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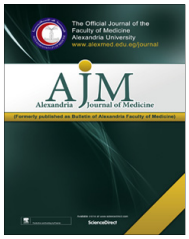

Impact of occult hepatitis B virus infection on antiviral therapy in chronic hepatitis C patients

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KEYWORDS

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Abstract *Background:* Occult HBV infection (OBI) can be defined by the presence of HBV-DNA in the serum of patients who are negative for HBsAg. The presence of OBI has been associated with a poor therapeutic response to alpha IFN in many, but not in all studies.
Objective: The aim of our study was to assess the prevalence of OBI in the serum of Egyptian patients with CHC, and to evaluate its impact on the response to treatment with a combination of Peg-IFNα and RBV.
Materials and methods: Fifty chronic HCV infected patients who were treated with Peg-IFNα once a week in combination with RBV for 48 weeks were included in this study. Patients were divided into two groups, group I which included 25 patients who achieved SVR and group II that included 25 patients who failed to achieve SVR (Non-SVR). Both patient groups were subjected to detailed questionnaire, clinical examination, routine laboratory investigations and virological studies.
Results: No statistical significant difference was found in sex distribution regarding SVR and Non-SVR. The frequency of patients with low viral load has a statistically significant association

Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; EDHS, Egyptian Demographic and Health Survey; Anti-HCV, antibody to hepatitis C virus; Anti-HBc, anti-hepatitis B core; HBsAg, hepatitis B surface antigen; PCR, polymerase chain reaction; OBI, occult HBV infection; CHC, chronic hepatitis C; Peg-IFN α and RBV, pegylated interferon alpha and ribavirin; SVR, sustained virological response; RNA, ribonucleic acid

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with SVR patients compared to Non-SVR patients. The frequency of anti-HBc seropositivity has a statistically significant association with the Non-SVR patients compared to SVR patients. Out of 11 anti-HBc positive samples, 10 (90.9%) were positive for the *pol* and *s* genes while 9 (81.81%) were positive for the *c* gene. About 17 (34%) out of 50 patients included in the study were HBV-DNA positive. The frequency of HBV-DNA positive HCV patients has a statistically significant association with Non-SVR patients compared to the SVR patients ($p < 0.05$).

Conclusion: The prevalence of OBI was higher in our CHCV patients. OBI was significantly associated with poor response to combined Peg-IFNα and RBV therapy.

1. Introduction

Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections represent the main causes of chronic liver disease worldwide, affecting 350–400 million and 170 million people, respectively.[1] HCV is currently the most significant public health problem in Egypt.[2] The recently published Egyptian Demographic and Health Survey (EDHS) in 2008 estimated an overall antibody to hepatitis C virus (anti-HCV) prevalence of 14.7% with an estimated 9.8% of resident Egyptian populations that are chronically infected.[3] Anti-hepatitis B core (anti-HBc) sero-positivity in the general population in Egypt is reported to be 10–13%.[4]

HBV and HCV share common modes of transmission, thus, simultaneous infection is quite frequent, particularly where both viruses are endemic as among people with a high risk for parenteral infections.[5] Diagnosis of HBV infection is usually based on the detection of hepatitis B surface antigen (HBsAg), and the disappearance of this antigen indicates the clearance of HBV.[6] The extensive application of sensitive molecular tests such as polymerase chain reaction (PCR) and real-time PCR has enabled HBV-DNA to be detected in specimens from individuals without serological evidence of chronic HBV infection.[7,8] Occult HBV infection (OBI) can be defined by the presence of HBV-DNA in the serum of patients who are negative for HBsAg.[5,9] In the last decade, OBI pattern has been documented and frequently identified in patients with chronic hepatitis C (CHC) infection.[8] Its prevalence, which varies according to HBV endemicity, ranges from 30% of CHC patients in Italy to 80% in Japan.[2]

The currently recommended therapy for CHC is the combination of pegylated interferon alpha and ribavirin (Peg-IFNα and RBV) that proved to be superior to standard IFNα and RBV. Several virological responses may occur, labeled according to their timing relative to treatment. The most important type of virological responses is the sustained virological response (SVR), defined as the absence of HCV-ribonucleic acid (RNA) from serum by a sensitive PCR assay 24 weeks following discontinuation of therapy. More than 50% of patients can achieve an SVR after 24 weeks (in genotypes 2 and 3) or after 48 weeks (in genotypes 1 and 4) of this combination therapy, making HCV a potentially curable disease.[10] The presence of OBI has been associated with a poor therapeutic response to alpha IFN in many,[1,5,9] but not in all[5,11–13] studies. However, few studies have documented the response rates to the current Peg-IFNα and RBV therapy. The aim of our study was to assess the prevalence of OBI in the serum of Egyptian patients with CHC, and to evaluate its impact on the response to treatment with a combination of Peg-IFNα and RBV.

2. Materials and methods

Fifty chronic HCV infected patients who were treated with 180 μg Peg-IFNα-2a or 1.5 μg/kg Peg-IFNα-2b once a week in combination with RBV (800–1400 mg/day) for 48 weeks were included in this study.[14,15] Those patients were selected from the outpatient clinic of the Medical Research Institute, Alexandria University who were eligible and fulfilling the criteria for treatment with combined Peg-IFNα and RBV and followed up for 48 weeks of treatment. PCR- HCV was done at 0 and 48 weeks of the treatment and after 24 weeks of the end of treatment where the patients were divided into 2 groups; group I which included 25 patients who achieved SVR and group II that included 25 patients who failed to achieve SVR (Non-SVR). Before enrollment in the study both patient groups were subjected to detailed questionnaire, clinical examination and the following laboratory investigations: (a) biochemical studies (ALT, AST and Bilirubin)[16] (b) hematological studies (Prothrombin activity and Platelets count)[17] (c) virological studies that included: Anti-HCV antibodies and HCV-RNA (0, 48 and 72 week). After selection of both patient groups other virological studies were done that included: serological detection of anti-HBc antibodies, Murex anti-HBc total (Murex Diagnostics, Chicago, IL)[18] and detection of HBV-DNA (*s* and *c* genes) by the SYBR Green technique,[19] *pol* gene by the nested PCR technique.[20]

Anti-HCV antibodies, third generation ELISA kits (MUREX ANTI-HCV (VERSION 4) MUREX BIOTECH.)[18]

HCV-RNA extraction was done using QIAamp viral RNA Mini kits (Qiagen) according to the manufacturer's protocol. For the quantitative detection of HCV-RNA, it was amplified by using (Artus HCV QS-RGQ PCR Kit) reagents based on the amplification and simultaneous detection of a specific region of the HCV genome using real-time RT-PCR with TaqMan probe assay. The amplification reaction was performed as follows; 6 μl of HCV RG Master A, 9 μl of HCV RG Master B and 10 μl of Qiagen extracted RNA were added to bring the reaction to a final volume of 25 μl. Real-time PCR was performed with the Mx3000P TM (Stratagene) real-time PCR system. The reaction took place under the following thermal profile; incubation at 50 °C for 30 min to transcribe viral RNA to complementary DNA (cDNA) by RT. This was followed by AmpliTaq gold activation at 95 °C for 10 min,

then 45 cycles of three PCR-step amplification, denaturation at 95 °C for 30 s, followed by annealing at 50 °C for 1 min and extension at 72 °C for 30 s, with end point fluorescence detection.

HBV-DNA extraction was done using QIAamp DNA Blood Mini kit (Qiagen) according to the manufacturer's protocol. The detection of HBV-DNA by SYBR Green real-time PCR (GeneON GmbH) assay was based on the specific amplification of HBV-DNA using primers targeting the *s* and *c*-gene and detection in real-time with SYBR Green dye (GeneON Gmb HSYBR Green PCR Kits). The amplification reaction was performed as follows; HBV *s* and *c*-gene were amplified by using 30 pmol of HBV *s* and *c*-gene sense and antisense primers with 12.5 µl SYBR Green universal PCR master mix 2-fold (GeneON GmbH) and 10 µl of Qiagen extracted DNA. H_2O was added to bring the reaction to a final volume of 25 µl. real-time PCR was performed using the Mx3000P TM (Stratagene) real-time PCR system. The amplification profile was done as the following; AmpliTaq activation at 95 °C for 10 min, followed by 40 cycles of PCR amplification, including denaturation at 95 °C for 15 s, annealing at 55 °C for 30sec and extension at 72 °C for 1 min. A melting curve analysis was done to determine the purity and specificity of the amplification product.

HBV-DNA extraction was done using QIAamp DNA Blood Mini kit (Qiagen). Detection of HBV-DNA was done by nested PCR using specific primers for *pol* genes. The outer primers (first round of PCR) used in this test amplify part (domain B and C) of the HBV polymerase gene. The amplified product of the first round PCR was targeted by nested (inner) primers. Nested amplification products were 341 bp long, analyzed on a 2% agarose gel, and visualized by staining with Ethidium Bromide (EtBr) at 302 nm. The outer amplification reaction components consisted of; 30 pmol of HBPr134 sense and HBPr135 antisense primers, 12.5 µl universal PCR master mix 2-fold (GeneOn GmbH), 10 µl of Qiagen extracted DNA and H_2O was added to bring the reaction to a final volume of 25 µl.

The nested amplification components contained 30 pmol of HBPr75 sense and HBPr 94 antisense primer, 12.5 µl universal PCR master mix 2-fold (GeneON GmbH), 3 µl of outer amplified products and H_2O was added to bring the reaction to a final volume of 25 µl. The thermal profile was performed as follows; outer amplification profile containing AmpliTaq activation at 95 °C for 10 min, followed by 40 cycles of PCR amplification, including denaturation at 95 °C for 30 s, annealing at 45 °C for 30 s and extension at 72 °C for 30 s. then the final elongation at 72 °C for 10 min. Nested amplification thermal profile was similar to the outer amplification profile (35 cycles) (see Tables 1 and 2).

3. Results

Among the 50 HCV patients included in this study 52% were males and 48% were females (Table 3). No significant difference was found in sex distribution regarding SVR and Non-SVR. Table 4 shows that HCV viral load before treatment (at 0 week) was significantly associated with SVR. Fifteen patients (60%) out of 25 SVR patients had a viral load below 800,000 IU/mL compared to eight (32%) of the Non-SVR patients. Table 5 shows that 11 (22%) out of 50 patients included in the study were anti-HBc positive. anti-HBc seropositivity was 36% of the Non-SVR HCV group compared to 8% of the SVR group, this was found to be statistically significant.

By comparing the detection of HBV-DNA by conventional nested PCR (*pol* gene) in relation to the real-time SYBR Green technique (*s* and *c* genes) we found that, out of 11 anti-HBc positive samples, 10 (90.9%) were positive for the *pol* and *s* genes while 9 (81.81%) were positive for the *c* gene (Table 6; Figs. 1 and 2). Among the 17 HBV-DNA positive HCV patients 70.6% were Non-SVR patients while the remaining 29.4% were SVR patients. This was found to be statistically significant (Table 7).

4. Discussion

There is general agreement that patients infected with HCV should be considered as a category of individuals with high prevalence of occult hepatitis B.[21] The incidence of OBI in HCV patients varies greatly, ranging from 0% to 52%.[12] In our study; 34% of HCV patients were positive for OBI. Our results were in agreement with those reported among Mediterranean countries since HBV-DNA was detectable in about one-third of HBsAg negative persons infected with HCV in the Mediterranean basin.[22,23]

Mrani et al.[1] reported that HBV-DNA was found in 1/4 of French chronic hepatitis C patients regardless of the presence of anti-HBc. Such an occult HBV co-infection was associated with more severe liver disease, higher HCV viral load and decreased response to antiviral therapy irrespective of HCV genotypes. Fukuda et al.[24] and Liu et al.[25] reported that the serum titer of HCV-RNA was visibly higher in patients with concurrent HBV and HCV infection than those with HCV mono-infection. Mrani et al.[1] also found that HCV viral load was significantly higher in HBV-DNA positive than in negative patients. Emara et al.[12] reported that this seemed to be applicable to genotype 4, where HBV-DNA positive patients in their study showed higher baseline HCV viral load than HCV mono-infected patients. Our results agree with these results as 82.4% of our HBV-DNA positive patients were associated with high HCV viral load compared to 39.4% of HBV-DNA negative and this was statistically significant.

Early studies reported a higher prevalence of HBV-DNA detection in anti-HBc-positive than in anti-HBc-negative patients.[24,26] In his study, Marusawa et al.[27] found that the prevalence of anti-HBc is almost 50% in patients with HCV related chronic liver disease. In the present study 34% of our HCV cases were positive for HBV-DNA of whom 58.8% were anti-HBc positive. Chemin et al.[28] reported that occult HBV infection was most frequently seen in patients with anti-HBc as the only HBV serological marker. On the opposite, Mrani et al.[1] found that OBI was not associated with the presence of anti-HBc in chronic HCV patients.

Ramia et al.[29] found that the overall rate of HBV-DNA in the HCV-infected patients was 16.3%, compared to the HBV-DNA rate of 41% in HCV-infected patients who were anti-HBc alone positive. Our results agree with Ramia et al.[29] since 22% of the 50 HCV patients were anti-HBc positive of whom 90.9% were HBV-DNA positive, on the other hand 78% were anti-HBc negative of whom only 17.9% were positive for HBV-DNA.

Table 1 Sequences of primer pairs used for PCR to detect HBV genome.

Primer name	Nucleotide sequence 5′ to 3′	Nucleotide position
Surface gene		
S-sense	AGAACATCGCATCAGGACTC	159–178
S-antisense	CATAGGTATCTTGCGAAAGC	642–623
Core gene		
C-sense	CTGGGAGGAGTTGGGGGA	1730–1747
C-antisense	GTAGAAGAATAAAGCCC	2503–2487

Table 2 Sequences of primer pairs used for PCR to detect HBV genome.

Primer name	Nucleotide sequence 5′ to 3′	SEQ ID No.
Outer primer		
HBPr134 sense	GCTGCTATGCCTCATCTTC	134
HBPr 135 antisense	CA(G/A)AGACAAAAGAAAATTGG	135
Nested primer		
HBPr 75 sense	CAAGGTATGTTGCCCGTTTGTCC	75
HBPr 94 antisense	GG(T/C)A(A/T)AAAGGGACTCA(A/C)GATG	94

Table 3 Sex distribution among the 50 HCV patients.

Sex	SVR (n = 25)		Non-SVR (n = 25)		Total	
	N	%	*N*	%	*N*	%
Female	11	44.0	13	52.0	24	48.0
Male	14	56.0	12	48.0	26	52.0

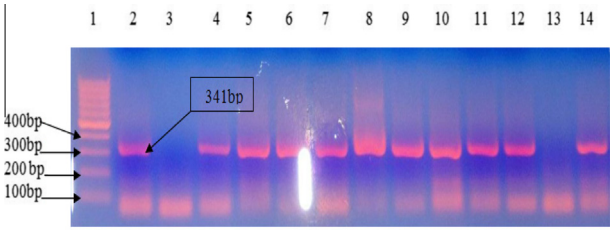

Figure 2 Gel electrophoresis of nested PCR of *pol* gene. The 15 µl of PCR product was run on 2% agarose gel at 100 V for 1 h. Lane 1: DNA ladder −100 bp, Lane 2: positive control, Lane 3: negative control, Lane 4–12 and 14: positive samples and Lane 13: negative sample.

The finding of OBI among anti-HBc positive persons supports the notation that OBI is frequently a late phase of overt chronic HBV infection or serologically recovered acute HBV infection. Another possible hypothesis for this finding is that HCV infection may block the circulating viral expression of

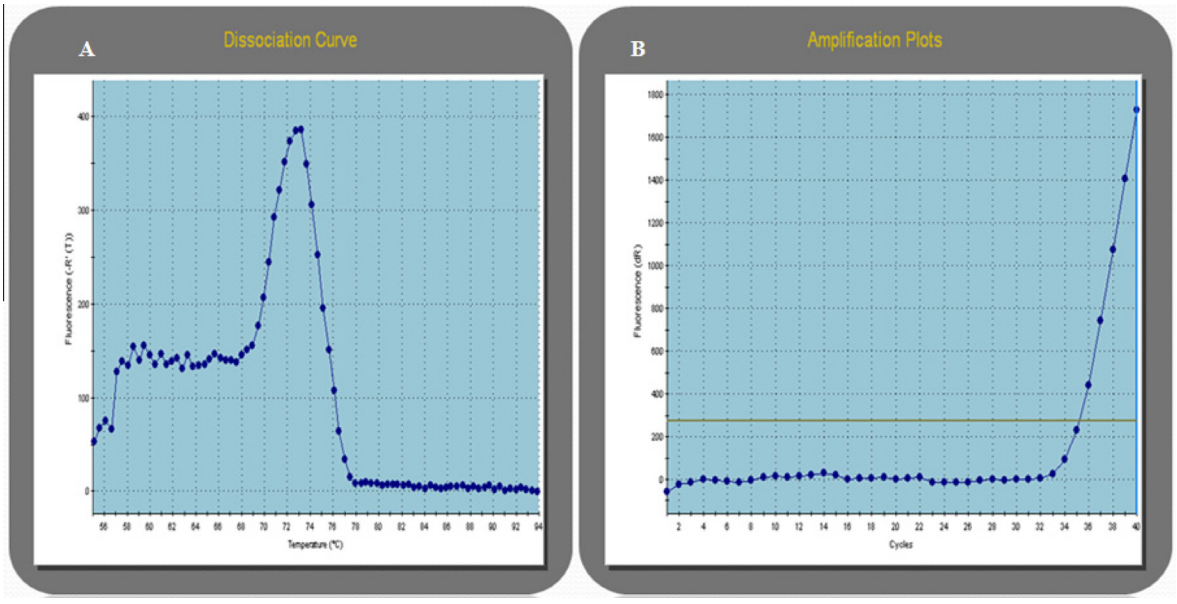

Figure 1 Detection of HBV *s* gene by SYBR Green. (A) Dissociation curve of *s* gene. (B) Amplification plot of *s* gene showing a Ct of 35 cycles.

Table 4 HCV viral load in SVR and Non-SVR HCV patients.

HCV viral load	SVR (n = 25)		Non-SVR (n = 25)		
	N	%	*N*	%	*P*
Low: <800,000 IU/mL	15	60.0	8	32.0	0.047*
High: ⩾800,000 IU/mL	10	40.0	17	68.0	

* $P < 0.05$ (significant).

Table 5 Distribution of anti-HBc among the 50 HCV patients.

	SVR (n = 25)		Non-SVR (n = 25)		Total		
	N	%	*N*	%	*N*	%	*P*
Anti-HBc							
Negative	23	92.0	16	64.0	39	78.0	0.017*
Positive	2	8.0	9	36.0	11	22.0	

* $P < 0.05$ (significant).

Table 6 Detection of HBV-DNA in 11 anti-HBc positive samples using conventional and real-time PCR techniques.

HBV-DNA	SYBR Green PCR				Conventional nested PCR	
	s gene		*c* gene		*pol* gene	
	n	%	*n*	%	*N*	%
Positive	10	90.90	9	81.81	10	90.90
Negative	1	9.09	2	18.18	1	9.09

Table 7 Distribution of HBV-DNA positivity among SVR and Non-SVR HCV patients.

	SVR (n = 25)		Non-SVR (n = 25)		Total		
	N	%	*N*	%	*n*	%	*P*
HBV-DNA							
Negative (n = 33)	20	80.0	13	52.0	33	66.0	0.037*
Positive (n = 17)	5	20.0	12	48.0	17	34.0	

* $P < 0.05$ (significant).

HBV but anti-HBc in the serum and HBV-DNA in the hepatocytes may persist.[30] Also persistence of low levels of HBV-DNA in the absence of detectable HBsAg may be due to host and viral factors suppressing viral replication and keeping the infection under control.[7]

Few studies however evaluated the impact of occult hepatitis B infection on the current standard treatment of HCV infection, that is, combination of Peg-IFNα and RBV.[21] Caviglia et al.[11] concluded that despite the high prevalence rate of liver HBV DNA in patients with CHC, SVR was not affected by occult HBV infection. Kao et al.[19] reported that the sustained rates of response to combined alpha IFN and RBV therapy were similar between chronic HCV patients with OBI and those without OBI, and thus, low-level HBV infection did not interfere with the response to combination therapy against HCV. Kishk et al.[31] reported that exposure to HBV or occult HBV infection in patients with chronic HCV does not affect the outcome of therapy at weeks 12 and 24.

By contrast, Mrani et al.[1] reported that sustained response to IFN and RBV was achieved in 11 (28%) of 40 HBV-DNA positive cases with chronic hepatitis C, compared with 65 (45%) of the 144 HBV-DNA negative cases ($P < 0.05$). Cacciola et al.[9] reported that the occult HBV was detected in 26 of 55 patients in whom IFN therapy was unsuccessful and in 7 of 28 patients in whom treatment was successful. In the present study, SVR was achieved in 60.6% of the HBV-DNA negative chronic HCV patients compared to only 29.4% of the occult HBV chronic HCV patients. This was statistically significant.

The serological pattern consisting of "anti-HBc alone", namely anti-HBc without both HBsAg and anti-HBs, has gained increased attention as a possible marker of OBI. The emerging generally accepted concept is that there is a significant, but not absolute, correlation between persistence of OBI and anti-HBc.[32] Our findings were in agreement with the previous investigations since out of the 11 anti-HBc positive CHCV patients 10 (90.9%) were OBI. Ocana et al.[33]

suggested that anti-HBc determination is useful in OBI diagnosis, even when HBV-DNA is available, because of the possibility of intermittent viremia. Also, the Taormina group recommended its use as a surrogate marker whenever an HBV-DNA test is not available to identify potential sero-positive OBI individuals.[33]

5. Conclusion

The prevalence of OBI was higher in our CHC patients. OBI was significantly associated with poor response to combined Peg-IFNα and RBV therapy.

Conflict of interest

None declared.

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