Occult Hepatitis B Infection in Patients with Chronic Hepatitis C

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Abstract: Occult hepatitis B virus infection (OBI) is characterized by the presence of HBV DNA in the liver tissue or in the serum of HBsAg negative individuals. Although OBI was detected frequently in patients with chronic hepatitis C, the clinical implication of this co-infection is still not fully clarified. The aim of the present study was to assess the prevalence and the possible clinical impact of occult HBV infection in patients with chronic hepatitis C. A total of 60 chronic HCV patients who were HBsAg negative, were enrolled into the study. Serum samples from the studied patients were tested for the presence of anti-HBs and total anti-HBc antibodies by ELISA technique and HBV DNA by real time PCR assay. The results showed that 8 (13.3%) patients were HBV DNA positive; 6 (75%) patients were anti-HBc positive while 3 patients (37.5%) were anti-HBs positive. There was no significant difference between chronic HCV patients with or without HBV DNA in duration of infection, ALT level, histological score or HCV viral load. In conclusion, a considerable proportion of patients with chronic hepatitis C had occult HBV infection. Occult HBV infection was significantly higher among anti-HBc positive patients. Occult HBV infection did not seem to modify the progression of chronic HCV-related liver disease.
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1. Introduction

Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are major public health problems worldwide. Egypt has the highest prevalence of hepatitis C virus (HCV) worldwide, ranging from 6-28% with an average of approximately 15% in the general population (Nafeh *et al.,* 2000; Mohamed, 2004 & El-Zanaty and Way, 2009) and intermediate prevalence of HBV (2-8%) (WHO, 2004).

Hepatitis B and hepatitis C viruses' co-infection may occur because of the common modes of viral transmission, particularly in areas where the two viruses are endemic (Liu and Hou, 2006). Patients with chronic HCV infection are at high risk of acquiring HBV infection in the absence of serological markers for HBV which called occult hepatitis B infection (OBI) (Cacciola *et al.,*1999 & Fukuda *et al.,*1999). In such patients, OBI may be associated with flare of the liver enzymes (Kannangai *et al.,*2007), increased incidence of cirrhosis (Cacciola *et al.,*1999 & De Maria *et al.,*2000), and hepatocellular carcinoma (HCC) (Branco *et al.,*2007 & Miura *et al.,* 2008). Occult HBV infection may also play a role in the poor response of HCV viremia to alpha interferon and rebavirin therapy irrespective of HCV genotype (Fukuda *et al.,*2001 & Mrani *et al.,* 2007).

Occult hepatitis B infection is simply defined by the presence of HBV-DNA in the liver tissue or in the serum of HBsAg negative individuals (Raimondo *et al.,*2008). Although, the exact pathogenesis of OBI is not fully understood, it is probably due to both host and viral factors which are important in suppressing viral replication and keeping the infection under control (Hollinger and Sood, 2010).

The gold standard for diagnosis of OBI is detection of HBV-DNA from liver tissue or serum (Urbani *et al.,*2010). It is strongly recommended to utilize a highly sensitive and specific approach based on ''nested'' or ''real time'' polymerase chain reaction (PCR) techniques and the use of oligonucleotide primers specific for different HBV genomic regions (Raimondo *et al.,*2010).

Occult HBV infection is world-wide spread however, its prevalence is closely related to the endemicity of HBV infection (Bréchot *et al.,*2001) and the characteristics of the studied population, being more common in patients with chronic liver disease and less common among healthy blood or organ donors (Conjeevaram and Lok, 2001). Herein, the frequency and the impact of occult hepatitis B infection in patients with chronic hepatitis C are still under discussion. The aim of the present study was twofold; to assess the prevalence of OBI in patients with chronic hepatitis C and to evaluate its possible impact on liver disease progression regarding the liver enzymes level and fibrosis activity.

2. Patients and methods:

Study design and patients

This is a cross sectional study conducted at Departments of Medical Microbiology & Immunology, Clinical Pathology and Internal Medicine of Soahg University hospital, Egypt, during the period from June to December 2011. The study included 60 chronic

HCV patients who were recruited from outpatient clinics of Internal Medicine Department, Sohag university hospital. All studied patients were HBsAg negative, HCV positive and without manifestations of hepatic decompensation. The diagnosis of chronic HCV infection was based on clinical, laboratory and histologic evaluation. Laboratory diagnosis of chronic HCV patients was based on detection of anti-HCV antibodies by ELISA at least twice within 6 months before enrollment of the patients, and HCV RNA by real time PCR. Patients with autoimmune or metabolic liver disease, schistosomiasis, hepatocellular carcinoma, serological evidence of HIV infection, history of hepatotoxic drugs or current antiviral therapy for HCV and those on hemodialysis were excluded. All eligible patients were subjected to full clinical assessment, routine laboratory investigations and abdominal ultrasonography. Signed written informed consents were obtained from the participants.

Liver biopsy

Liver biopsy was performed for all studied patients as a part of pre-treatment evaluation for HCV infection. The degree of hepatic fibrosis and portal inflammation was evaluated according to the METAVIR scoring system. The stage of fibrosis varied from 0 to 4 (F0 = no fibrosis; F1 = portal fibrosis without septa; F2 = portal fibrosis with few septa; F3 = septal fibrosis, without cirrhosis; F4 = cirrhosis). The grade of inflammatory activity classified into; none, mild, moderate and severe (METAVIR, 1994).

Serodiagnosis of HBV

Anti-HBs and total anti-HBc antibodies were detected by ELISA technique according to the manufacturers' instructions (DiaSorin diagnostic kits, Italy).

HBV DNA amplification and detection by real time PCR assay

HBV DNA was extracted from patients' serum samples by QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. DNA was extracted from 200 µl serum samples, eluted in 200 µl of buffer and stored at –20°C. HBV-DNA was determined quantitatively by real-time PCR assay using Rotor-Gene Q instrument (Qiagen, Germany). Oligonucleotide primers were selected from highly conserved regions of the HBV S gene; FOR 4 (5′-CCTATGGGAGTGGGCCTCA- 3′, nucleotides 639–657) and HBV REV 7 (5′-CCCCAATACCACATCATCCATATA-3′, nucleotides 761–738) (Invitrogen, USA) yielding a 123-bp. The probe sequence was selected within a conserved region of the 123 bp amplicon (5′ - CACTGAACAAATGGCACTAGTAAACTGAGCCA - 3′) (Zanella et al., 2002). The PCR reaction mix for real-time quantification contained 10 µl of the extracted DNA, 1×TaqMan Universal PCR Master Mix (Applied Biosystems, USA), 45 pmol of each primer and 10 pmol of probe, in a final volume of 50 µl. The TaqMan Universal PCR Master Mix contains AmpErase® uracil-N-glycosylase (UNG) to prevent the reamplification of carryover PCR products by removing any uracil incorporated into single- or double-stranded DNA, AmpliTaq Gold, PCR buffer, dNTPs and the rhodamine derivative ROX as a passive reference dye. Thermal cycling conditions were as follows: initial activation of AmpErase UNG at 50°C for 2 min followed by activation of Taq Gold at 95°C for 10 min. Subsequently, 45 cycles of amplification were performed at 95°C for 30 sec and 59°C for 1 min (Zanella *et al.,*2002). Standard curves were done using the Rotor-Gene Q Series Software version 2.0.2.4 (Qiagen, Germany). A sample result is accepted only when the internal control is amplified.

Statistical analysis

Statistical analysis was done using Statistical package for social sciences version 10 (SPSS Inc., Chicago, IL, USA). Data were presented as median and range or as an absolute number and percentage. Chi-square test was used for categorical data. Quantitative data with uneven distribution were analyzed with the Mann-Whitney U test. $P < 0.05$ was considered statistically significant.

3.Results

The study group comprised of 60 patients with chronic HCV, 45 males (75 %) and 15 females (25 %). The median age of the participants was 45 years and a range from 22-59 years. According to METAVIR score, 70% of the studied patients have moderate inflammatory activity while about 47% showed portal, without septal, fibrosis (F1). Patients' characteristics were shown in Table 1.

Normal values of AST (up to 40 IU/L), ALT (up to 41 IU/L), Total biliruben (up to 1 mg/dL), Albumen (3-5 g/dL), Alkaline phosphatase (up to 256 IU/L)

Out of 60 chronic HCV patients included in the study, 27 (45 %) patients were positive to total anti-HBc antibodies and 6 (10 %) patients were positive to anti-HBs antibodies (Table 2).

Among the studied patients, the serum samples of 8 patients (8/60; 13.3%) were positive for HBV DNA by the real time PCR assay, documenting an occult HBV infection. Out of 8 OBI/HCV dually infected patients, 6 (75%) patients were anti-HBc positive while 3 patients (37.5%) were anti-HBs positive. This result was statistically highly significant (P=0.001). There was no significant difference between chronic HCV patients with or without occult HBV infection in terms of clinical characteristics including gender distribution, age, histological score, liver function tests and HCV viral load (Table 3).

Table 1: Patients' characteristics

Characteristics	Patients (N= 60)
Age; (years)	45 (22 - 59)
Gender; n (%)	
Male	45 (75 %)
Female	15 (25 %)
BMI (kg/m^2)	27.6 (21.5 – 30.4)
Duration of HCV infection; (years)	6 (2 – 18)
Liver function tests:	
Serum albumin (g/dL)	4.2 (3.3 – 6)
Liver enzymes (IU/L)	
ALT	50.5 (8 – 134)
AST	41.5 (18 -102)
Total bilirubin (mg/dL)	0.9 (0.5 – 1.4)
Alkaline phosphates (IU/L)	111.5 (57 -281)
Basal HCV RNA load (IU/ml)	272,669 (1747 - 5,230,677)
METAVIR Score inflammatory activity	
Mild	16 (26.7 %)
Moderate	42 (70 %)
Severe	2 (3.3 %)
Fibrosis	
F1	28 (46.7 %)
F2	22 (36.7 %)
F3	10 (16.6 %)
F4	0 (0 %)

Data were presented as median (range) or number (%)
Abbreviations: BMI; Body mass index, ALT; Alanine transaminase, AST; Aspartate transaminase.

Table 2: HBV Serological patterns of the studied patients

Serological markers	Anti-HBc (-) Anti-HBs (-)	Anti-HBc (+) Anti-HBs (-)	Anti-HBc (-) Anti-HBs (+)	Anti-HBc (+) Anti-HBs (+)
N (%)	29 (48.3 %)	25 (41.7 %)	4 (6.7 %)	2 (3.3 %)

4. Discussion

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 (Saravanan *et al.,* 2009). Occult HBV infection has been frequently reported in patients with chronic HCV with a prevalence ranging from 0-52% (Fukuda *et al.,* 1999 and Goral *et al.,* 2006). Considerable data suggested that occult infection may contribute to chronic liver damage, poor response to antiviral therapy, and the development of HCC (Miura *et al.,* 2008 and Mrani *et al.,* 2007).

The present study reported that 13.3% of patients with chronic hepatitis C had detectable HBV DNA in the serum, despite the absence of circulating HBsAg. This result was consistent with other reports which revealed prevalence of OBI ranging from 11%-14.8% among chronic HCV patients (Zignego *et al.,* 1997; Kao *et al.,* 2002; Silva *et al.,* 2004 and Selim *et al.,* 2011). However, a wide variation of the prevalence of occult HBV infection in patients with chronic hepatitis C has been reported. Some authors failed to detect HBV-DNA in both serum and liver samples of HCV patients (Pontisso *et al.,* 1993 and Goral *et al.,* 2006) while others reported an exceedingly high prevalence reaching up to 90% among HCV-infected patients (Uchida *et al.,* 1997 and Koike *et al.,* 1998). This dissimilarity among studies might be due to the geographic variations regarding the HBV prevalence, the number of patients' samples investigated in each study or the different sensitivities of the assays used to detect HBV-DNA and the different types of specimens used to detect the presence of HBV (serum or liver).

Table 3: **Characteristics of OBI/HCV dually infected and HCV mono-infected patients**

Characteristics	OBI/HCV dual infection(N= 8)	HCV mono-infection (N= 52)	*P* value
Age; (years)	45 (24 – 56)	44.5 (22 -59)	0.90
Gender; n (%)			
Male	5 (62.5%)	40 (76.9%)	0.38
Female	3 (37.5%)	12 (23.1%)	
BMI (kg/m^2)	25.6 (21.9 – 30.2)	27.7 (21.5 – 30.4)	0.50
Duration of HCV infection; (years)	5 (2 -12)	6 (2 -18)	0.32
Liver function tests:			
Serum albumin (g/dL)	4.6 (3.3 – 4.8)	4.1 (3.4 – 6)	0.60
Liver enzymes (IU/ml)	48.5 (30-134)	50.5 (8 – 132)	0.90
ALT			
AST			
Total Bilirubin (mg/dl)	48.5 (28 – 68)	37 (18 – 102)	0.47
Alkaline phosphatase (IU/ml)	1 (0.6 – 1.1)	0.9 (0.5 -1.4)	0.35
	87 (80 – 198)	126 (57 -281)	0.47
Basal HCV RNA load (IU/ml)	276,939 (250,334 – 4,956,255)	260,459 (1747 – 5,230,677)	0.24
METAVIR Score inflammatory activity			
Mild	3 (37.5%)	13 (25.0%)	0.67
Moderate	5 (62.5%)	37 (71.2%)	
Severe	0 (0.0%)	2 (3.9%)	
Fibrosis			
F1	3 (37.5%)	25 (48.1%)	0.18
F2	5 (62.5%)	17 (32.7%)	
F3	0 (0.0%)	10 (19.2%)	
Serological markers for HBV			**0.001***
Anti-HBc(-)/Anti-HBs (-)	1 (12.5%)	28 (53.9%)	
Anti-HBc(+)/Anti-HBs (-)	4 (50.0%)	21 (40.4%)	
Anti-HBc(-)/Anti-HBs (+)	1 (12.5%)	3 (5.8%)	
Anti-HBc(+)/Anti-HBs (+)	2 (25.0%)	(0.0%)	

Data expressed as median (range) or number (%)
*Significant when HBV DNA positivity in anti-HBc(+) was compared with anti-HBc(-) patients

In agreement with some reports, the present study suggested that occult HBV could be predicted by serological markers of HBV infection (Cacciola *et al.,* 1999; Kao *et al.,* 2002 and El-sherif *et al.,* 2009). Seventy five percent of the patients with detectable HBV DNA had anti-HBc antibodies. This proportion constituted 22.2% (6/27) of the total anti-HBc positive patients indicating that about a quarter of the patients with positive anti-HBc definitely had OBI. Meanwhile, 3 (37.5%) patients had anti-HBs antibodies. This could be explained by the notion that occult HBV infection is frequently a late phase of overt chronic HBV infection or serologically recovered acute HBV infection while titres of anti-HBs decreases over years to undetectable levels, anti-HBc antibodies only persists. Another possible hypothesis is that HCV infection may block the circulating viral expression of HBV but anti-HBc in the serum and HBV DNA in the hepatocytes may persist (Zollner *et al.,* 2006). On the other hand, other studies found no association between prevalence of HBV DNA and HBV serological markers (Khattab *et al.,* 2005; Emara *et al.,* 2010 and Selim *et al.,* 2011).

Most cross sectional studies that addressed the issue of OBI did not report a strong correlation between ALT/AST levels and occult hepatitis B (Silva *et al.,* 2004; Torbenson *et al.,* 2004). Many studies, including our failed to demonstrate a relationship between occult HBV infection and high aminotransferases levels in chronic HCV patients (Georgiadou *et al.,* 2004; Branco *et al.,* 2007; Chen *et al.,* 2010 and Emara *et al.,* 2010). From the other point of view, a relationship between OBI/HCV co-infection and high aminotransferases levels has been suggested by few studies (Fukuda *et al.,* 1999; Kannangai *et al.,* 2007; Saravanan *et al.,* 2009 and Selim *et al.,* 2011). Because of these inconsistent data, it was evidenced that aminotransferases levels in patients with chronic HCV could not predict the presence of OBI.

Several studies showed an association of occult HBV infection with progressive HCV-related liver disease evidenced by the presence of biochemical activity, increased HCV viral load or increased histological activity and fibrosis (Cacciola *et al.,* 1999; Fukuda *et al.,* 2001 and Branco *et al.,* 2007). The authors of these studies explained this finding by accelerated inflammation of the hepatic cells induced by OBI due to increased HBV DNA replication, immune activation, and subsequent liver injury. On the other hand, the present study and some other studies did not find any association between occult HBV infection and virologic or histologic data (Kazemi-Shirazi *et al.,* 2000; Kao *et al.,* 2002; Silva *et al.,* 2004 and Georgiadou *et al.,* 2004). Collectively, the present study showed no correlation between clinical outcome and severity of HCV- related liver disease and silent HBV infection. This finding could be attributed to the small number of co-infected patients or that ALT levels correlate well with the inflammatory activity on biopsy, consequently, the ALT level was comparable to the reported mild to moderate degree of inflammation. Another possible explanation is that most of these studies were cross sectional which have the drawback of evaluating the disease at certain point of time and therefore, longitudinal studies should be designed to evaluate the real impact of OBI on liver disease progression.

Conclusion

This study reported that a considerable proportion of patients with hepatitis C had occult HBV infection. The study also supported the use of anti-HBc (either alone or with other markers of previous HBV infection) as a surrogate marker for occult HBV infection in patients with chronic hepatitis C and HBsAg should not be used alone as the golden marker for the diagnosis of HBV infection. Patients who may be at risk for acquiring and/or transferring occult HBV should be examined for the presence of HBV DNA by PCR. There was no correlation between occult HBV infection and the severity of the liver disease. However, further studies including larger number of patients, are needed to clarify the clinical significance of occult HBV for accelerating the natural course of chronic HCV infection.

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