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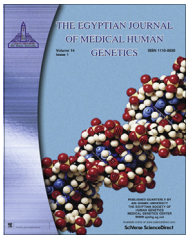

ORIGINAL ARTICLE

Detection of occult HBV infection by nested PCR assay among chronic hepatitis C patients with and without hepatocellular carcinoma

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HBV-DNA;
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Abstract Occult hepatitis B virus infection (OBI) has been reported among patients with chronic hepatitis C virus (HCV) infection and hepatocellular carcinoma (HCC). This study aimed to evaluate the prevalence of OBI in chronic hepatitis C patients with and without hepatocellular carcinoma. A total of 40 chronic hepatitis C patients who were negative for HBsAg, were enrolled into the study. They were classified into two groups: Group I which included 20 patients with chronic hepatitis C only whereas Group II included 20 patients with chronic hepatitis C and HCC. Detection of HBV DNA was done by nested-PCR using two sets of primers specific for surface and X viral genomes in serum and liver tissue of patients. Genotyping system based on PCR using type-specific primers was done for HBV-DNA positive intrahepatic samples. OBI was detected in the liver tissue in 23/40 (57.5%) of chronic HCV infected patients, 18 (78.3%) of whom belonged to group II, conferring a 90% prevalence of this group. In serum, HBV-DNA was detected in 9/40 patients (22.5%), 7 (77.8%) of whom belonged to group II. Among the 23 positive intrahepatic HBV-DNA samples studied, HBV-genotype D (34.8%) and B (26.1%) were more predominant, whereas HBV-genotype C (13.1%) and A (8.7%) infections were the least observed, respectively; mixed genotypes were detected in 17.4% (n = 4), 3 of them were with HBV-genotype D and B, and 1 was with HBV-genotype C and A.

In conclusion OBI is a fact in our community; it was detected in liver tissue of chronic HCV-infected patients, especially in cases of chronic HCC. In addition, OBI might be related to severity of necroinflammatory activity and fibrosis. Large studies are needed to confirm that co-infection could determine a worse progress of chronic liver disease in this population. Also, detection of intrahepatic HBV-DNA is more useful in diagnosis of OBI.

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1. Introduction

Hepatitis B and C viruses (HBV and HCV) are the main etiological agents of chronic hepatitis related to the emergence of liver cirrhosis and hepatocellular carcinoma (HCC). Despite careful investigations into the etiological factors for chronic li-

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ver disease, in 5–10% of cases no etiological factor is detected. However, this index may be smaller if molecular tests are performed in search of occult infection with HBV in this population [1].

Occult hepatitis B virus infection (OBI) is defined as the presence of hepatitis B virus (HBV) DNA in the liver of patients with negative results of hepatitis B surface antigen (HBsAg) test with or without serological markers of previous viral exposure [2]. In most cases, OBI is related to low level HBV infection with sub-detectable levels of HBsAg and not infection with HBV variants that cannot express S proteins or produce S proteins with aberrant epitopes which are not detected by conventional serological assays [3].

OBI may be involved in several clinical contexts; reactivation of the infection and consequent development of the HBV-related liver disease; transmission of the "occult" virus mainly through blood transfusion and orthotopic liver transplantation with consequent hepatitis B in the recipient; the effect on occurrence and progression of the chronic liver disease; and the role in hepatocarcinogenesis [4].

OBI has been found in individuals without HBV serological markers, past HBV infection, and with HCC patients with or without chronic hepatitis C (CHC) [5]. OBI has been found with a high prevalence in patients with chronic hepatitis C (CHC), probably because both HBV and hepatitis C virus (HCV) share the same parenteral way of transmission. In particular, HBV DNA is detectable in about one-third of CHC patients with negative results of HBsAg test in the Mediterranean basin [6]. OBI in patients with chronic HCV infection may be associated with more severe liver damage and even the development of HCC [7].

Despite evidence that co-infection with HBV and HCV may accelerate the progress to liver disease [8], be related to the emergence of HCC [9], and adversely influence the response to HCV treatment [6], there is still a debate on the consequences of occult HBV infection in patients with chronic liver disease by HCV. Studies are warranted to understand the significance of occult HBV infection better, and to clarify the possible role exerted by this cryptic infection on the outcome of liver disease and hepatocellular carcinoma development [10].

It has been reported that in individuals with OBI, the DNA level in peripheral blood is usually fewer than 10^3 copies/mL and as low as 1000 copies per 1 μg of DNA extracted from liver tissue. Advances in detection technologies in the past decade have allowed greater insight into OBI [11]. The frequency of detection of HBV DNA is higher in liver tissue than in serum [12].

The molecular basis of OBI is related to the long-lasting persistence in the nuclei of hepatocytes of the viral covalently-closed-circular DNA (cccDNA) [13]. Almost all OBI cases are infected with replication-competent HBV showing strong suppression of replication and gene expression, probably due to host immune-surveillance and epigenetic factors [12].

On the basis of HBV antibody profile, OBI may be distinguished in seropositive-and seronegative-OBI; the former is positive for hepatitis B core and/or s antibodies, the latter is negative for all markers of HBV infection besides very low amount of HBV DNA (<200 IU/ml) [14]. Seronegative-OBI cases may have either progressively lost HBV specific antibodies after the resolution of an acute infection or, theoretically, had negative results of tests from the beginning of infection, similar to what has been observed in the woodchuck model of hepadnavirus infection with the woodchuck hepatitis virus [15], where a low-dose infection was insufficient to allow the maturation of an antiviral protective memory response [16].

Although OBI status is significantly associated with the presence of antibodies to HBV [5], the analysis of liver DNA extracts represents the gold standard for occult HBV evaluation [14]. Hence, serum analysis must be taken into account only in the absence of liver specimens. In any case, it is strongly recommended to use a highly sensitive nested polymerase chain reaction (PCR) or real time PCR with oligonucleotide primers specific for different HBV genomic regions and complementary to highly conserved nucleotide sequences [17].

The purpose of this study was to assess the prevalence of OBI among chronic hepatitis C patients with and without hepatocellular carcinoma.

2. Subjects and methods

2.1. Study design and population

This cross-sectional study was conducted on forty Egyptian patients with chronic HCV infection in the period from January 2012 to February 2013. The study was held in the Internal Medicine Clinic of Ain Shams University (Cairo, Egypt). It was approved by the Hospital Ethics Committee of Ain Shams University Hospitals (ASUHs) and an informed consent was obtained from all subjects participating in this study. Inclusion criteria were: an age of 18 years or over, proven HCV infection based on viral load and HCV antibody (Ab), negative for hepatitis B surface antigen (HbsAg) and negative for HIV antibody. Exclusion criteria: history of alcohol abuse, the use of potentially hepatotoxic drugs, patients with autoimmune hepatitis, hemochromatosis, alpha-1-antitrypsin deficiency or Wilson disease.

Diagnosis of chronic HCV infection was based on standard serological assays and abnormal serum aminotransferase levels in some patients ($\geqslant 1.5$ times the upper limit of normal) for at least 6 months [18]. All patients were positive for HCV antibodies by the third generation enzyme linked immunosorbant assay (Orth. HCV 3.0 Orth., Raritan, NJ, USA) with infection confirmed by polymerase chain reaction and was quantitated in all patients using the amplicor HCV monitor assay (Roche, Diagnostic Systems, Branchburg, NJ, USA) then categorized as follows: weak viremia patients had 600–200,000 IU/ml, those with moderate viremia had 200,000–2 million IU/ml and those with high viremia had more than 2 million IU/ml.

The study population was classified into two main groups:

- **Group I**: 20 patients with chronic hepatitis C who were in pre-treatment evaluation with no evidence of hepatic mass lesions suspicious for HCC.
- **Group II**: 20 patients with chronic hepatitis C and HCC evaluated on admission for surgical resection of tumor, or liver nodule biopsy.

For all subjects in the study the following was done:

1. Detailed history taking and thorough clinical examination, symptoms and signs of chronic liver disease (Jaundice, fatigue, encephalopathy).

2. Laboratory investigations: complete blood count (CBC), liver function tests [serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), albumin, and bilirubin] and also α fetoprotein.
3. Abdominal ultrasound, abdomen computed tomography or magnetic resonance imaging to detect hepatic mass lesions suspicious for HCC.

2.2. Specimens

- A peripheral venous blood sample was collected into a tube containing EDTA.
- At the same time, ultrasound guided percutaneous liver biopsy was performed as a part of pre-treatment evaluation for HCV infection for histopathological analysis of the lesions and for further analysis of HBV DNA by PCR. Biopsy specimens were fixed in buffered formalin and embedded in paraffin.

The degree of inflammation and fibrosis were assessed and graded according to Franciscus [19]. Patients were divided according to the necroinflammatory score of the liver biopsy into three groups: patients with mild necroinflammatory activity (Score 1–8), patients with moderate necroinflammatory activity (Score 9–12), and patients with severe necroinflammatory activity (Score 13–18). They were also subdivided as regards fibrosis stage of the liver biopsy into: patients with mild fibrosis (Stage 0–1), patients with moderate fibrosis (Stage 2–4) and patients with severe fibrosis (Stage 5 or 6).

2.3. Detection of occult HBV DNA by nested PCR

In our study detection of HBV DNA was done by nested-PCR using two sets of primers (Table 1) specific for surface and X viral genomes regions, respectively, in both serum and tissue specimens as previously described by Kazemi-Shirazi et al. [20]. Usually, the genes amplified by PCR are S and X; the former has been found to be the most sensitive in serum, and the second has been described as the most sensitive in the liver [6]. The preferred lower limit of detection (LLOD) for HBV is 5 IU/mL [21].

2.3.1. DNA extraction

DNA was extracted from the serum by QIAGEN viral DNA extraction kit (Qiagen GmbH, Hilden, Germany) using 200 μl of the patient serum following the manufacturer's instructions. DNA was extracted from liver tissue by DNAase Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In brief as regards DNA extraction from liver tissue, 50 mg tissue specimens were homogenized in buffer (150 mmol/L NaCl, 50 mmol/L Tris–HCl (pH7.4), 10 mmol/L EDTA, 1% SDS), and incubated overnight with proteinase K (800 μg/mL) at 37 °C. After extraction with phenol/chloroform the nucleic acids were precipitated with ethanol. Nucleic acids were then resuspended and digested with pancreatic ribonuclease (100 μg/mL) followed by extraction with phenol/chloroform, and re-precipitation in pure cold ethanol. The DNA was resuspended in 10 mmol/L Tris–HCl (pH 7.4), 1 mmol/L EDTA, and its concentration was determined by using a UV spectrophotometer (Nanodrop). Amplification of the β-actin gene was done to test the presence of artifacts and to set a baseline for tissue sample that enables the evaluation of the target genes in the HBV PCR. The amplified products were visualized on an ethidium bromide stained 2% agarose gel.

2.3.2. Determination of HBV genotypes

In this study we investigated the presence of HBV DNA genotypes A–F. The DNA extracted from liver tissues of patients positive for HBV DNA (n = 23) was subjected to nested PCR according to the described method by Naito et al. [22]. The sequences of PCR primers used in this study are shown in Table 2. In brief, the first and second- round PCR primers were designed on the basis of the conserved nature of nucleotide sequences in regions of the pre-S1 through S genes, irrespective of the six HBV genotypes. P1 and S1–2 were universal outer primers. B2 was used as the inner primer with a combination called mix A for genotypes A–C. The first PCR was carried out in 40 ul of a reaction mixture containing 100 ng of each outer primer, a 200 mM concentration of each of the four deoxynucleotides, 2.5 U of Taq DNA polymerase (Promega, France) 1 × PCR buffer containing 50 mM KCl, 10 mM Tris pH 8.3) and 1.5 mM $MgCl_2$. The cycling protocol included one cycle of 5 min at 95 °C, followed by 40 cycles consisting of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min. Two second-round PCRs were performed for each sample, with the common universal sense primer (B2) and mix A for types A through C and the common universal antisense primer (B2R) and mix B for types D through F. A 1 ul aliquot of the first PCR product was added to two tubes containing the second sets of each of the inner primer pairs, each of the deoxynucleotides, Taq DNA polymerase, and PCR buffer, as in the first reaction. These were amplified for 40 cycles with the following parameters: preheating at 95 °C for 5 min, 30 cycles of amplification at 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1.30 min. Genotypes of HBV for each sam-

Table 1 Sequences of primer pairs used for HBV PCR detection.

Gene	Sequence	Amplified segment
Surface gene	S-1s: 5′AGAACATCGCATCAGGACTC3′	159–178
	S-2a: 5′CATAGGTATCTTGCGAAAGC3′	642–623
	S-3s: 5′AGGACCCCTGCTCGTGTTAC3′	181–200
	S-4a: 5′AGATGATGGGATGGGAATAC3′	619–600
X gene	X1s: 5′CTAGCCGCTTGTTTTGCTCG3′	1282–1301
	X2a: 5′TTATGCCTACAGCCTCCTAG3′	1666–1647
	X3s:5′GGTCTTACATAAGAGGACTC3′	1518–1537
	X4a: 5′GTTCACGGTGGTCTCCAT3′	1625–1608

s: sense; a: antisense.

Table 2 HBV genotyping primer sequence.

Primer sequence	(Position, specificity, and polarity)
First PCR	
P1 5′-TCA CCA TAT TCT TGG GAA CAA GA-3′	(nt 2823–2845, universal, sense)
S1-2 5′-CGA ACC ACT GAA CAA ATG GC-3′	(nt 685–704, universal, antisense)
Second PCR	
Mix A	
B2 5′-GGC TCM AGT TCM GGA ACA GT-3′	(nt 67–86, types A–E specific, sense)
BA1R 5′-CTC GCG GAG ATT GAC GAG ATG T-3′	(nt 113–134, type A specific, antisense)
B1R 5′-CAG GTT GGT GAG TGA CTG GAG A-3′	(nt 324–345, type B specific, antisense)
BC1R 5′-GGT CCT AGG AAT CCT GAT GTT G-3′	(nt 165–186, type C specific, antisense)
Mix B	
BD1 5′-GCC AAC AAG GTA GGA GCT-3′	(nt 2979–2996, type D specific, sense)
BE1 5′-CAC CAG AAA TCC AGA TTG GGA CCA-3′	(nt 2955–2978, type E specific, sense)
BF1 5′-GYT ACG GTC CAG GGT TAC CA-3′	(nt 3032–3051, type F specific, sense)
B2R 5′-GGA GGC GGA TYT GCT GGC AA-3′	(nt 3078–3097, types D–F specific, antisense)

ple were determined by identifying the genotype-specific DNA bands. The two different second-round PCR products from one sample were visualized on an ethidium bromide-stained 3% agarose gel.

2.4. Statistical analysis

Analysis of data was done by using a personal computer software package (Statistical version 5, Stat Soft Inc. USA 1995). The data obtained were expressed as descriptive statistics (mean ± standard deviation). Statistics included two tailed unpaired *t*-test for comparison between two groups. Chi-square test (χ^2) was used for comparing and correlating categorical parameters, respectively. A p value < 0.05 was considered statistically significant and $p > 0.05$ was considered non significant.

3. Results

From the forty patients enrolled in the present study, 24 were males (60%) and 16 were females (40%) with mean age of 49.78 ± 5.04 years.

In serum, HBV DNA was detected in nine out of 40 patients (22.5%). As regards intrahepatic HBV DNA, it was detected in 23 out of 40 (57.5%), 4/40 of them had surface (S) gene (10%), 11/40 had X gene (27.5%), while 8/40 had both X and S genes (20%). X gene was detected in four patients within group I and in 15 patients within group II.

Furthermore, HBV DNA-positive was found more frequently in patients within group II (HCC) as we analyzed the prevalence of occult infection in this group and found that 7/9 of serum positive patients (77.8%) were in this group while 18/23 of intrahepatic positive samples (78.3%) were also in this group.

Among the 23 positive intrahepatic HBV-DNA samples studied, the distribution of HBV genotypes showed that, OBI are predominantly caused by HBV-genotype D and B that constituted (n = 8, 34.8%) and (n = 6, 26.1%), respectively. Whereas HBV-genotype C and A were the least detected and constituted (n = 3, 13.1%) and (n = 2, 8.7%) respectively, while; mixed genotypes were detected in 17.4% (n = 4), three cases with HBV-genotype D and HBV-genotype B and one case with HBV-genotype C and HBV-genotype A. No other genotypes were detected (Fig. 1).

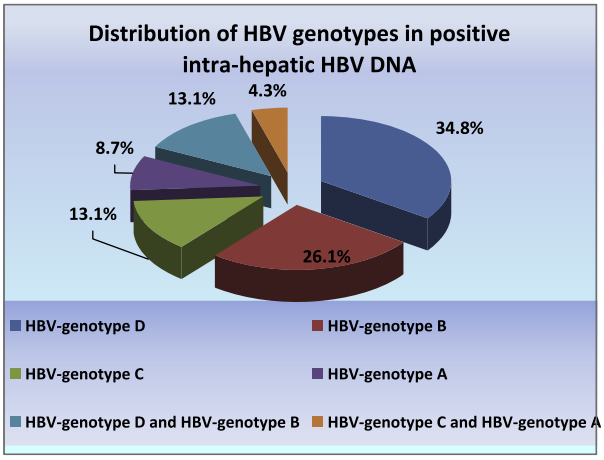

Figure 1 Distribution of HBV genotypes in positive intrahepatic HBV-DNA samples.

Among the various clinical characteristics, comparative study between the HBV DNA-positive and DNA-negative patients revealed statistically highly significant difference as regards the age, prothrombin time and the platelet counts ($p < 0.001$). While, total bilirubin, direct bilirubin, serum albumin and serum HCV RNA levels showed significant difference. However, there was non significant difference regarding gender, ALT, AST and ALP as shown in Table 3.

Comparison between patients with different grades of necroinflammatory activity and stages of fibrosis according to the detection of intrahepatic HBV DNA was done as shown in Tables 4 and 5. We noticed a higher percentage of positive intrahepatic HBV-DNA samples among patients with severe necroinflammatory activity (n = 15) than among patients with moderate and mild necroinflammatory activity (n = 6 & 2 respectively) (Fig. 2). Also, a higher percentage of positive intrahepatic HBV-DNA samples was observed in severe fibrosis (n = 19) than in moderate and mild fibrosis (n = 3 & 1 respectively) (Fig. 3).

4. Discussion

There is an increasing interest in OBI due to the increasing evidence of its clinical relevance. This can be divided into four

Table 3 Characteristics of HCV-infected patients (with and without occult HBV infection).

Laboratory tests	Positive Intrahepatic HBV DNA (n = 23)	Negative Intrahepatic HBV DNA (n = 17)	P value	Significance
Age (years)	51.74 ± 3.89	47.12 ± 5.29	0.0029	HS
Gender				
Male (n = 24)	14	10	0.8961	NS
Female (n = 16)	9	7		
ALT(IU/L)	82.22 ± 35	63 ± 32.13	0.0836	NS
AST(IU/L)	136.21 ± 84.11	102.02 ± 64.04	0.1694	NS
ALP (IU/L)	115.11 ± 31.74	97.35 ± 47.84	0.1661	NS
Total bilirubin (mg/dL)	1.79 ± 0.970	1.16 ± 0.34	0.0147	S
Direct bilirubin (mg/dL)	0.864 ± 0.314	0.541 ± 0.445	0.0104	S
Prothrombin time (%)	13.884 ± 1.65	12.21 ± 1.421	0.0018	HS
Albumin (g/dL)	3.646 ± 0.355	4.061 ± 0.743	0.0242	S
Platelets (/mm^3)	112.333.33 ± 68.291.65	183.691.89 ± 82.505.63	0.0049	HS
Serum HCV RNA (copy/ml)	22.652.73 ± 10.376.61	14.603.33 ± 8.604.63	0.0131	S

Table 4 Comparison of degree of necroinflammatory activity regarding presence of OBI.

Necro-inflammatory activity index	Occult HBV positive (n = 23)	Occult HBV negative (n = 17)
Mild (Score 0–8)	2 (8.6%)	3 (17.6%)
Moderate (Score 9–12)	6 (26.2%)	6 (35.3%)
Severe (Score 13–18)	15 (65.2%)	8 (47.1%)

Table 5 Comparison of degree of fibrosis regarding presence of OBI.

Fibrosis stages	Occult HBV positive (n = 23)	Occult HBV negative (n = 17)
Mild (Stage 0–1)	1 (4.3%)	5 (29.4%)
Moderate (Stage 2–4)	3 (13.1%)	4 (23.5%)
Severe (Stage 5 or 6)	19 (82.6%)	8 (47.1%)

categories: contagiousness of occult HBV infection, reactivation of occult HBV infection, its link to chronic liver diseases, and its link to hepatocellular carcinoma [23].

As HBV and HCV share similar transmission routes, co-infection with the two viruses is not a rare event in areas where the two viruses are endemic and among subjects with high risks of parenteral infections [24]. The importance of an association between HBV and HCV lies in the fact that although HBV viral load in occult infection is often low, a greater likelihood of progression to cirrhosis and to HCC is observed when these patients are compared with those infected only with HCV [1].

The prevalence of the intrahepatic HBV-DNA in the current study among CHC patients was 57.5% while it was 22.5% in serum samples. Similarly, OBI prevalence in HCV patients is reported to be 28.1–50% in liver tissue [11,25,26]. While, it was reported that 26.2–43.6% had detectable HBV DNA in serum [27–29]. In the study of Fang et al. [30], 28.3% of cryptogenic chronic liver disease patients and 70.4% of HBsAg-negative HCC patients were positive for HBV DNA in serum. Similarly, Bréchot et al. [2] reviewed some studies and concluded that about 20–30% of serum and 40–50% of liver tissue showed HBV DNA positivity in anti-HCV positive patients. Also, Hassan et al. [31] reported that intrahepatic occult HBV-DNA was detected in 62.5%, whereas; serum occult HBV-DNA was detected in only 22.5% of HCC patients.

However, other reports revealed prevalence of OBI ranging from 6.7–14.8% among chronic HCV patients [32–34]. While, Ferreira et al. [35] observed a lower rate (2.7%) in non- injecting drug users in Brazil. In the study of Rossi et al. [36], OBI was observed in 10% among patients with chronic lymphocytic leukemia. Whereas in the study of Roman et al. [37], OBI was detected in 14.2% of the native population sample in Mexico. Also, De Matos et al. [38] detected serum occult HBV DNA in 12.7% of injecting drug users in Brazil.

The patterns of local endemicity for HBV infection in the populations of the different geographic areas, the patients' sample investigated in each study, and the methodological differences between studies may explain the observed discrepancies in the rates of OBI among populations. Furthermore, among certain patient groups, the prevalence of occult HBV infection would likely be significantly higher.

In this study, HBV DNA-positive was found more frequently in patients within group II (HCC) as 7/9 (77.8%) of serum positive patients and 18/23 (78.3%) of intrahepatic positive samples were in this group. X gene was detected in four patients of group I and in 15 patients within group II.

Growing evidence suggests a high prevalence of occult B infection in HCV-infected patients with HCC as there is observed high proportion of HCV-related HCC cases show OBI [39–42]. Similarly, the prevalence of OBI was evaluated in CHC patients with and without HCC and found that 77.7% of them were with HCC [1]. Furthermore, other studies from Japan and Italy showed the reported frequency of HBV DNA detection in the liver of HBsAg-negative, anti-HCV-po-

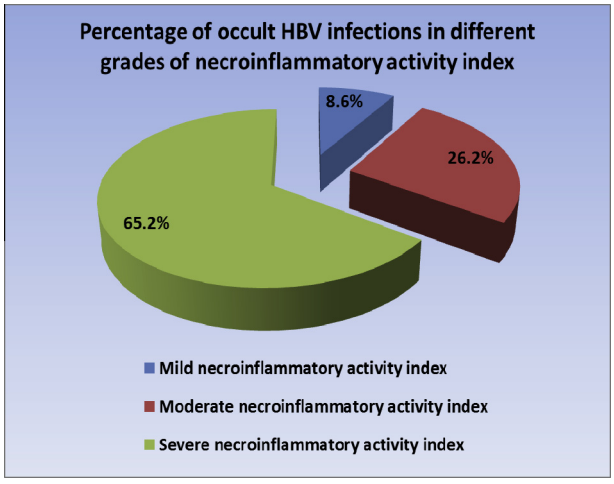

Figure 2 Percentage of occult HBV infection in different grades of necroinflammatory activity.

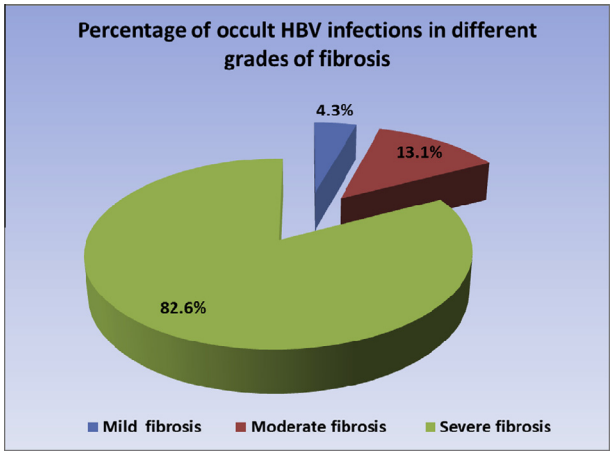

Figure 3 Percentage of occult HBV infection in different grades of fibrosis.

sitive patients ranged from 15% to 49% [43] for patients without HCC and up to 73% among patients with HCC [44].

In the current study, no follow up was done for group I patients who were co-infected with chronic HCV and OBI to detect its association with development of HCC. However, most [21,28,40,45,46] but not all [9,34,47,48] studies from Asia and Europe have found that patients with hepatitis C who had detectable HBV DNA in the liver or serum had an increased risk of HCC. One of the markers in HCC cases, is the presence of the HBV-X gene expression. Since the HBV-X protein positivity in liver tissue in several studies, reached half of the liver tissues specimens and X gene deregulates cell cycle control, interferes with cellular DNA repair and apoptosis, and plays an important role in interaction with p53 and Rb gene [49]. These findings suggested that OBI might contribute to hepatocyte transformation, playing a direct oncogenic role through both its integration into the host genome and a maintained transcriptional activity, allowing the synthesis of proteins with potential pro-oncogenic properties.

In the current study, the predominant OBI genotypes were HBV-genotype D and B, while HBV-genotype C and A were less predominant and mixed genotypes were much less predominant. Similar results were found in the study of Hassan et al. [31]. Also, Zekri et al. [50] reported that the prevalence of mixed genotype infections was 15.7% especially those with chronic active hepatitis. However, Vivekanandan et al. [51] found that the predominant genotype was genotype A followed by genotype D. Roman et al. [37] studied OBI genotypes among native Mexican populations (Nahuas and Huichol) and found that Nahuas had HBV/H the predominant genotype followed by genotypes D, C, B, A while Huichol had HBV/A as the predominant genotype followed by D, G and H. Furthermore, Candotti et al. [52] found that HBV genotypes B and C were dominant. De Albuquerque et al. [53] and Matos et al. [38] found that Genotype D was the most common, followed by genotype A among OBI patients.

In this study, comparative study between characteristics of the HBV DNA-positive and DNA-negative patients revealed statistically highly significant difference as regards the age, prothrombin time and the platelet counts ($p < 0.001$). While, total bilirubin, direct bilirubin, albumin and serum HCV RNA levels showed significant difference. However, there was non significant difference regarding gender, ALT, AST and ALP.

In agreement with previous reports by Fukuda et al. [54] and Mrani et al. [55] who showed that serum HCV-RNA load was significantly higher in CHC OBI-positive patients than in negative ones. Also, Matsuoka et al. [28] reported that only the age of the HBV DNA-positive and DNA-negative patients and their platelet counts differed significantly, while, ALT, AST, ALP did not differ significantly. Studies that were done by Silva et al. [33] and Torbenson et al. [56] did not report a strong correlation between ALT/AST levels and occult hepatitis B. In addition, Sheneef et al. [57] reported that there was no significant difference between chronic HCV patients with or without occult HBV infection in terms of clinical characteristics including gender, AST, ALT and ALP.

In contrast with other studies [28,34,58–61] no statistically significant differences were found as regards serum HCV-RNA load in CHC OBI-positive and negative patients. Hollinger and Sood [21] reviewed several cross-sectional studies where it was suggested that HBV replication accounts for many of the ALT flares that occur in patients with HCV. Also, Sheneef et al. [57] reported that there was no significant difference between chronic HCV patients with or without occult HBV infection as regards age, serum albumin, total bilirubin and HCV viral load.

Comparison between patients with different grades of necroinflammatory activity and stages of fibrosis regarding presence of OBI was done in this study. We noticed that there was an increase in the percentage of positive intrahepatic HBV-DNA samples with severe necroinflammatory activity and severe fibrosis.

Similar observations were made by other authors [8,23,61,62]. Similarly, OBI accelerates the progression of cirrhosis, hepatic decompensation and HCC as reported by other studies [63,64]. Cirrhosis is considered as an important risk factor for the development of HCC [60,65]. In addition, OBI may favor neoplastic transformation in HCV-infected patients through its contribution to cirrhosis. Many epidemiologic and molecular studies indicate that persistent HBV infection may have a critical role in the development of HCC in HBsAg-negative patients [66].

However, other studies that were done by Georgiadou et al. [27], Kao et al. [34] and Fabris et al. [58] did not find any association between OBI and severity of chronic liver disease. Also, Chen et al. [59] reported that patients with both OBI and HCV infection had lower liver histology activity index and fibrosis scores than those with HCV monoinfection.

In conclusion OBI is a fact in our community; it was detected in liver tissue of HCV-infected patients, especially in cases of HCC. In addition, OBI might be related to severity of necroinflammatory activity and fibrosis. Large studies are needed to confirm that co-infection could determine a worse progress of chronic liver disease in this population. Also, detection of intrahepatic HBV-DNA is more useful in diagnosis of OBI.

5. Recommendation

Intrahepatic HBV-DNA detection is recommended for diagnosis of occult HBV infection. However, if a liver biopsy specimen is not available, analysis of serum samples should be performed with a highly sensitive and specific approach based on nested PCR or real time PCR. It is recommended to perform serum HBV detection at different time points because the analysis of only one sample, drawn before therapy, could not be sufficient to detect OBI if virus replication is intermittent. A closer follow-up is recommended in patients with positive results of OBI test for the potential risk of HCC development.

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