Occult hepatitis B virus among patients with chronic hepatitis and hepatocellular carcinoma

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Background Hepatitis B virus (HBV) infection is diagnosed when the circulating HBV surface antigen (HBsAg) is serologically detected. Occult HBV infection is defined as the infection state negative for HBsAg serology, but it has shown viral genome persistence in infected individuals. The aim of the study is to determine the prevalence of occult HBV among patients with chronic hepatitis negative to HBsAg in the presence or absence of hepatitis C virus (HCV) infection.

Patients and methods This study was conducted on a total number of 55 patients with chronic hepatitis (liver cirrhosis in 44 cases, nonalcoholic fatty liver in six cases) and hepatocellular carcinoma in five cases. All studied cases were subjected to routine liver function tests, HBsAg, HBsAb, hepatitis c virus immunoglobulin G (HbcIgG), α-fetoprotein, HCV RNA, and HBV DNA detection.

Result All cases were negative to HBsAg and HBsAb in the presence or absence of HCV infection. HBV DNA detection by real-time RT-PCR confirmed the positivity of HBV infection [occult hepatitis b infection (OBI)] in two (4.5%) out of 44 cases of cirrhotic liver and represented 3.6% of the total cases studied with a viral DNA of 116 and 159 copies/ml, respectively. One case of OBI had a high level of α-fetoprotein (392 Iu/ml) and the second case had high copies of HCV RNA 127 000 copies/ml, that is coinfection. HbcIgG was positive in 31.8% in cirrhotic patients (including one out of the two positive OBI). HCV RNA was negative in 100.0% of nonalcoholic fatty liver, positive in 39 (one was positive OBI) cases with cirrhosis with a median value of 45 000 copies and in four out of the five hepatocellular carcinoma cases with a median value of 1.85E+08. This is statistically significant (P=0.01). We come to the conclusion that occult HBV do exist in our community. The diagnosis of OBI should be based on high sensitivity of HBsAg and HBV DNA testing.

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Introduction

Hepatitis B virus (HBV) infection is a global health problem, and more than 400 million people worldwide are chronic carriers of the virus, where Egypt is considered as an area of intermediate endemicity [1]. The infection is associated with a large spectrum of clinical manifestations ranging from acute or fulminant hepatitis to various forms of chronic infection, including asymptomatic carriers, chronic hepatitis, cirrhosis, and hepatocellular carcinoma (Said *et al.*, 2013; [2,3]).

In general, HBV infection is diagnosed when the circulating HBV surface antigen (HBsAg) is serologically detected [4]. Occult HBV infection is manifested by the presence of very low levels (<200 IU/ml) of HBV DNA in the blood and the liver while exhibiting undetectable HBsAg (Martinez *et al.*, [5]). It is important to detect high-risk groups for occult HBV infection to prevent transmission. The detection of viral DNA reservoir in hepatocytes provides the best evaluation of occult HBV prevalence in a defined set of patients. However, this diagnostic approach is obviously unsuitable; blood detection of occult hepatitis B requires assays of the highest sensitivity and specificity with a lower limit of detection less than 10 IU/ml for HBV DNA and less than 0.1 ng/ml for HBsAg (Ocana *et al.*, [6]).

Occult hepatitis B is an independent risk factor in hepatocellular carcinoma (HCC) development in anti-hepatitis C virus (HCV)-negative patients. A synergistic or additive role in the occurrence of HCC in HCV-coinfected patients is more problematic due to the HCC risk attributable to HCV alone, especially in patients with advanced fibrosis and cirrhosis [7].

On the other hand, patients with chronic hepatitis C, patients with cryptogenetic liver disease, and patients undergoing hemodialysis, and immunosuppressed patients, must be investigated for occult hepatitis b

infection (OBI) because of its possible influence on the development of these diseases (Ocana *et al.*, [6]).

Mutation of HBsAg are known to contribute to HBV occult infections via the reduction of virion secretion [8,9]. So other reports have highlighted the importance of 'a' determinant mutations such as G145R as a major mechanism underlying occult infection [4].

Patients and methods

Ethical consideration: the purpose, nature and potential risk of the study will be explained to all patients. This study was conducted on a total number of 55 patients with chronic hepatitis (liver cirrhosis in 44 cases, nonalcoholic fatty liver in six cases) and hepatocellular carcinoma in five cases:

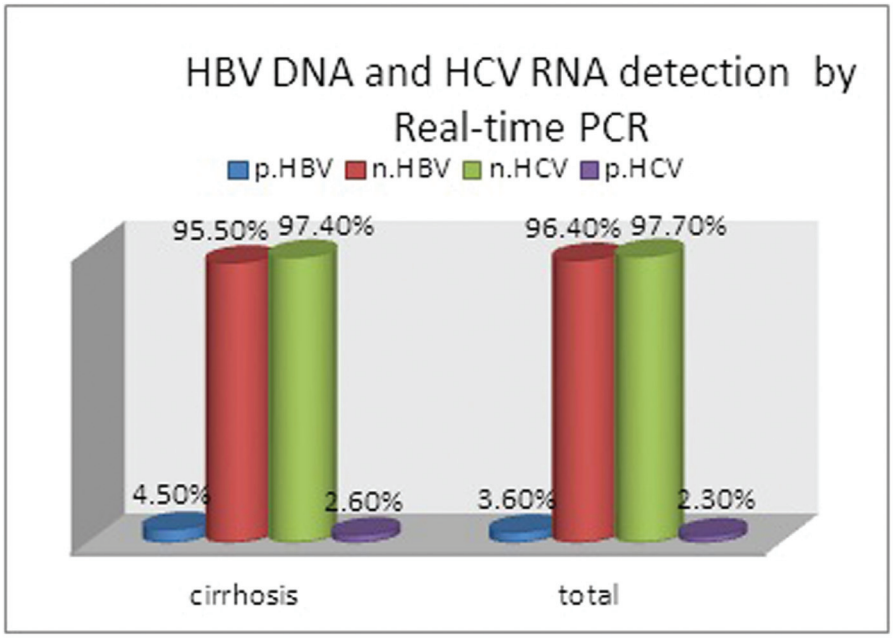
(1) All studied cases were subjected to routine liver function tests hepatitis c virus immunoglobulin G (HbcIgG) HBsAg, HBsAb, and α-fetoprotein (AFP) detection using Cobas e 411 device (Roch, Germany) (using ELISA based on electrochemiluminescence technology.
(2) HBV DNA extraction and PCR amplification using real-time PCR (RT-PCR). Viral DNA was extracted from serum using QIAamp Viral DNA Mini Kit (Qiagen, Dusseldorf, Germany), and then the extract was added to Brilliant QRT-PCR 1-step Master Mix, which is based on TaqMan technology (Stratagene, La Jolla, California, USA) and real-time RT-PCR was done by Stratagene Mx3000P device.
(3) HCV RNA extraction and PCR amplification were done using RT-PCR. Viral RNA was extracted from serum using QIAamp Viral DNA Mini Kit (Qiagen).

Results

This study was conducted on 55 patients with chronic hepatitis (liver cirrhosis in 44 cases, nonalcoholic fatty liver in six cases) and hepatocellular carcinoma in five cases attending at El-Menoufia University and Ain Shams hospitals.

(1) They were 38 men (32 cirrhotic cases including the two positive OBI, five cases of HCC, one of nonalcoholic fatty liver) and 17 women (12 cirrhotic cases and five of nonalcoholic fatty liver). The median value of age ranged from 58.5 to 70 years.
(2) HBsAg, HBsAb were negative in all studied cases (100.0%).
(3) HBV DNA detection by RT-PCR confirmed positivity of HBV infection (OBI) in two that represent 4.5% out of 44 cases with liver cirrhosis 3.6% of the total cases studied and confirmed negativity in 95.5% of cirrhotic patients, and 96.4% of all cases studied as shown in Fig. 1. The viral load was 116 copies/ml in one

Figure 1



Number and percent of patient with positive and negative OBI.

of them that was also positive for HCV, and was 159 copies/ml in the other positive case.

Results of liver function test, HBsAg, HBsAb, HbcIgG, HCV RNA, and AFP in the different studied groups showed the following:

(1) Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and bilirubin median values increased in the studied cases and their marked increase was in HCC cases while the hemoglobin level was decreased in HCC cases.
(2) AFP level was increased in 10 of cirrhotic cases with a median serum value of 1860 IU/ml and in the five HCC cases with a median value of 1000 IU/ml. The obtained previous results were statistically insignificant ($P>0.05$) (Table 1).
(3) HCV RNA was negative in 100.0% of nonalcoholic fatty liver. It was detected in 39 of cirrhotic cases (88.6%) with a median value of 45 000 copies/ml. One case was positive OBI, that is, coinfection with HBV; it represents 2.6% of the total 39 positive HCV cirrhotic patients and 2.3% of the total positive HCV cases (Fig. 1). It was also detected in four (80%) cases of HCC patients with a median value of 1.85E+08. This was statistically highly significant ($P=0.01$) (Tables 1 and 2).
(4) Hbc.IgG was negative in 100.0% in nonalcoholic fatty liver patients and positive in 31.8% in cirrhotic patients and in 40.0% HCC patients. This was statistically insignificant ($P=0.234$) (Table 3).
(5) Comparing the result of routine liver function tests, Hb, AFP, and HCV-PCR among positive and negative OBI are shown in Table 4.
(6) The obtained result revealed that the median value of ALT, AST, and bilirubin was higher in positive. OBI was statistically insignificant ($P=0.857$, 0.418, 0.2) subsequently. There was also no statistically significant difference in hemoglobin level between positive and negative OBI ($P= 0.857$).
(7) Also AFP was high in one case and HCV RNA was high in the other positive OBI cases (392 IU/ml) and in 127 000 copies subsequently. But this was statistically insignificant ($P=0.102$, 0.809) subsequently.

Table 1 Comparison of the results of liver function tests, hemoglobin, α-fetoprotein, and hepatitis C virus RNA in the studied cases

	Median	25%	75%	*H*	*P*	Significance
AST (IU/ml)						
Cirrhosis (*n*=44)	83.5	63.5	128			
HCC (*n*=5)	178	51.5	214			
Nonalcoholic fatty liver (*n*=6)	72.5	63.5	108.75			
				0.996	0.608	NS
ALT (IU/ml)						
Cirrhosis (*n*=44)	79	55.75	104.75			
HCC (*n*=5)	120	51.5	137.5			
Nonalcoholic fatty liver (*n*=6)	94.5	64	112.25			
				1.041	0.594	NS
Bilirubin (ml/dl)						
Cirrhosis (*n*=44)	2.15	1.3	3.375			
HCC (*n*=5)	3.9	0.8	4.2			
Nonalcoholic fatty liver (*n*=6)	1.05	0.875	1.375			
				598.90%	0.05	NS
Hb (g/dl)						
Cirrhosis (*n*=44)	10.6	9.4	11.65			
HCC (*n*=5)	9.3	8.85	11.3			
Nonalcoholic fatty liver (*n*=6)	10.95	9.825	11.55			
				1.19	0.552	NS
AFP (IU/ml)						
Cirrhosis (*n*=10)	1860	879.5	6130.25			
HCC (*n*=5)	1000	1000	50 500			
				0.137	0.711	NS
HCV RNA						
Cirrhosis (*n*=39)	45 000	1700	530 000			
HCC (*n*=4)	1.85E+08	1.45E+08	2.25E+08			
				6.72	0.01	S

AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Hb, hemoglobin; HCC, hepatocellular carcinoma; HCV, hepatitis C virus.

Table 2 Comparison of the result of hepatitis C virus RNA in the studied patients

	Cirrhosis	HCC	Nonalcoholic fatty liver	Total
HCV-PCR				
N				
Count	5	1	6	12
%	11.4	20.0	100.0	21.8
P				
Count	39	4	0	43
%	88.6	80.0	0.0	78.2
Total				
Count	44	5	6	55
%	100.0	100.0	100.0	100.0

HCC, hepatocellular carcinoma; HCV, hepatitis C virus.

Table 3 Comparison of the result of HbcIgG in the studied patients

	Cirrhosis	HCC	Nonalcoholic fatty liver	Total
HbcIgG				
N				
Count	30	3	6	39
%	68.2	60.0	100.0	70.9
#9619;*P*				
Count	14	2	0	16
%	31.8	40.0	0.0	29.1
Total				
Count	44	5	6	55
%	100.0	100.0	100.0	100.0
Value	*P*=0.234			

HCC, hepatocellular carcinoma, HbcIgG, hepatitis c virus immunoglobulin G.

Table 4 Comparison between negative and positive OBI as regards liver function tests, hemoglobin, α-fetoprotein, and hepatitis C virus PCR among the studied cases

	HBV-PCR	*n*	Median	25%	75%	*Z*	*P*	Significance
Age	Negative	53	61	53	67			
	Positive	2	55.5	43		–0.563	0.574	NS
AST (IU/ml)	Negative	53	81	62.5	128			
	Positive	2	167.5	71		–0.809	0.418	NS
ALT (IU/ml)	Negative	53	81	56.5	109			
	Positive	2	168.5	36		–0.18	0.857	NS
Bilirubin	Negative	53	1.9	1.15	3.35			
	Positive	2	3.7	2.50		–1.28	0.2	NS
Hb (g/dl)	Negative	53	10.6	9.35	11.50			
	Positive	2	10.4	8.50		–0.18	0.857	NS
AFP (IU/ml)	Negative	14	1533	1000.00	6130.25			
	Positive (159 IU/ml)	1	392	392.00	392.00	–1.64	0.102	NS
HCV-PCR (copies/ml)	Negative	42	68 500	3162.50	747 500.00			
	Positive (116 IU/ml)	1	127 000	127 000.00	127 000.00	–0.24	0.809	NS

AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Hb, hemoglobin; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; OBI, occult hepatitis b infection.

Discussion

HBV infection is a global health problem, and more than 400 million people worldwide are chronic carriers of the virus, where Egypt is considered as an area of intermediate endemicity [1].

Occult hepatitis B infection is one of the most challenging topics in the field of viral hepatitis. Occult HBV infection is defined as the infection state negative for HBsAg serology, but it has shown viral genome persistence in infected individuals [10].

A large body of evidence has demonstrated that HBV occult infection is highly prevalent, particularly in HBV endemic areas, and also it was reported in healthy blood donors, in viral reactivation following immunosuppression, and accidental transmission through transplantation. In particular, it is significantly related to severe forms of liver disease, such as cirrhosis and as a risk factor in HCC [2,11].

Although the exact mechanism of occult HBV has not been proved recently it is contributed to the persistent synthesis of minute undetectable amounts of virus by HBV covalently closed to circular DNA or other viral transcript which maintains the HBV-specific memory T-cell response [12].

The prevalence of OBI varies from region to region worldwide (1–87%) [10]. This variability relies on the sensitivity of HBV DNA detection assays, the sample size, and the detection of HBV DNA in liver tissue or serum [14]. It also relies on the endemicity of HBV infection, geographical variations, power of the study, and composition of study population [15].

Occult HBV infection diagnosis is based on the detection of HBV DNA when HBsAg is absent. It is important to define the optimal methodology to test this marker to prevent false positive results, depending on the HBsAg assay sensitivity. The virus

can express many different viable HBsAg mutants (the so-called escape mutants). Some mutants might lead to a loss of detection in commercially available HBsAg assays. The electrochemiluminescence immunoassay HBsAg assay was especially developed in order to detect a multitude of these mutants. The cutoff sensitivity of this technique is less than 0.1 IU/ml. The 1 IU is equivalent to 0.43 ng (Ocana *et al.*, 2011).

Occult HBV infection was detected in this study in two out of 44 cases with cirrhosis representing 4.5% of cirrhotic patients and 3.6% of total cases studied. This result is more than that has been found in the US and some other western countries where only 0.1–2.4% of HBsAg negative, anti-HBc-positive blood donors were found to have HBV DNA [16]. Also it is higher than in Iran where the prevalence of OBI has been reported to be two in 50 000 blood donors [17].

However, our result is consistent with other studies done in Egypt, where it varied from a low 4.1% to high 26.8% in hemodialysis patients [18]. This wide range of OBI may be related to different study designs as well as different HBV DNA detection methods. Moreover, it may relate to the liver disease severity and the immunity of the studied patients. Also in our study the prevalence is much less most likely because of the small number (55) of patients with chronic liver disease in whom we detected HBV DNA. In Italy HBsAg-negative/anti-HBc-positive donors were assayed for antibodies to HBsAg (anti-HBs) and for HBV-DNA using COBAS Ampliscreen HBV (Roche) on individual blood donations. The prevalence of anti-HBc positive subjects was 4.85% which is nearly similar to what we found (Manzini *et al*. [19]).

The current study did not show significant difference between the studied groups as regards age. For patients with negative OBI the median age is 61 and for patients with positive OBI it is 55.5. This is consistent with the result observed by Said [20]. This was also reported by Allian and Cox (2011) who found that OBI donors were generally older than 45 years [21].

Considering the fact that concurrent detection of anti-HBc and anti-HBs might be assessed as an indicator of past exposure and recovery, it has been reported that OBI is associated with the presence of HBcAb or HBsAb. In some cases, neither HBcAb nor HBsAb can be detected. So the data did not suggest HBcAb test as a screening tool in the study setting [15]. The detection of serum anti-HBc has been used as a surrogate marker for the presence of liver HBV DNA to detect OBI in numerous investigations exploring the correlation between OBI and liver fibrosis in patients with HBV. The prevalence of cirrhosis in the anti-HBc-positive subgroup was significantly higher than in the anti-HBc-negative patients suggesting the role of OBI in fibrosis progression [22].

We found that Hbc.IgG was positive in 31.8% in cirrhotic patients including one of the two positive OBI, which may reflect the role of hepatitis B infection in the progression of fibrosis. Also it was positive in 40.0% of HCC patients, but these percentages were statistically insignificant due to the low number of cases studied for reliable statistical analysis.

Liver enzyme alterations may signal liver damage as well as alterations in bile flow. It may either be the accompanying biochemical picture in a patient with symptoms or signs of liver disease or in patients with OBI. In healthy people, conjugated bilirubin is virtually absent from the serum mainly because of the rapid process of bile secretion. Levels increase when the liver has lost at least half of its excretory capacity. Therefore, evaluation of alteration in bilirubin levels is a useful test for monitoring liver synthetic activity and as a sign of liver disease [23]. In our study, the median levels of ALT, AST, and bilirubin were higher in positive OBI than in negative OBI. (168.5 vs. 81), (167.5 vs. 81), and (3.7 vs. 1.9), respectively (Table 4). An increase in ALT serum levels is more specific for liver damage since AST is also diffusely represented in the heart, skeletal muscle, kidneys, brain, and red blood cells [24]. In our study these increase in transaminases and total bilirubin was statistically insignificant due to the low number of cases. The determination of bilirubin is very useful in the diagnosis of hepatopathies.

In our study, HBV DNA was (116 and 159 copies/ml) by RT- PCR. Current technologies used for DNA detection are: nested-PCR, real-time PCR, and transcription-based mediated amplification. Using these assays, it is possible to decrease the lower detection limit (<5 IU/ml of HBV DNA). This is particularly important in OBI, because the HBV DNA levels vary from less than 200 copies/ml. However, the false negative and positive rates are around the cutoff level due to the Poisson distribution of the virions and blank specimens [16]. This assay (RT-PCR) has been used to lower the detection limit (<5 IU/ml of HBV DNA). This is particularly important in OBI, because HBV DNA levels vary

from less than 10 to 425 copies/ml. Usually, when a blood sample is positive for HBV DNA, the liver sample is also positive; however, HBV DNA from the liver can be detected even when HBV cannot be detected in the serum. Patients with undetectable HBV DNA in the liver has also undetectable levels of HBV DNA in peripheral blood. However, there are no standardized assays and liver specimens are not routinely obtained. Commercial assays use serum or plasma to determine the presence and the amount of HBV DNA (Ocana *et al*., 2011). The detection of HCV RNA in 42 patients out of the 55 studied cases is not surprising, since HCV infection is high in Egyptian people (14.7%.) [25]. Occult HBV infection in chronically HCV-infected patients has been associated with an increased incidence of fibrosis [26].

In our study, OBI that associate HCV infection represented 2.3% of the HCV infection and 2.6% of HCV cirrhotic patients. OBI prevalence in Egyptian HCV-positive patients is 1.85–38.3% according to the available data [10,27]. Also one case of OBI in our study had high level of AFP (392 Iu/ml) and this patient must be followed up. Serum concentration of AFP is increased in most patients with HCC so this increase is considered a risk factor in patients with chronic liver lesions for HCC development [28–30].

Conclusion and recommendations

(1) We come to the conclusion that occult HBV do exist in our community.
(2) The problem of contamination and false positives in nucleic acid amplification assays is eliminated in automated real-time PCR assays, which is sufficiently sensitive, specific, accurate, reproducible, and cost-effective for the detection of HBV DNA.
(3) It is important to screen for OBI in patients with chronic liver disease.
(4) ALT elevations may be also important in the diagnosis and follow-up of patients with OBI
(5) Further work on a large number of cases is needed to clarify the clinical significance of OBI, infectivity, possible transmission and its pathogenic consequences, reactivation, and progression to chronic liver disease or hepatocellular carcinoma in the presence or absence of HCV infection.

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Conflicts of interest
There are no conflicts of interest.

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