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Correlation of Hepatitis C Virus NS-4with Different Liver Diseases.

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ABSTRACT

Quantitation of hepatitis C virus antigens is a useful tool for diagnosis and treatment. We aimed to identify the relationship betweennon-structural antigen-4concentration and liver different liver diseases. Three hundred and fiftyserum sampleswere divided as 50 asymptomatic individuals as controls (GI), 200 patients with liver fibrosis (GII); 50 liver cirrhosis (GIII) and 50 hepatocellular carcinoma(GIV).Our antigen was identifiedin patients' sera using sodium dodecyl sulphate polyacrylamide gel electrophoresis and western blot. Enzyme linked immunosorbent assaywas designed to quantifythe antigen in patients' sera.The antigen concentrationswere (21.3±5.2 ng/l) in GI, (47.7±3.2 ng/l) in GII, (62.5±7.6 ng/l) in GIIIand (74.4±7.8 ng/l) in GIV. There wasan extremely significant difference (P<0.0001) in antigenconcentration amongall groups.In conclusion,Quantitation of hepatitisC virus non-structural antigen-4 concentration using enzyme linked immunosorbent assaycould be used as a useful tool for assessingdifferent liver diseases hepatitis C virus positive patients.
Keywords: Hepatitis C Virus, HCV-NS4,SDS, Western blotting.

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INTRODUCTION

Hepatitis C virus (HCV) was identified in 1989 [1]and is now considered to be endemic worldwide. Hepatitis C virus is one of the main infectious causes ofhepatitis, a liver injury associated with an influx of acute or chronicinflammatory cells into the liver [2]. It is estimated that, about170 million people infected worldwide [3]. The clinical picture of HCV infection is often an acute stage, followed by chronic infection in about 80% of people initially infected[4]. The laboratory abnormalities may provide the initial clue to suggest a diagnosis of acute HCV infection. Alanine aminotransferase (ALT) is largely concentrated in the liver and consequently serves as a fairly specific indicator of current liver status and the most important indicator of HCV activity [5,6]. The degree of enzyme elevation may be important in acute phase of the disease but of limited importance in chronic liver disease [7,8].

HCV genome encodes for a single polyprotein that is subsequently cleaved into 10 mature proteins, with the structural proteins located near the 5′ end of the polyprotein and the nonstructural proteins located near the 3′ end. Both structural (core, E1, E2, and p7) and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B)are cleaved by viral proteases NS2-3 and NS3-4A[9,10].The molecular weight of the HCV-NS4 antigen, 27 kDa, is similar to that of the HCV-NS4B protein [11,12]. The most widely suggested function for NS4b is the creation of a platform in the cell that concentrates the virus template, replication and host cell proteins, thereby increasing the efficiency of replication [13,14]. Alternatively, distortion of cellular membranes can reduce the transport of cell surface proteins in infected cells in order to escape from the host immune response [15]. Other functions attributed to NS4b are inhibition of host as well as viral protein translation [16, 17] and modulation of NS5ahyper-phosphorylation [18]. Clearly, NS4b is involved in a wide range of activities, which seem to point to a role in modulating the host cell environment either for evasion of the host response or optimizing the setting for viral replication.

MATERIALSAND METHODS

Materials

The present study included 350 serum samples from asymptomatic and different liver diseases patients. Samples were classified into four groups: 50 asymptomatic individuals (38 males and 12 females, mean age 39.5±5.4 year) as controls, GI; 200 patients with liver fibrosis (139 males and 61 females, mean age 43.0±4.5years), GII; 50 liver cirrhosis (37 males and 13 females, mean age 49.4±6.1 years), GIII; and 50 hepatocellular carcinoma (36 males and 14 females,mean age 53.1±5.6years), GIV. In addition, there were 20 serum samples from healthy individuals who are negative for HCV. Blood samples were collected from Mansoura University Hospitals, Mansoura, Egypt. All patients were positive for anti-HCV antibodies and were negative for hepatitis A, B and HIV antibodies. The infection with HCV was confirmed by screening for the presence of anti-HCV antibodies in sera of all patients and absence in normal controls using the HCV-Ab ELISA (Biotec Laboratories Ltd., Suffolk, UK) and positive serum test for HCV-RNA using the qualitative polymerase chain reaction (COBAS Ampliprep/COBAS

TaqMan, Roche Diagnostics, Pleasanton, USA) to assess viral load. All individuals were informed and approved for participating inthe present study.

Methods

Laboratoryparameters**.** Liver functions including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin (TB), albumin,Prothrombin time and INR were measured by COBAS INTEGRA® 400 plus (Roche Diagnostics GmbH, USA). Blood platelets count is included ina complete blood count (CBC) using the COULTER® LH 500 Hematology Analyzer(Beckman Coulter Inc., California, USA).

Gel electrophoresis and electroelution

Selected serum samples of HCV infected individuals were subjected to analytical sodium dodecyl sulphate polyacrylamide gel electrophoresis(SDS-PAGE), at 25µg/lane, using vertical slabs of 12% or 16% polyacrylamide gel [19]. Standard molecular weights (BioRad Laboratories, CA) were run in parallel. In preparative slab gel electrophoresis, the running condition was adapted to reduce smear of proteins and to enable a considerable long migration distance between bands in the 27-kDa region in serum sample according to the prestained molecular weight marker. In each run, a lane from electrophoresed preparative gel was Coomassie blue and immunoblotted to identify the 27-kDa band. In the unstained preparative gel, the adjacent band was then cut and the 27-kDa antigen electroeluted from polyacrylamide gel at 200 V for 3 h in a dialysis bag (Sigma). After dialysis, the electroeluted antigen was concentrated using polyethylene glycol and then precipitated using 40% trichloroacetic acid (TCA), then centrifuged at 6500-×g for 15 min. The pellet was washed twice using diethyl ether to remove the excess of TCA. The excess diethyl ether was removed by drying and the pellet was reconstituted in PBS, pH 7.2.The protein content was measured [20], then aliquoted and stored at -70 C until used.

Western blotting

The same serum samples were separated on SDS-PAGE and electrotransferred onto nitrocellulose (NC) membrane (0.45 µm pore size, Sigma) in a protein transfer unit [21]. The NC membrane was blocked using 2% (w/v) skimmed milk dissolved in 0.05 M Tris-buffered saline (TBS) containing 200 mM NaCl (pH 7.4), rinsed in TBS and incubated with mono specific antibody directed against HCV-NS4 antigen diluted in blocking buffer with constant shaking. The NC membrane was washed 3 times (30 min each) in TBS, followed by incubation for 2 h with anti-mouse IgG alkaline phosphatase conjugate (Sigma) diluted 1: 500 in TBS. After washing 3 times with TBS (15 min each), the NC membrane was soaked in alkaline phosphatase substrate (5-bromo-4-chloro-3-indolyl phosphate [BCIP], nitro blue tetrazolium [NBT], 0.1 M Tris buffer, pH 9.6) (Kir-kegaard and Perry Laboratories, Gaithersburg, Maryland,USA). The color was observed within 10 min, and the reaction stopped by dipping the NC membrane in distilled water.

Standardization of ELISA technique and determination of HCV-NS4 antigen cut-off

Diluted serum samples in coating buffer were allowed to bind overnight to wells of ELISA plates. Mono-specific antibodies to HCV-NS4 antigen were added separately per well in PBST20. The antigen-antibody binding was allowed to proceed for 2 hours at 37°C. Alkaline phosphatase-conjugated goat anti-rabbit IgG, diluted in conjugate buffer, were added. The amount of coupled conjugate was determined by incubation with 1mg/ml P-Nitrophenyl phosphate in substrate buffer. The enzyme converts a substrate (chromogen) to a colored product, indicating the presence of Ag-Ab binding. The reaction stopped by 3M Sodium hydroxide (NaOH) and the absorbance was readied at 490 nm usinga micro titer plate reader.The cut-off level was calculated as the mean ELISA optical densities of serum samples from 16 individuals non-infected with HCV.The cut-off level was set at 0.28. The value above or below the cut-off was considered positive or negative, respectively. In addition, we used four serum samples from patients with chronic HCV and four from patients with HCC as positive controls.

Dose curve and quantitation of HCV-NS4 antigen

Dose-response curve for HCV-NS4 antigen in the ELISA as a function of the concentration of antigen (ng/L) in serum samples of controls and HCV infected patients was done. Serial concentrations of purified 27-kDa HCV-NS4antigen ranged from 0 to 800 ng/L anddiluted serum samples in coating buffer were allowed to bind overnight to wells of ELISA plates. Mono-specific antibodies to HCV-NS4 antigen were added separately per well in PBST20. The antigen-antibody binding was allowed to proceed for 2 hours at 37°C. Alkaline phosphatase-conjugated goat anti-rabbit IgG, diluted in conjugate buffer, were added. The amount of coupled conjugate was determined by incubation with 1mg/ml P-Nitrophenyl phosphate in substrate buffer. The enzyme convertsa substrate (chromogen) to a colored product, indicating the presence of Ag-Ab binding. The reaction stopped by 3M Sodium hydroxide (NaOH) and the absorbance was read at 490 nm using a micro titer plate reader. The concentration of HCV-NS4 antigen in serum samples were calculated from the dose curve.

Statistical analysis

All statistical analyses were done by a Statistical Package for the Social Sciences (SPSS); version 15.0 on Microsoft Windows XP (SPSS Inc., Chicago, IL,USA). Descriptive results were expressed as mean±SD and range or number (percentage) of patients witha condition. Differences in continuous variables were assessed using Student's t-test or analysis of variance (ANOVA) and X^2 test for categorical variables. All tests were two-tailed and statistical significance was assessed at the 0.05 level.

RESULTS

Laboratory parameters of patients with different liver diseases

The study of bio-laboratory parameters for three groups of samples showed extremely highly significant increases (P<0.0001) in the values of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and Prothrombin-INR and total bilirubin values. While it showed that; extremely highly significant decrease (P<0.0001) of albumin values and platelet count as summarized in Table-1.

Table 1: Laboratory data of asymptomaticand patients samples

Laboratory variables	Mean ± SD				**P value
	Asymptomatic (n=50)	Fibrosis (n=200)	Cirrhosis (n=50)	HCC (n=50)	
Age (years)	39.5±5.4	43.0 ± 4.5	49.4 ± 6.1	53.1 ± 5.6	< 0.0001
ALT (U/mL)*	54.64±29	69.5 ± 12.8	63.5 ± 15.9	73.0 ± 21.3	< 0.0001
AST (U/mL)*	52.31± 23	55.7 ± 15.3	67.8 ± 16.1	87.9 ±17.8	< 0.0001
ALP (U/L)*	45.59 ± 19	81.3 ± 14.5	122.0 ± 27.8	189.4± 30.6	< 0.0001
Total bilirubin (mg/dL)*	1.1± 0.32	0.8 ± 0.24	1.5 ± 0.32	2.7 ± 0.46	< 0.0001
Albumin (g/L)*	39.64±6.8	42.5 ± 5.3	37.1 ± 6.1	31.7 ± 4.3	< 0.0001
Platelet count x 10^9/L*	349±58	192 ± 33.9	147 ± 29.9	145 ± 28.5	< 0.0001
Prothrombin-INR*	1.1 ± 0.31	1.1 ± 0.25	1.2 ± 0.23	1.4 ± 0.45	< 0.0001

***Normal values:** Alanine aminotransferase (ALT) up to 45 U/mL; aspartate aminotransferase (AST) up to 40 U/mL; alkaline phosphatase (ALP) 22-92 IU/L; total bilirubin up to 1 mg/dL; albumin 38–54 g/L; platelet count 150–400 (x10^9/L) and Prothrombin-INR(international normalized ratio) 1.
**P < 0.05 considered significant.
P < 0.0001 considered extremely significant

Identification of HCV-NS4 using western blotting

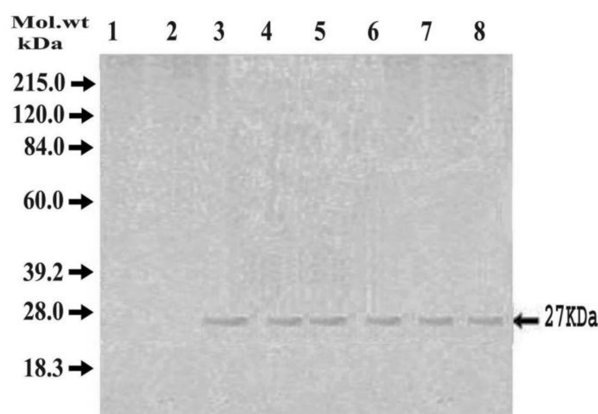

Figure 1: Identification of HCV-NS4 in serum samples of chronic hepatitis C and HCC patients using western blot.

A single band was identified at 27 kDa in serum samples from patients with different liver diseases [liver fibrosis (lane 3-4), liver cirrhosis (lane 5-6) and HCC (lane 7-8)]. No specific reaction was observed with the sera from non-infected individuals as a control group (lane 1-2)as shown in Figure-1.

Reactivity of mono-specific antibody against the purified HCV-NS4 antigen

The results showed that the TCA precipitate revealed a polypeptide band at 27-kDa. The rabbit mono-specific anti-27 kDa identified HCV-NS4 antigen in samples from chronic hepatitis C patients, hepatocellular carcinoma and in the TCA precipitate at 27-kDa. No specific reactions were observed with the TCA supernatants of the purified fraction from sera of chronic hepatitis C patients and hepatocellular carcinoma as shown in Figure-2.

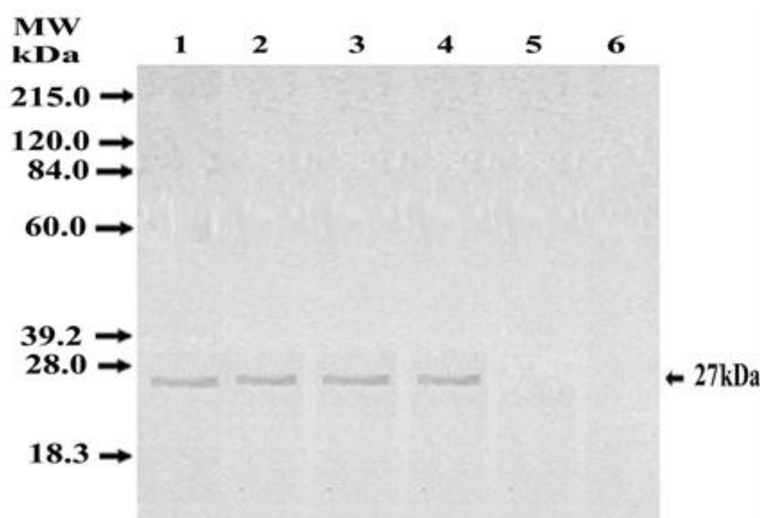

Figure 2: Immunoblots of serum from CHC patient, HCC patient, TCA precipitate fraction and TCA supernatant fraction of HCV-NS4 purified antigen from CHC patients and TCA precipitate fraction and TCA supernatant fraction of HCV-NS4 purified antigen from HCC patients.Lane (1): Serum sample from CHC patient, Lane (2): Serum sample from HCC patient. Lane (3): TCA precipitate of the purified fraction from sera of CHC patients, Lane (4): TCA precipitate of the purified fraction from sera of HCC patients, Lane (5): TCA supernatant of the purified fraction from sera of CHC patients, Lane (6): TCA supernatant of the purified fraction from sera of HCC patients. Lane (Mr): Molecular weight marker (Sigma) was included myosin (215.0 kDa), phosphorylase B, (120.0 kDa), Bovine serum albumin (84.0 kDa), Ovalbumin (60.0 kDa), carbonic anhydrase (39.2 kDa), trypsin inhibitor (28.0 kDa), and lysozyme (18.3 kDa).

Quantitation of HCV-NS4 antigen using ELISA

The mean ± standard error (SE) of serum HCV-NS4 antigen concentration were (21.3 ± 5.2 ng/l) in asymptomatic individuals, (47.7 ± 3.2 ng/l) in liver fibrosis cases, (62.5 ± 7.6 ng/l) in liver cirrhosis cases and (74.4 ± 7.8 ng/l) in HCC cases. There was an extremely significant difference ($P<0.0001$) in values of HCV-NS4antigen concentration between the four groups as

mentioned in Table-2. Concentrations of HCV-NS4 in serum samples were calculated from the dose curve as shown in Figure-3.

Table 2: HCV-NS4 antigen concentration (ng/L) in sera of patients with different liver diseases in comparison with asymptomatic individuals using ELISA

Group	No.	Mean ± SE (ng/L)	*P value
Asymptomatic	50	21.3 ± 5.2	<0.0001
Liver fibrosis	200	47.7 ± 3.2	
Liver cirrhosis	50	62.5 ± 7.6	
HCC	50	74.4 ± 7.8	

*P < 0.05 considered significant.
P < 0.0001 considered extremely significant

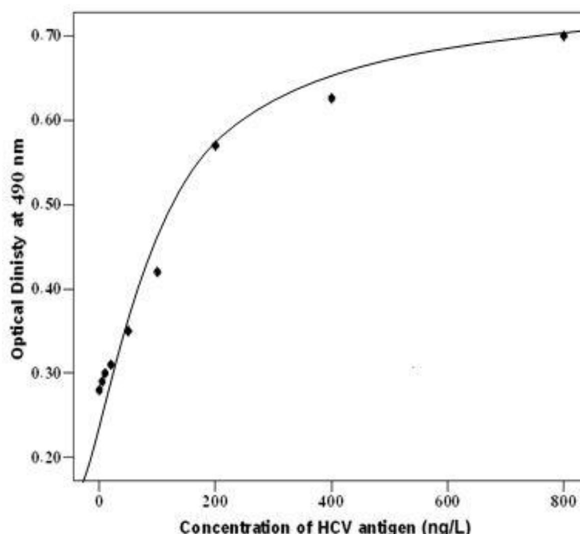

Figure 3: Dose curve of purified 27-kDa HCV-NS4 antigen using ELISA.

DISCUSSION

Chronic viral hepatitis C is a public health problem worldwide, leading to liver fibrosis, cirrhosis, and hepatocellular carcinoma. Nowadays, the golden standard method to assess liver pathology is a liver biopsy for fibrosis scoring systems [22, 23]. However, biopsy is an invasive technique and prone to sampling error leading to over or under staging of liver pathology [24]. This procedure also has significant morbidity, including post procedure pain, major bleeding, infections and ascites leakage and can lead to mortality [25]. Moreover, it is contraindicated in patients with coagulation disorders. This hampers the primary determination of liver pathology and optimal management of therapy. Consequently, there is a need for non-invasive methods to accurately diagnose the stage of liver pathology.

The present work was designed to identify the relationship between HCV-NS4 antigen and severity of liver diseases. Our results revealed that, there were significant differences in the serum levels of HCV-NS4 antigen in HCV patients with different liver diseases. Quantitation of HCV-NS4 antigen was found to have a good discriminative value in differentiation of different liver diseases in HCV patients.

The current study demonstrated that, laboratory parameters of liver function tests for study groups showed that; extremely significant increases (P<0.0001) in the mean values of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and Prothrombin INR and total bilirubin values among the studied groups. While, we found an extremely significant decrease (P<0.0001) in serum albumin values and blood platelet count.

The serum activities of liver enzymes, which are located in hepatocytes, have been clinically used for the assessment of liver function or injury [26]. Liver disease is often clinically silent until late in its course. For this reason, laboratory tests are usually needed for recognition and characterization of the type of liver injury present. In general, serum activities of transaminases are increased in various liver diseases, for example hepatitis, cirrhosis, infections, carcinoma, or alcohol abuse. Serum activities of aminotransferases, however, are only of limited prognostic value and do not reflect the extent of liver cell necrosis appropriately [27].

We observed that, HCV-NS4 antigen was identified at 27 kDa in serum of HCV patients with different liver diseases (fibrosis, cirrhosis or HCC) but no reaction with serum samples of healthy control individuals. In our study, HCV-NS4 was quantified in serum by using the dose curve of the purified antigen in sera of patients with different liver diseases using ELISA technique. The results showed that, there were extremely significant differences (P<0.0001) in HCV-NS4 antigen concentrations among all groups. These results agree with a previous study which studied the presence of anti-HCV antibodies and HCV non-structural proteins among patients with chronic HCV infection [28]. They found that, the non-structural protein, NS4B was immunogenic in 85% of the cases. They found that, the molecular weight of the HCV-NS4 antigen, 27 kDa, is similar to that of the HCV-NS4B protein [12,29]. This antigen was ahead before the other non-structural proteins, NS3, NS5A and the structural protein, E2. Also, they noticed remarkable differences in the frequency of anti-HCV antibody responses as well as there was a lot of variation in antibody titers against individual HCV proteins. Also, our findings are consistent with those who found an association between the severity of liver disease and HCV proteins in liver tissues [12,30]. These findings may indicate that patients with HCC maintain an efficient intrahepatic hepatitis C virus replication even at the end-stage disease [31]. The obtained high level of HCV-NS4 in our patients with HCC could be attributed to tumorigenic potentiality of HCV-NS4B as suggested by another study [32] which showed that the HCV-NS4B has *in vitro* and *in vivo* tumorigenic potentiality.

In conclusion, detection of HCV-NS4 antigen using ELISA could be applied for mass screening of HCV infection. Moreover, quantitation of HCV-NS4 antigen concentration could be used as a useful tool for assessing HCV patients with different liver diseases.

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