obtaining nontumorous tissues to identify OBI in cryptogenic HCC patients.

In addition to the qualitative detection of HBV DNA in HCC patients with CHB and OBI using nested PCR, intrahepatic and serum HBV DNA and pgRNA were quantified in the present study using real-time PCR and real-time RT-PCR, respectively. To our knowledge, this is the first study to quantify HBV pgRNA levels in HCC patients with OBI. The levels of pgRNA in CHB HCC patients determined in this study were comparable to that reported in hepatitis B e-antigen-negative CHB patients without HCC, whereas cryptogenic HCC patients with OBI had lower levels of intrahepatic total HBV DNA, cccDNA, and pgRNA than CHB patients. Furthermore, the number of copies of pgRNA produced by each cccDNA molecule in cryptogenic HCC patients with OBI was significantly lower than that of HCC patients with CHB, suggesting that the HBV transcriptional activity was low in cryptogenic HCC patients with OBI. However, because the range of pgRNA/cccDNA was quite wide, especially in CHB patients, this finding needs to be supported by future studies. Previous evidence has shown that HBV transcriptional activity is suppressed in CHB patients with low HBV viral load. In this present study, our data provide the first indirect evidence that HBV transcriptional activity may also be suppressed in cryptogenic HCC patients with OBI. However, even a low level of HBV replication may still be the crucial factor for the development of HCC in these OBI patients in whom the hepatitis B infection was acquired within the first 2 years of life. Although the HBsAg status before their presentation with HCC was not known, it is highly possible that these cryptogenic HCC patients were CHB patients who had undergone HBsAg seroclearance. As demonstrated in our previous studies, patients with HBsAg seroclearance can still develop HCC.

Another important finding observed in the present study is that five out of nine patients with alcohol-related HCC had OBI. In an early study by Bréchot et al., all 20 HCC patients in France with alcoholic liver diseases had detectable HBV DNA in the liver. Although it is well known that alcoholic liver diseases per se can cause liver cirrhosis and HCC, the present study found that more than 50% of patients with alcohol-related HCC were found to have OBI. The exact carcinogenic contribution from these two risk factors, i.e., HBV and alcohol, should be worked out in more detail in future studies. There is a possibility that the hepatocarcinogenic risk increases synergistically in patients with OBI and alcoholic liver diseases, just as alcohol is proven to cause synergistic damage to the liver in CHB patients.

In conclusion, the present study identified that 73% of cryptogenic HCC patients had OBI in a highly endemic area for HBV infection. Although HBV replication activities (intrahepatic HBV DNA, cccDNA, and pgRNA) in cryptogenic HCC patients with OBI...
were lower than HCC patients with CHB, a low replicative level in the liver tissues might already be adequate to predispose patients to develop HCC.

References