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Circulating levels of collagen III and MMP-1 in patients with chronic hepatitis C co-infected with hepatitis B virus

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ABSTRACT

Background: There is controversial data in the literature about the characteristics and features of dual hepatitis B and hepatitis C infection. This work is concerned with estimating the extent to which HBV could influence circulating levels of hepatitis C viral nonstructural-4 (HCV-NS4) in addition to some direct fibrosis markers in chronic hepatitis C.

Methods: Thirty-eight HCV mono-infected and 87 HCV/HBV co-infected patients constituted this study. Western-blot and ELISA were used for identifying HCV-NS4, hepatitis B surface antigen (HBsAg), collagen III and matrixmetalloproteinase-1 (MMP-1) in patients' sera.

Results: Hepatitis B surface antigen (HBsAg) provided area under curve (AUC) of 0.97 for identifying HBV-patients with 89% sensitivity and 94% specificity, while HCV-NS4 antigen provided an AUC of 0.95 for identifying HCV-patients with 89% sensitivity and absolute specificity (100%). In general, patients with significant fibrosis (F2–F4) showed significantly higher concentration of collagen III ($P = 0.009$) and lower concentrations of MMP-1 ($P = 0.007$) when compared to patients with minimal fibrosis (F1). However, HCV/HBV co-infected patients with F1 and F2–F4 did not show any significant difference ($P > 0.05$) from HCV mono-infected patients with respect to HCV-NS4, collagen III and MMP-1. These results indicate that HBV does not influence the rate of HCV-NS4 synthesis and the deposition of extracellular matrix in HCV/HBV co-infected patients and subsequently does not affect the progression rates of hepatic fibrosis.

Conclusion: HCV/HBV co-infected and HCV- mono-infected patients had similar clinical characteristics and there is no effect of HBV co-infection on the progression rates of liver fibrosis in chronic hepatitis C patients.

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Chronic hepatitis C; co-infection; fibrosis markers; HCV-NS4; hepatitis B; Western-blot

Introduction

Liver fibrosis is a major cause of morbidity and mortality worldwide due to chronic viral hepatitis.[1] The most common causes are hepatitis B virus (HBV) and hepatitis C virus (HCV) that cause liver inflammation and subsequently chronic liver disease.[2] Globally, HBV infects at least 350 million people and every year one million people die due to liver cirrhosis.[3] HCV infection is a global health burden affecting approximately 160 million people worldwide.[4] Hence, the liver has become a focal point of pathogenic insult and subsequent pathological damage for both HCV and HBV. The influence of co-infection with these two hepatotropic infectious agents on progression rate of liver fibrosis is a matter of great controversy. Some studies suggested that patients co-infected with HBV and HCV have a greater rate of progression to advanced liver disease compared with patients infected with HBV or HCV alone.[5] However, others found that liver disease in patients with dual infection was not more severe than in patients with single HBV or HCV infection. In other words, no significant differences were found between such dual and single infections.[6–8]

This work is concerned with solving the controversy regarding the impact of HCV/ HBV co-infection on liver fibrosis progression. This was determined by identification and quantitative determination of hepatitis C viral nonstructural-4 (HCV-NS4) in addition to direct fibrosis markers, which are directly involved in deposition and removal of extracellular-matrix (ECM). To do so, we recruited patients with chronic hepatitis C (CHC) with and without HBV co-infection and estimated the extent to which HCV/HBV co-infection could influence the degree of liver fibrosis.

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Materials and methods

This work was conducted at Biotechnology Research Center in Egypt with sera recruited from the Tropical Medicine Department, Mansoura University hospitals, Mansoura, Egypt. A total of 125 individuals [90 with minimal fibrosis (F1), 35 with significant fibrosis (F2–F4)] constituted the present study. Informed consents were obtained from all participants and they were fully informed concerning the diagnostic procedures involved and nature of the disease. The study protocol conformed to ethical guide-lines of 1975 Helsinki Declaration. Histopathological classification for liver fibrosis and cirrhosis was performed according to the METAVIR score.[9] In METAVIR system; the stage score represents the amount of fibrosis based on a 5-point scale: F0, no fibrosis; F1, portal fibrosis alone; F2, portal fibrosis with rare septae; F3, portal fibrosis with many septae; F4, cirrhosis. Needle liver biopsy specimens were obtained with an 18-gauge or larger needle. To be considered as adequate for scoring, the liver biopsies have to measure at least 15 mm and/or contain at least five portal tracts, except for cirrhosis for which no limitation was required.

Patients were then classified into two groups. The first group included 38 patients who have HCV mono-infection. This cohort comprised 29 men and 9 women with a mean (±SD) age of 41.6 (±7.6) years. The second groups included 87 patients who have HCV/HBV co-infection. This group comprised 60 men and 27 women with a mean (±SD) age of 44.2 (±8.7) years.

HCV mono-infected patients had no history or laboratory evidence of previous or current *Schistosoma mansoni* infection and negative for other causes of chronic liver disease including viral hepatitis A and B. Exclusion criteria for the study were hepatocellular carcinoma, prior antiviral or immunosuppressive therapy, decompensated liver disease (ascites, jaundice, variceal bleeding, or encephalopathy), evidence of coexistent liver disease and liver transplantation.

Liver function tests (albumin, total bilirubin, aspartate aminotransferase [AST], alanine aminotransferase [ALT] and alkaline phosphatase [ALP]) were all measured on fresh serum on an automated biochemistry analyzer (A15, Biosystem, Barcelona, Spain). Patients with HCV were confirmed for the presence of HCV-RNA using polymerase chain reaction assay (COBAS Ampliprep/ COBAS TaqMan, Roche Diagnostics, Pleasanton, U.S.A.). Patients with HBV were also confirmed for the presence of HBV-DNA using polymerase chain reaction assay (QIAGEN GmbH, Hilden, Germany).

SDS-PAGE was carried out in 0.75 mm-thick, 12% vertical slab gels according to the method of Laemmli.[10] Serum samples were mixed with the sample buffer (0.125 M Tris base, 4% sodium dodecyl sulfate, 20% glycerol, 10% β-mercaptoethanol and 0.1% bromophenol blue as a tracking dye).

Following electrophoretic separation, Western electroblotting was used to transfer the separated protein bands onto a nitrocellulose membrane (0.45 mm pore size, Sigma, St. Louis, U.S.A.) in a protein transfer unit according to Towbin et al. [11]. They were then immunostained using respective antibodies (ABC Diagnostics, New Damietta, Egypt) corresponding to HCV-NS4, hepatitis B surface antigen (HBsAg), collagen III and MMP-1, separately. Finally, bands of the aforementioned markers were cut and electroeluted separately from preparative polyacrylamide gels at 200V for three hours in a dialysis bag (Sigma). The protein content of the purified bands was determined [12] and the remainder was stored at −20 °C.

The ELOSA protocol was as follows. First, diluted serum samples (1:250) in coating buffer (50mM carbonate/bicarbonate buffer, pH 9.6) were tested (50 µl/well) for HBsAg bound on a 96-well microtiter plate at 4 °C overnight. After blocking with 0.5% BSA in coating buffer (200 µL/well), 50 µL/well of a monoclonal antibody corresponding to HBsAg at a dilution 1:200 in PBS was added and incubated at 37 °C for 2 h. Next, 50 µL/well of anti-mouse IgG alkaline phosphatase-conjugated (Sigma), diluted 1:500 in 0.2% BSA in PBS-T20, was added. The plate was washed with PBS + 0.5% Tween 20 after every step. Finally, an enzyme detection system composed of nitrophenyl phosphate substrate (50 µL/well) was added. The absorbance was read at 450 nm after 10 min using a microtiter plate reader (Σ960, Metretech Inc, Germany). Colour intensity was proportional to the amount of bound conjugate and, therefore, is a function of the concentration of HBsAg present in the serum sample. Similarly, the aforementioned quantitation methods was performed in respect of HCV-NS4, collagen III and MMP-1 using the same quantities and intervals but in different concentrations as the following: sera dilution in coating buffer (1:250 for HCV-NS4; 1:50 for collagen; 1:50 for MMP-1), blocking with BSA in coating buffer (0.5% for HCV-NS4; 0.5% for collagen III; 0.6% for MMP-1), respective antibodies was diluted in PBS (1:200 for HCV-NS4; 1:200 for collagen III and 1:500 for MMP-1) and alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma) was diluted in 0.2% BSA in PBS-T20 (1:450 for HCV-NS4; 1:700 for collagen III; 1:50 for MMP-1).

Statistical analyses were performed by SPSS software version 15.0 (SPSS Inc., Chicago, IL). Continuous variables were expressed as mean ± standard deviation. Statistically, significant differences between groups were determined using ANOVA, Student *t*-test and Mann–Whitney *U* test. A value of $P < 0.05$ is considered statistically significant. The diagnostic value of HBsAg and HCV-NS4 antigen were estimated by calculating the area under the receiver operating characteristic curves. Based on the receiver-operating characteristic analysis, the best cut-off points were selected and diagnostic performances (sensitivity, specificity, accuracy, positive predictive value and negative predictive value) were determined.

Results

The mean age of the patients included in this study was 43.2 ± 8.7 years, 89 (71.2%) were men, while 36 (28.2%) were women. Overall, 90/125 (72.0%) patients had mild fibrosis (F1), 21/125 (16.8%) had moderate fibrosis (F2), 10/125 (8%) had sever fibrosis (F3) and 4/125 (3.2%) patients had cirrhosis (F4). Laboratory characteristics of HCV infected patients with and without HBV were assessed and presented in Table 1. HCV mono-infected and HCV/HBV co-infected patients had similar clinical characteristics and there was no significant difference with respect to any assessed variables except for total bilirubin in F2-F4 patients who have HCV with and without HB as seen in Table 1.

SDS-PAGE followed by Western blotting was used to identify the target HBsAg, HCV-NS4, collagen III and MMP-1 in patients' sera. A single immunoreactive band was shown at 24-, 27-, 70- and 245-kDa, respectively, due to their binding with their respective antibodies. No specific reaction was observed in sera of healthy individuals under these conditions.

In order to estimate the diagnostic accuracy of HBsAg, ROC curve was used. As a result, this antigen enabled the correct identification of patients with HBV infection with an AUC of 0.97. Based on ROC analysis, the best cut-off point of 0.30 was chosen for the optimal prediction of HBV infection. A cut-off point of optical density (OD) = 0.30 gave a sensitivity of 89%, specificity of 94% and accuracy of 92% for discriminating patients with HBV infection from healthy individuals.

The diagnostic accuracy of HCV-NS4 antigen was estimated based on ROC analysis giving an AUC of 0.95. HCV-NS4 antigen yielded 89% sensitivity and 95% accuracy for detecting HCV infection. Based on this method, an absolute specificity (100%) was obtained for differentiating HCV-infected patients from healthy individuals. In addition, at this point, HCV-infection could be excluded with an NPV of 90%, i.e. 10% of HCV-infected patients would be classified falsely. Moreover, HCV infection could be confirmed with an absolute PPV (100%).

The levels of HCV-NS4 in relation to METAVIR fibrosis stages in CHC-patients with and without HBV are presented in table 1 and Figure 1.

We estimated the circulating levels of both collagen III and its degrading enzyme MMP-1 in CHC patients with and without HBV infection in order to examine the extent to which could HBV affect the progression rates of liver fibrosis in chronic hepatitis C patients. Collagen III and MMP-1 were chosen because they are directly involved in deposition and removal of extracellular matrix and they have been already proposed as predictors of liver fibrosis.[13] Regardless of the presence or absence of HBV, patients with significant fibrosis (F2–F4) were found to have higher concentration of collagen III than those who developed minimal fibrosis (F1) ($P = 0.009$) (Figure 2(A)). On the contrary, MMP-1 was decreased with the progression of liver fibrosis being lower in patients with F2–F4 ($P = 0.007$) (Figure 2(B)).

Patients with F2–F4 and F1 were further classified according to HCV co-infection with HBV into two subgroups for evaluating the impact of HBV on the concentration of collagen III and its degrading enzyme. As a result, F2–F4 patients with HCV/HBV co-infection did not show any significant difference ($P > 0.05$) from HCV mono-infected patients with respect to both collagen III and MMP-1. The same goes for F1 patients who have HCV with and without HBV co-infection (Figure 2).

Discussion

Hepatitis C is known for its tendency to cause chronic infection in approximately 75% of acutely infected adults.[14] Every year many people die and are subjected to complex hospitalisation and medical assistance due to HCV infection.[15] The long-term impact of hepatitis C is highly variable, ranging from minimal changes to extensive fibrosis and cirrhosis.[16] However, many factors have been observed to influence the natural history of liver disease.[17] The data in the literature about the characteristics and features of dual hepatitis B and hepatitis C infection is controversial. Several studies have compared the histological

Table 1. Patients' characteristics at the time of liver biopsy.

Variables	Reference values	Minimal fibrosis (F1); $n = 90$			Significant fibrosis (F2–F4); $n = 35$		
		Group I	Group II	*P* value	Group I	Group II	*P* value
Number of patients		25	65		13	22	
Sex (male/ female)		21/4	46/19		8/5	14/8	
Age (years)		39.0 ± 8.1	43.8 ± 10.1	0.044	44.8 ± 5.1	45.4 ± 4.9	0.733
ALT (U/L)	< 45	64.2 ± 33.9	68.6 ± 37.1	0.609	68.0 ± 66.5	80.1 ± 46.8	0.539
AST (U/L)	< 40	45.3 ± 18.5	55.1 ± 27.6	0.105	56.3 ± 34.8	74.7 ± 39.9	0.180
T. bilirubin (μmol/L)	< 17.1	12.1 ± 6.5	12.8 ± 5.9	0.583	12.1 ± 5.5	16.4 ± 8.4	0.045
Albumin (g/L)	38–54	42.9 ± 2.7	42.6 ± 3.0	0.667	43.0 ± 2.3	39.7 ± 7.2	0.137
ALP (U/ml)	40–150	72.1 ± 51.5	78.4 ± 36.9	0.668	86.2 ± 47.8	66.1 ± 23.4	0.229
Platelet count (10^9/L)	150–450	209.8 ± 50.2	193 ± 48.3	0.177	177.1 ± 38.5	188.3 ± 70.5	0.602
HCV-NS4 (μg/mL)		75.8 ± 30.3	103.3 ± 48.6	0.061	92.2 ± 31.2	167.9 ± 196.0	0.123
Collagen III (μg/mL)		10.1 ± 9.0	10.0 ± 7.1	0.904	14.9 ± 10.0	14.0 ± 6.4	0.633
MMP-1 (μg/mL)		7.6 ± 8.0	9.2 ± 8.8	0.444	6.1 ± 8.0	3.4 ± 3.0	0.175

Group I: HCV mono-infection; Group II: HCV/HBV co-infection. ALT: alanine aminotransferase; AST: aspartate aminotransferase; T. bilirubin; ALP: alkaline phosphatase.

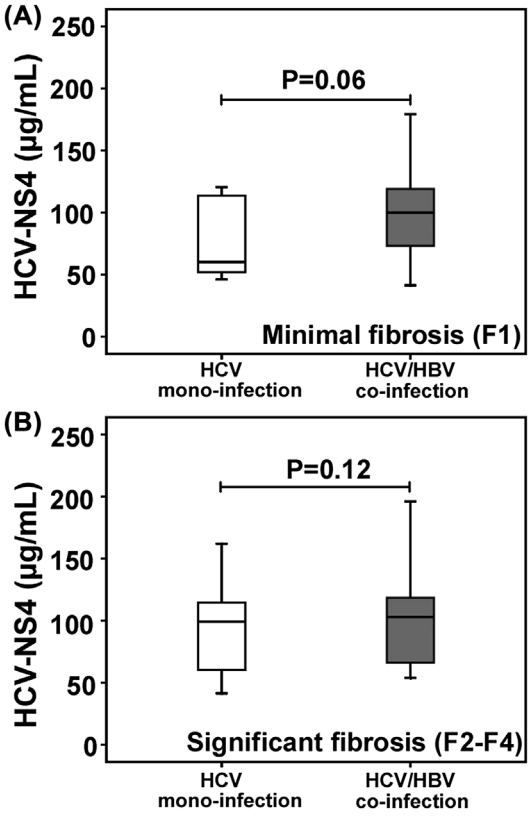

Figure 1. Levels of hepatitis C viral nonstructural-4 in absence and presence of HBV in (A) Patients with minimal fibrosis (F1); (B) Patients with significant fibrosis (F2–F4).

findings between HCV/HBV co-infection and single viral infection. Zarski et al. found that liver injury was more severe in HCV/HBV co-infection than in HCV mono-infection.[18] Similar results were obtained by Sagnelli et al. [19] and Lee et al. [20] who found liver histology to be more severe in chronic hepatitis C patients with HBV co-infection than those with HCV mono-infection. In spite of these studies which suggested that HCV/HBV co-infected patients had more severe liver disease, other studies have not supported this finding.[21,22]

We set out to examine if there is an impact of HBV co-infection with HCV on hepatitis C viral proteins in different METAVIR fibrosis stages. Herein, Western-blot analysis revealed that mono-specific antibody reacted against HCV-NS4 at an apparent molecular weight of 27-kDa in sera. Indeed, HVC-NS4 is one of HCV-proteins that was previously proved to suppress T helper-1 responses, [23] thereby hindering cellular and antiviral immunity. Bataller et al. [24] demonstrated that HCV core and nonstructural proteins directly stimulate the inflammatory and fibrogenic actions of HSCs which subsequently secrete large amounts of extracellular matrix. [25] In the present study, when the levels of HCV-NS4 were determined in CHC-patients with and without HBV, the differences were found statistically not significant. This means that HBV co-infection does not stimulate the synthesis of HCV proteins and subsequently does not promote the secretion of extracellular-matrix.

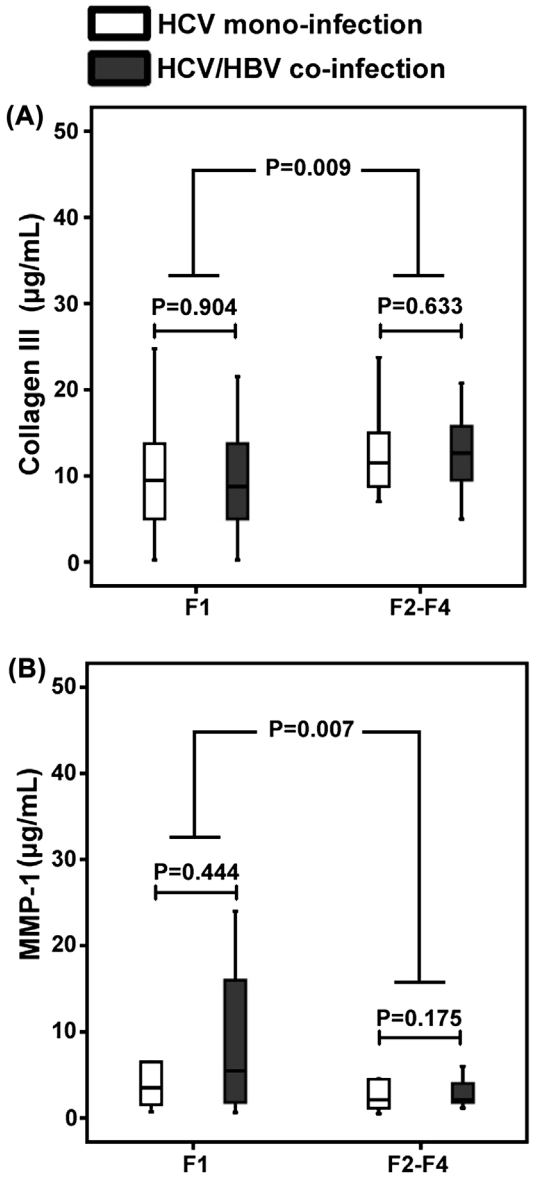

Figure 2. Levels of collagen III and matrix metalloproteinase-1 (MMP-1) in absence and presence of hepatitis B virus co-infection. The box represents the interquartile range, the line across the box indicates the median value.

We next estimated circulating levels of collagen III and MMP-1 in patients with minimal fibrosis (F1) versus those with significant fibrosis (F2–F4) in CHC patients with and without HBV. Collagen is considered to be the main component of connective tissue, and is the most abundant protein in mammals.[26] There is only five of the many collagen subtypes described have been detected in liver. They are types I, III, IV, V and VI.[27] Because of serum levels of collagen III is directly related to the hepatic fibrogenic process, it could be used as surrogate marker of liver fibrogenesis.

Patients with F2–F4 were associated with higher collagen content than patients with F1. The latter result may be explained by the fact that fibrogenesis is closely related to activation of the main type of fibro-competent cells in the liver: hepatic stellate cells (HSCs).[28] These HSCs and other portal fibroblasts, and myofibroblasts

have been identified as the main collagen producing cells in the liver.[29] Upon hepatic injury, HSCs become activated and secrete large amounts of extracellular matrix, resulting in progressive thickening of the septa and chemical cross-linking of collagen.[30] On the other hand, matrix metalloproteinases (MMPs) are a family of zinc-dependent neutral proteases which is responsible for regulating the degradation of extracellular matrix. MMP-1 in one of these metalloproteinases that have a role in degrading and denaturing interstitial collagens types I, II and III.[31] Several studies suggested that MMP activity decreases with the progression of hepatic fibrosis due to an over expression of their specific inhibitors (TIMPs).[32] Our results showed that the mean value of MMP-1 was found to decrease in patients with significant fibrosis when compared to patients with minimal fibrosis. This may be explained by the fact that the increased expression of hepatic TIMPs, which are co-expressed with MMPs and contribute to the regulation of local metalloproteinase activity, may lead to reduced metalloproteinase activity and is thought to be important for hepatic fibroproliferation.[33] The latter result was obtained in CHC patients regardless of the presence or absence of HBV.

Conceptually, the higher collagen content in any fibrosis stage may indicate their increased susceptibility for progressing to subsequent liver fibrosis stage. Surprisingly, our results showed that patients who developed F1 and co-infected with HCV/HBV did not show any significant difference in collagen III and its degrading enzyme 'MMP-1' when compared to those with F1 patients mono-infected with HCV. The same results were obtained in patients with significant fibrosis (F2–F4). This means that HCV/HBV co-infection does not influence the deposition of extracellular matrix and subsequently does not affect the progression rates of hepatic fibrosis. Consistent with our results, Villari et al.[21] reported that histological examination of liver tissue of patients with HCV/ HBV co-infection showed no typical patterns or evidence that this group of co-infection was a more severe than that caused by a single virus infection. Others [7,8] concluded that HBV/HCV co-infection and HBV-mono-infected patients had similar clinical characteristics.

Further prospective studies involving a greater number of patients are warranted to determine whether HCV/HBV co-infection is associated with more severe liver disease than mono-infection in chronic HCV or HBV. This work represents an advance in biomedical science as it provides evidence that HCV/HBV co-infection does not influence either the deposition of extracellular matrix or the progression rates of hepatic fibrosis.

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Disclosure statement

No potential conflict of interest was reported by the authors.

Summary table

What is known about this topic

- Several studies were done for investigating the relationship between HCV and HBV regarding fibrosis progression rate
- However, there is controversial data in the literature about the characteristics and features of dual hepatitis C and hepatitis B infection

What this work adds

- HBV has no impact on the concentration of HCV-NS4, which was previously proved to stimulate fibrogenic actions of HSCs
- Co-infected patients did not show any significant difference from HCV mono-infected patients with respect to both collagen III and MMP-1

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