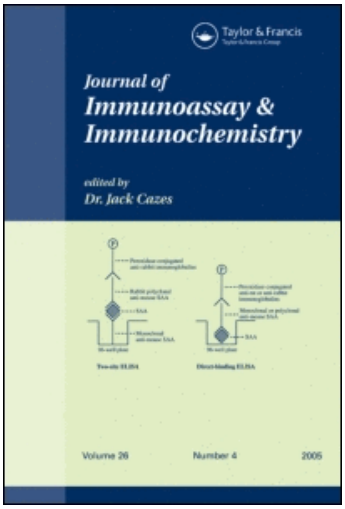

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Abstract: Serum tests measuring the dynamic processes of fibrogenesis and fibrolysis may reflect the severity of liver disease. Fibronectin plays a role in liver fibrosis. The aim of this study was to assess the diagnostic value of fibronectin in chronic HCV infection among Egyptian patients. Fibronectin was identified using specific monoclonal antibody and Western blot at 90-kDa in sera of HCV infected patients with liver fibrosis. The purified serum fibronectin showed one peak at 8 min when analyzed by capillary zone electrophoresis. Fibronectin was quantified in serum using ELISA. The mean ($\pm$SD) serum level of fibronectin (mg/L) in liver fibrosis patients were 450.9 ($\pm$170.3) and 230.5 ($\pm$90.3) in control individuals, respectively. There was a significant correlation between METAVIR score and serum fibronectin ($r = 0.401$; $P < 0.0001$). The area under the receiver operating characteristic (ROC) curve of fibronectin for discriminating patients with liver fibrosis from those with no fibrosis livers and its p value were 0.78 and $P < 0.0001$. The efficiency of fibronectin for discriminating patients with liver fibrosis from those with non fibrosis livers was 75%. In conclusion, serum fibronectin can differentiate HCV infected patients with liver fibrosis from patients with non fibrosis.

Keywords: HCV, Fibrosis, Biomarkers, Fibronectin

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INTRODUCTION

The major etiologies of liver fibrosis are viral associated hepatitis, alcohol abuse, non-alcoholic steatohepatitis, and autoimmune disease.[1] Hepatitis C virus is a major cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma worldwide.[2] Liver fibrosis is characterized by the accumulation of an extracellular matrix, which distorts the hepatic architecture.[3] Serum tests measuring the dynamic processes of extracellular matrix synthesis (fibrogenesis) and extracellular matrix degradation (fibrolysis) reflect the amount of extracellular matrix present, the degree of fibrosis or the ongoing process of architectural change of the liver.[3] various serum markers have been investigated and correlations with liver biopsies and severity of liver diseases have been found.[4,5] Previous studies have suggested that serum levels of extracellular matrix proteins may be used to assess the severity and progression of liver fibrosis. Accumulation of extracellular matrix proteins results from both increased synthesis and decreased degradation.[6] Fibronectin is multifunctional high molecular weight glycoprotein and acute phase reactant present in the blood and in the extracellular matrix proteins of tissues.[7,8] Fibronectin is biologically classified into two forms, namely plasma and cellular fibronectin.[9] The cellular form is generally insoluble and is deposited in tissues, while the plasma form is soluble and is widely deposited in connective tissue, blood vessel walls, and basement membranes, being a major non-collagenous component of all organ stroma.[10] As a major component of the extracellular matrix, it is considered to have an important role in chronic inflammatory periodontal disease.[11] Fibronectin also plays important roles in the development and pathogenesis of many disorders, including cancer.[12–14] In the present study, serum fibronectin was identified using Western blot, quantified using ELISA and its diagnostic accuracy of fibronectin for discriminating patients those with liver fibrosis from those without fibrosis was assessed.

EXPERIMENTAL

Samples

Serum samples and liver biopsies of 126 HCV infected individuals (91 males, 35 females; aged 23–72 year) were collected from Internal Medicine University Hospital, Mansoura University, Mansoura. The HCV infection was diagnosed based on biochemical, serologic, and histological criteria. Patients with chronic HCV infection diagnosed on a positive test for anti-HCV antibody (Biotec Laboratories Ltd., Suffolk, UK) and a positive ELISA test for HCV-NS4 antigen.[15] The HCV-NS4 antigen was quantified in serum using ELISA.[16] All individuals were negative for other causes of chronic liver disease including viral hepatitis A and B. None of the patients

had other causes of chronic liver injury, a history of habitual alcohol consumption, or hepatocellular carcinoma. Needle liver biopsy specimens were examined by a pathologist who was unaware of the clinical and laboratory results of each sample examined. METAVIR score was used to stage the fibrosis (F0 to F4).[17] The patients were pathologically classified into two groups: 12 patients with no liver fibrosis (F0, considered as controls) and 114 patients with fibrotic liver (F1-F3). Liver function tests, including aspartate aminotransferase and alanine aminotransferase, albumin, and bilirubin, were measured using standard methodologies (bioMérieux SA, Marcy l'Etoile, France). All serum and tissue samples were obtained with informed consent.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out in 0.75 mm-thick, 16% vertical slab gels according to the method of Laemmli.[18] Serum samples were mixed with the sample buffer (0.125 M Tris base, 4% sodium dodecyl sulfate, 20% glycerol, 10% mercaptoethanol, and 0.1% bromophenol blue as a tracking dye) and immediately boiled for three minutes. A mixture of reference proteins (Sigma Chemical Co., St. Louis, MO, USA) was run in parallel. Gels were then stained with Coomassie blue.

Western Blot

The sodium dodecyl sulfate–polyacrylamide gel electrophoresis resolved samples were electro-transferred onto nitrocellulose membrane (0.45 μm pore size, Sigma) in a protein transfer unit according to Towbin et al.[19] The nitrocellulose membrane was blocked using 2% (w/v) bovine serum albumin (BSA) dissolved in 0.05% M Tris-buffered saline (TBS), containing 200 mM NaCl (pH 7.4), rinsed in TBS, and incubated with fibronectin mouse monoclonal antibody (ABC Diagnostics, New Damietta, Egypt) diluted (1:100) in 1% BSA dissolved in TBS with constant shaking. The nitrocellulose membrane was washed three times (30 min each) in TBS followed by incubation separately for 2 h with goat anti-mouse IgG alkaline phosphatase conjugate (Sigma) diluted (1:500) in TBS. The dilutions of monoclonal antibody and conjugate were adjusted to eliminate the background, i.e., the presence of fibronectin in the healthy control individuals. After washing 3 more times with TBS (30 min each), the nitrocellulose membrane exposed to alkaline phosphatase substrate [5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro-blue tetrazolium (NBT) in 0.1 M Tris buffer, pH 9.6, [Sigma] for 10 min. The reaction was stopped using distilled water.

Gel Electroelution

The target fibronectin band (90-kDa) was cut and electroeluted from preparative polyacrylamide gels at 200 volts for 3 h in a dialysis bag (Sigma). After dialysis, the electroeluted fibronectin band was concentrated using polyethylene glycol and 40% trichloroacetic acid, then centrifuged at 6,500 × g for 15 min. The precipitate was washed twice using diethyl ether. The excess diethyl ether was removed by gentle drying and the pellet was reconstituted in phosphate buffered saline (PBS, pH 7.2). The protein content of the purified fibronectin band was determined[20] before remainder was stored at −20°C.

Capillary Zone Electrophoresis

Analysis was performed on a Prince autosampler model 1-LIFT (Prince Technologies, Handelsweg, Emmen, The Netherlands), according to Attallah et al.[21] The instrument was connected with Lambda 1010 variable UV (Deuterium lamp)/VIS (Halogen lamp) detector (Metrhom Herisau, Switzerland). Fused silica capillary coated with polyimide film (Prince Technologies) was used. The instrument was controlled by an IBM compatible computer fitted with WinPrince software, version 5 (Prince Technologies). Detection was performed and signals analyzed using the Dax software, version 5 (Prince Technologies). The purified fibronectin (25 μg per one mL of distilled water) separated with 100 mM borate buffer, pH 8.3, on a 65 cm × 75 μm capillary, 30 kV, 20°C and UV detection at 200 nm.

Detection of Fibronectin in Serum using ELISA

Fifty μL of serum diluted 1:10 in coating buffer (50 mM carbonate/bicarbonate buffer, pH 9.6) were allowed to bind overnight to wells of ELISA plates. Serial concentrations of purified fibronectin (200–1600 mg/L) were tested in parallel to establish a dose-response curve as a function of the concentration (mg/L) in serum samples. After five washes with phosphate buffered saline-Tween 20 (PBS-T 20), the wells were blocked with 0.2% BSA in coating buffer. After five washes with PBS-T20, fifty μL of mouse monoclonal antibodies to fibronectin (ABC Diagnostics) were added separately per well at dilution 1:100 in PBS. The antigen-antibody binding was allowed to proceed for 2 hours at 37°C. The plates were washed five times with PBS-Tween 20 (0.05%) and 50 μL/well of alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma), diluted 1:500 in 0.2% BSA in PBS-T20, were added. After 1 hour, the plates were washed five times with PBS-T20; the amount of coupled conjugate was determined by incubation with 1 mg/mL p-nitrophenyl phosphate in substrate buffer for 30 min at 37°C. The reaction was stopped by addition of 25 μL/

well of 3 M NaOH and the absorbance was read at 405 nm using a microtiter plate reader (Σ960, Metretech Inc, Germany).

Statistical Analyses

All statistical analyses were done with a statistical software package "SPSS 12.0" for Microsoft Windows, SPSS Inc.). Numerical data were expressed as mean $\pm$ SD and the analyses were considered statistically significant at a two-sided $P < 0.05$. The level of fibronectin was analyzed by the Mann-Whitney U-test and was used for comparisons between independent groups. The Spearman correlation test (r) was used to investigate the relation between each two variables among each group. To assess the ability of serum fibronectin for differentiating liver fibrosis (F1–F3) from non liver fibrosis (F0), we calculated the sensitivity and specificity for fibronectin, then constructed an ROC curve by plotting the sensitivity against the reverse specificity (1 minus specificity) at each value. The diagnostic value of fibronectin was assessed by the area under the ROC curve. An area under the curve (AUC) of 1.0 is characteristic of an ideal test, whereas 0.5 indicates a test of no diagnostic value. The nearer a curve shifted to the top left-hand corner of the graph, the more useful marker was for the diagnosis. We determined the turning point of the curve to the best cut-off value for the diagnosis, and it was also a maximal value at the sum of the sensitivity and specificity. The diagnostic accuracy was calculated by sensitivity, specificity and efficiency.[22,23]

RESULTS

Identification of Fibronectin using Western Blot

Serum samples of liver diseased patients (F0–F3) were resolved by SDS-PAGE, transferred onto nitrocellulose, and stained with monoclonal antibody to fibronectin. The molecular weight of serum fibronectin was 90 kDa, respectively, as shown in Figure 1. No reaction was observed in serum samples of controls under this condition.

Purification of Fibronectin Antigen using Electroelution Technique

The reactive epitope of fibronectin (90-kDa) was purified from serum samples of fibrotic patients using an electroelution technique from preparative slab gels. The purified fibronectin showed one peak when analyzed by capillary zone electrophoresis at 8 min, Figure 2.

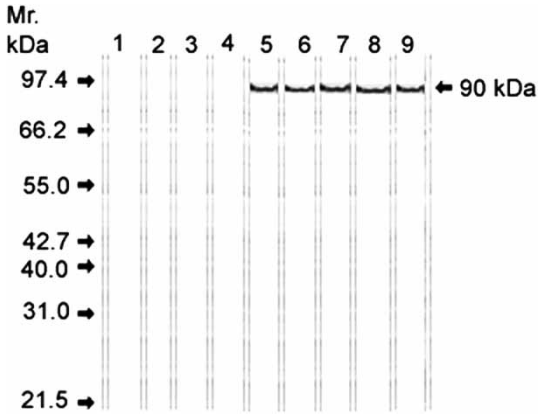

Figure 1. Western blot analysis of sera from normal controls and fibrotic patients with Chronic Hepatitis C. Lanes 1–4: Sera of normal individuals as negative controls. Lanes 5–9: Sera of fibrotic patients with chronic hepatitis C. Molecular weight markers (Mr.) include: Phosphorylase B (97.4 kDa), Bovine serum albumin (66.2 kDa), Glutamate dehydrogenase (55.0 kDa), ovalbumin (42.7 Da), aldolase (40.0 kDa), Carbonic anhydrase (31.0 kDa) and Soybean trypsin inhibitor (21.5 kDa).

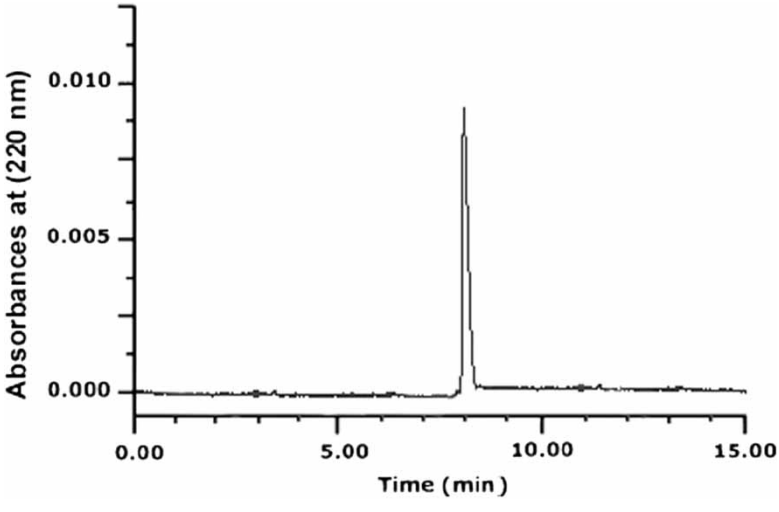

Figure 2. Capillary electrophoresis electropherogram of purified 90-kDa fibronectin from fibrotic patients with chronic HCV. The purified fibronectin from serum of liver fibrosis patients showed a single peak when analyzed by capillary zone electrophoresis at 8 min. The purified 90–kDa fibronectin (25 μg per one ml of distilled water) separated with 100-mM borate buffer, pH 8.3, on a 65-cm × 75-μm capillary, 30 kV, 20°C and UV detection at 220 nm.

Detection of Fibronectin using ELISA

A dose-response curve for purified fibronectin in the ELISA as a function of the concentration (mg/L) in serum samples of controls (F0) and liver fibrosis patients (F1–F3) was done. The values of serum fibronectin increased in the patients with HCV-associated liver disease according to the stage of liver fibrosis (F0–F3). The median, and mean ± SD (mg/L), of serum fibronectin in fibrotic liver patients were 420.0, 450.9 ± 170.3 and 200.0, 230.5 ± 90.3 in control individuals; respectively, (Figure 3). Laboratory values of liver function tests in different stages of liver fibrosis (F0–F3) are summarized in Table 1 for the 126 individuals. The values of ALT, AST, and bilirubin increased but albumin decreased in the patients with HCV-associated liver disease according to the stage of liver fibrosis (F0–F3). There is no significant difference between the value of fibronectin in females and males, but there is a positive significant correlation between fibronectin and age ($r = 0.522$; $P < 0.0001$). Correlation was observed between serum fibronectin level and the severity of liver disease as judged by the

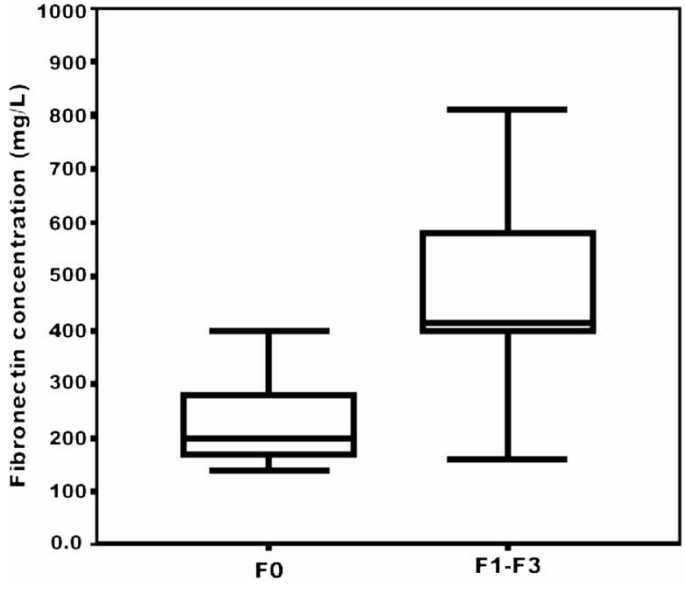

Figure 3. Box plots for level of fibronectin (mg/L) in patients with non-fibrotic (F0), fibrotic liver (F1–F3). The level of fibronectin (mg/L) with respect to stage of liver fibrosis (F0–F3). The box represents the interquartile range. The whiskers indicate the highest and lowest values, and the line across the box indicates the median value. Overall significance of differences among the 2 groups was determined by ANOVA for fibronectin level. A significance differences $p < 0.0001$) were shown between non-fibrotic liver (F0), liver fibrosis (F1–F3).

Table 1. Comparison between biochemical markers of chronic hepatitis C patients with non fibrotic liver (F0) and liver fibrosis (F1–F3)

Variable[a]	F0 (n = 12)	F1–F3 (n = 114)	P value[b]
ALT (U/mL)	26.7 ± 2.4	71.2 ± 51.2	<0.0001
AST (U/mL)	29.2 ± 6.4	64.9 ± 35.6	<0.0001
Bilirubin (mg/dL)	0.7 ± 0.14	2.7 ± 2.3	<0.01
Albumin (gm/L)	4.6 ± 0.41	3.6 ± 0.83	<0.0001
Fibronectin (mg/L)	230.5 ± 90.3	450.9 ± 170.3	<0.0001

[a]Normal values: alanine aminotransferase (ALT) up to 45 U/mL; aspartate aminotransferase (AST) up to 40 U/mL; bilirubin up to 1-mg/dL; albumin 3.8–5.4 g/L; fibronectin 250–400 mg/L according to Fortunato et al.[27]

[b]$p > 0.05$ is considered not significant, $p < 0.05$ considered significant; $p < 0.001$ considered very significant, $p < 0.0001$ is considered extremely significant.

values of ALT ($r = 0.372$; $P < 0.0001$), AST ($r = 0.312$; $P < 0.001$), bilirubin ($r = 0.554$; $P < 0.0001$) and albumin ($r = -0.497$; $P < 0.0001$). There was significant correlation between the METAVIR score and serum fibronectin ($r = 0.401$; $P < 0.0001$). The area under the ROC curve of fibronectin for discriminating patients with liver fibrosis (F1–F3) from those with no fibrosis livers (F0) and (p value) were 0.78 ($P < 0.0001$), (Figure 4). The sensitivity, specificity, and efficiency of fibronectin for discriminating patients with liver fibrosis (F1–F3) from those with no fibrosis livers (F0) were 75%, 82%, and 75%, respectively.

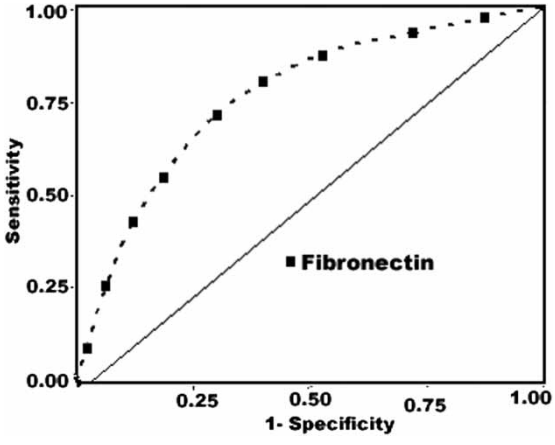

Figure 4. ROC curve of fibronectin for discriminating patients with liver fibrosis from those non liver fibrosis in chronic viral hepatitis C. The areas under the curve is 0.78 ($P < 0.0001$).

DISCUSSION

Fibronectin plays crucial roles in various cellular functions, including cell adhesion, migration, proliferation, and differentiation.[24] According to previous reports, plasma fibronectin is mainly produced by hepatocytes and cellular fibronectin in liver is produced, at least in part, by endothelial cells, fat-storing cells, and, to a lesser extent, by hepatocytes. It has also been mentioned that fibronectin plays important roles in hepatic fibrogenesis.[8,25] There is a need for accurate biochemical markers to aid in early diagnosis of liver fibrosis.[26] Several connective tissue substances have been studied as noninvasive markers for liver cirrhosis, e.g., hyaluronic acid, type III procollagen N-terminal peptide, laminin, and fibronectin[27] and have been proposed to represent hepatic fibrosis, focusing particularly on the diagnosis of hepatic cirrhosis. In the present study, Western blot analysis revealed that monoclonal antibody reacted against fibronectin at an apparent molecular weight of 90 kDa in serum samples of patients with liver fibrosis. Fibronectin was proteolytically cleaved and, after 20 minutes, four major fragments of 150, 120, 90, and 80 kDa were identified by Western blot analysis.[28] We performed an indirect immunoperoxidase technique with monoclonal fibronectin antibody, and localized fibronectin in the cytoplasm and extracellular spaces of liver fibrosis tissues (data not shown). Several authors detected fibronectin on the surface, in membranes, in cytoplasm, and between hepatocellular carcinoma.[29–31] Fibronectin has been purified from human plasma by methods combining precipitation steps, molecular-exclusion chromatography, and ion-exchange.[32] Fibronectin was purified from serum samples of liver fibrosis patients using an electroelution technique from preparative slab polyacrylamide gels. The purified fibronectin showed one peak when analyzed by capillary zone electrophoresis at 8 min. The fibronectin has been detected by using ELISA in serum samples which required purified fibronectin for quantitation. The dose-response curve for purified fibronectin in the ELISA as a function of the concentration in serum samples of controls and liver fibrosis patients was done. A few studies measured concentration of serum fibronectin in patients with chronic viral and alcoholic liver disease.[27,33,34] The level of serum fibronectin increased in our fibrotic patients with HCV infection agrees with Fortunato et al.[27] Increased amounts of fibronectin are significant in the development of early liver fibrosis and fibronectin may act as a chemotactic factor for collagen producing cells and as a skeleton for the new collagen formation.[35] In the present study, the area under the ROC curve of fibronectin for discriminating patients with liver fibrosis from those with no fibrosis livers was 0.78. ROC curve analysis showed comparable diagnostic accuracies of tissue inhibitor of matrix metalloproteinase-1 (0.32), laminin (0.46), type IV collagen (0.58), platelet count (0.71), matrix metalloproteinase-1 (0.73), hyaluronic acid (0.73), type III procollagen (0.73), age-platelet index (0.74), matrix metalloproteinase-1 mRNA, AST-to-platelet ratio index (0.80),

(0.88), platelet derived growth factor-BB (0.99) for prediction of fibrosis.[36,37] The sensitivity and specificity of fibronectin for discriminating HCV infected patients with liver fibrosis from those with no liver fibrosis livers were 75%, 82%, and 75%, respectively. The sensitivity and specificity of alpha 2-macroglobulin were (75% and 67%), bilirubin (61% and 53%), gamma glutamyl transferase (57% and 55%), apolipoprotein (26% and 50%) and haptoglobin (20% and 79%) for predicting fibrosis.[38] In conclusion, serum fibronectin showed satisfactory reproducibility and may be suitable for routine use to differentiate HCV infected patients with liver fibrosis from patients with non fibrosis.

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