Clinical significance of serum laminin and INR ratio to diagnose fibrosis in patients with chronic active Hepatitis C

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Abstract
Chronic hepatitis C (HCV) is the most prevalent agent causing hepatic fibrosis in human. Laminin (LA) and INR have been related to liver fibrosis and subsequent development of portal hypertension. The aim of this study was to evaluate the clinical utility of serum laminin and INR ratio and to create a score which helps in the diagnoses of hepatic fibrosis in order to reduce the frequent biopsy. This study included three groups: group I included 20 voluntaries persons as a control, group II included 22 patients infected with HCV without fibrosis and group III included 50 patients infected with HCV with fibrosis. Serum laminin was assayed in different groups by ELISA and routine blood analysis included ALT, GGT, INR and platelets count. Significantly higher mean level for serum LA, ALT, GGT, INR and Platelets count in fibrotic and non-fibrotic patients were compared with control group. The discriminate score (DS=0.03 laminin + 4.2 INR - 7.3) result of ≥ 0 suggests that the patients had fibrosis and the sensitivity of this score is 90% and specificity is 77.3% and the accuracy is 86.1%. DS could be considered as an easy method for the diagnosis of hepatic fibrosis in the patients suffering from chronic active hepatitis C. However, this study is preliminary and it is recommend that investigation is carried out on large scale.

Keywords: liver fibrosis, laminin, HCV.

INTRODUCTION
Liver fibrosis results from excessive accumulation of extracellular matrix in response to chronic liver inflammation. The accurate staging of liver fibrosis and the extent of inflammation in chronic liver diseases, especially the early diagnosis of liver cirrhosis is crucial for prognostic comments on the course of disease. Comments on the evaluation of a response to a therapy of chronic viral hepatitis as well as the occurrence of complications of chronic liver disease/liver cirrhosis such as bleeding of varices, ascites, hepatic encephalopathy and liver cell carcinoma are based on accurate and early staging (Afdhal et al., 2004; Fallowfield et al., 2006). It is characterized by excessive deposition of extra cellular matrix (ECM) proteins which include three large families of proteins, glycoproteins, collagens, and proteoglycans (Schuppan, 1990). Fibrosis occurs as a result of repeated cycles of hepatocytes injury and repair. The cascade of events that establish hepatic fibrosis is complex, and is influenced by how different cell types in the liver interact in response to injury. Activation of hepatic stellate cells (HSC) is the central event (McHutchison et al., 2000). Liver fibrosis is a dynamic process. It is usually secondary to hepatic injury and inflammation, and progresses at different rates depending on aetiology of liver disease and is also influenced by environmental and genetic factors (Friedman, 2003; Bataller et al., 2003). Hepatic fibrosis is a common response to chronic liver injury from many causes including hepatitis B (HBV) and hepatitis C (HCV) infection and increasing prevalence of non-alcoholic steatohepatitis (NASH) and fatty liver disease (NAFLD) and alcoholic steatohepatitis (ASH). They are major causes of chronic inflammatory liver disease resulting in the destruction of liver parenchyma and its replacement by scar tissue (Fibrosis). Rare etiologies are autoimmune hepatitis, parasitic infections (Schistosomiasis), and genetic disease such as hemochromatosis, α1-antitrypsin deficiency (Afdhal et al., 2004). Fibrosis is
characterized by the excess deposition of (ECM) involving molecular and histological re-arrangement of various types of collagens, proteoglycans, structural glycoprotein and hyaluronic acid (hyaluronan). It is a hallmark of liver cirrhosis and contributes significantly to the deleterious outcome of chronic liver disease (Olav et al., 2007). Chronic HCV is a slow progressing inflammatory disease of the liver that can lead to fibrosis and cirrhosis and their complications. Chronic hepatitis develops in at least half of persons acutely infected with HCV. Ten to 25% of these patients develop liver cirrhosis (Regev et al., 2002).

The complete evaluation of a patient with diffuse liver diseases requires clinical evaluation, laboratory tests, and pathological examination. The liver biopsy is regarded as the historical ‘gold standard’ for diagnosis and assessment of prognosis in chronic liver diseases (Bravo et al., 2001; Campbell et al., 2004). At least three scoring methods are commonly used to stage liver fibrosis: the Knodell, Ishak, and METAVIR scores (Knodell et al., 1981; Desmet et al., 1994). The Knodell and METAVIR score fibrosis from stage 0–4, with stage 4 as cirrhosis, whereas Ishak scores fibrosis from 0–6 where 5 is incomplete or early cirrhosis and 6 indicates established cirrhosis (Brunt, 2000). These methods are semi-quantitative and the invasiveness of liver biopsies with its associated life-threatening risks and morbidity make it a poor choice when considering assessment of liver fibrosis progression or regression. Furthermore, there is the issue of sampling error, defined as variable levels of fibrosis throughout the liver, with biopsy only examining a small (1/50,000 portion of the liver (Dienstag, 2002; Bedossa et al., 2003). Liver biopsy has been shown to have significant inter and intra observer variability among pathologists, with an average 20% error rate in the staging of fibrosis. The minimum suitable length of liver tissue needed for assessing liver fibrosis reliably is 25 mm (Regev et al., 2002).

Biochemical markers of liver fibrosis should be the ideal diagnostic tool to assess the grade of fibrosis. They provide accurate and reliable results in a simple, fast and cost-effective manner. Indeed, much effort has been dedicated in the past years to investigate non-invasive markers which discriminate between low fibrotic stages such as Metavir <F2 and higher fibrotic stages F2–F4, in order to identify patients who are at the risk of clinically relevant fibrosis progression. A panel of routine laboratory markers, which are widely used in clinical practice and novel direct and indirect biochemical markers of hepatic fibrosis have been evaluated. However, none of the tests met the expectations and their superiority to standard clinical evaluation is still questionable (Ulrike et al., 2009).

The development of direct markers of hepatic fibrosis is closely linked to pathophysiological understanding of fibrogenesis. Fibrosis is a dynamic process with increased deposition or removal of extracellular matrix (ECM); both of which may yield higher levels of circulating ECM components or their fragments in the peripheral blood. Direct markers can be classified according to their molecular structure as follows: the glycoproteins hyaluronic acid (HA), laminin and human cartilage glycoprotein (YKL-40), the collagen family (including procollagens I and III, type IV collagens and type IV collagen 7S domain) (Gabrielli et al., 1997; Mohamadnejad et al., 2006). The indirect non-invasive markers comprise routinely available test (Platelet count, ALT/AST ration, INR ratio, γGT, Age, Cholesterol) (Bonacini et al., 1997; Forms et al., 2002; Koda et al., 2007; Wai et al., 2003). More complex and expensive indirect non-invasive tests are (NIT) (Fibro test and α2-macroglobulin and haptoglobin and Gamma globulin and Apo lipoprotein) (Lok et al., 2005; Imbert-Bismut et al., 2001; Naveau et al., 1994). Direct non-invasive markers have been used singly or in combination with other indirect NIT increase the accuracy (Suzuki et al., 2005; Patel et al., 2004).

Laminin (LA) is a major basement membrane–associated non-collagenous glycoprotein of ECM and is deposited in the space of disse during capillarization (Friedman, 1993). It is comprised as a large complex of three long polypeptide chain arranged in the shape of a cross and held together by disulfide bonds (Gabrielli et al., 1997). LA is easily detectable in serum and has been related to liver fibrosis and its subsequent development to portal hypertension (He et al., 2002). The aim of this study is to evaluate a new SCORE for the diagnosis and differentiation between liver fibrosis and liver cirrhosis in order to avoid the hazier of liver biopsy in these patients.

PATIENTS AND METHODS

The study was conducted on 92 subjects with age range of 40 ± 8.5 years. 50 females (54%) and 42 males (46%) were subjected to investigation and the samples were collected in the period from January 2008 to January 2009 from Tropical department in Mansoura University. Subjects were divided into three groups:

- First group included 20 healthy individuals who were matched for age and sex and served as control subjects.
- Second group included 22 patients from Medical Department in University of Mansoura Hospital had chronic HCV and liver biopsy showing evidence of liver non fibrosis scoring (0).
- Third group included 50 patients from Medical Department in University of Mansoura Hospital who had chronic HCV and liver biopsy showing evidence of liver fibrosis scoring from 1 to 3. Complete medical
history was taken from all patients the inclusion criteria were as follow: seropositivity for antibody to HCV (EIA 2; Abbott Laboratories), positivity for HCV RNA by PCR using Cobas Amplicor HCV assay version 1 (Roche) and liver biopsy samples showing evidence of chronic hepatitis or fibrosis. The duration of disease was defined as the interval between the probable times of acquisition of HCV infection. Patients enrolled in the study had no serological markers for hepatitis A, B, D, cytomegalovirus infection or other hepatic or intestinal parasites (Bilharzias); Jaundice; Ascitis and Oedema of lower limb. None of the patients or controls drank alcohol or were smokers.

Histological assessment
Liver biopsy fragments were fixed in 10% neutralized formaldehyde, embedded in paraffin and then stained with hematoxylin and eosin, while reticulation fibrosis stain and the Sirius red method were used especially for staining fibrous tissue components. Liver biopsy samples were read in a blind fashion using the METAVIR scoring system which defines fibrosis scoring from 0 to 4 (Desmet et al., 1994) as follow : 0=no fibrosis; 1=mild fibrosis (portal fibrosis without fibrous septum formation); 2=moderate fibrosis (fibrous septa extending into lobules; but not reaching terminal hepatic (venules and other portal tracts); 3=sever fibrosis (fibrous septa extending to adjacent portal tracts and terminal hepatic venules); and 4=cirrhosis.

Sampling
10 ml of fasting blood samples were drawn from each subject. For detecting INR, 1.8 ml blood in tubes containing 0.2 sodium citrate to separate the plasma after centrifuging for 10 min and for detecting Platelet count 3 ml blood in tube containing EDTA. The other 5 ml blood was left to clot at room temperature to separate sera after centrifuging for measuring other parameters. The plasma and sera were stored at −70°C till the time of analysis.

All participants were subject to the following:
1. Determination of serum alanine transaminase (ALT) and serum gamma glutamyle transferase (γGT) by using the method recommended by the Committee on Enzymes of the Scandinavian Society for Clinical Chemistry and Clinical Physiology. The test was performed using commercially available kit from Boehringer-Mannhiem Company, Germany (1974).

2. Prothrombin time and concentration (INR) by using Coaglumeter instrument from Boehringer-Mannhiem Company, Germany.

3. Determination of serum laminin by using EIA kit enzyme immunoassay for quantitative determination of human laminin in serum, by Takara Bio Inc. (Japan). The laminin EIA kit is a solid phase EIA based on sandwich method that utilizes two mouse monoclonal anti-LN antibodies to detect LN (Burgeson et al., 1994).

Statistical Methods
Results were expressed as mean ± SD and were analyzed by using student’s T-test and ANOVA test, as appropriate. Correlation between different parameters was performed using Pearson’s correlation test. P≤0.05 was considered to be significant. All statistical procedures were performed using SPSS software ver. 15 for windows.

RESULTS
This study was conducted on 72 patients who were divided into two groups according to the presence of fibrosis, besides 20 subjects served as control group. The mean age was 40±9.1 years and 33 of the subjects were males and 59 were females. Table 1 [Supplementary data] and Fig. 1- Fig. 3 show significant increase of all studied parameters in group 3 and group 2 when compared with group 1 (control group). Table 2 shows the comparison between different parameters and different groups which shows highly significant increased in laminin and INR when compare between control and group 3 or group 2, and between group 3 with group 2. While ALT, GGT and platelet count show significant increase when we compare control with group 2 and group 3, but they showed non significant increase when compared between group 2 and group 3.

By running stepwise discriminate analysis; only two variables were found to be significant in discriminating fibrosis from non-fibrosis. These variables were laminin and INR. The standardized canonical discriminate function coefficients were: 0.867 for laminin (more discriminatory) and 0.685 for INR (less discriminatory). A discriminant Score (DS) can be calculated for any particular patient by substituting in the following equation.

\[
DS = 0.03 \text{Laminin} + 4.2 \text{INR} – 7.0
\]

If DS is equal or more than zero, the patient has fibrosis, and if less than zero, the patient dose not have fibrosis. This equation correctly diagnosed 45 of the 50 patients with fibrosis (90% sensitivity) and 17 of the 22 non-fibrosis patients (77.3% specificity). The overall accuracy of this equation is (86.1%). ROC curves were plotted for the five studied parameters (Fig. 4). Laminin
Clinical significance of serum laminin and INR ratio showed the largest area under ROC; AUROC=0.883 (SE=0.048) followed by INR (AUROC=0.724, SE=0.061) as shown in Table 3, AUROC of GGT was (0.658, SE=0.063), slightly different from 0.5 (0.5 is the AUROC of a non-discriminatory variable). The other 2 parameters did not differ significantly from 0.5 (p=0.375 for ALT and 0.246 for platelet count). By using 48 U/L as a cut off for laminin, sensitivity for diagnosing fibrosis was found to be 88.0%, specificity=63.6% and accuracy=80.6%.

**Figure 1:** Mean ± SD for ALT and GGT in different group.

**Figure 2:** Mean ± SD for laminin in different group.

**Figure 3:** Mean ± SD for INR in different groups.

By applying this DS on the patients in the groups 2 and 3 and comparing the results with the stage of fibrosis by liver biopsy a positive correlation between DS and the stage of fibrosis by liver biopsy is seen.

**Figure 4:** ROC Curve for all studied parameters.

**DISCUSSION**

In patients with liver disease, the assessment of fibrosis is currently considered of paramount relevance for prognosis implementation of follow-up, and need for therapy (Perillo, 1997; Jouet et al., 1994). Chronic hepatitis is accompanied with progressive deposit of hepatic fibrosis, which may lead to cirrhosis (Geroge, 2000). HCC is a major cause of mortality in patients with chronic viral hepatitis. In patients with chronic hepatitis C, the incidence of HCC increases within 20 years in approximately 20–30% of patients according to the stage of liver fibrosis (Yi et al., 2004). Non-invasive evaluation of liver fibrosis is thus of great clinical interest. Many parameters of non-invasive diagnosis of liver fibrosis are studied extensively in the previous communications. Myers et al. (2003) evaluated an index for fibrosis: fibrotest, which included an index total bilirubin, γGT, α2-macroglobulin, apolipoprotein A1 and haptoglobin. Recently, Mohamadnejad et al. (2006) found that liver fibrosis originated from HBV and was best predicted using HBV DNA level, ALP, ALB and PLT. However, some of the predictive markers in these models are not readily available in most clinical practices especially the molecular markers (Liu et al., 2007). Pissaia et al. (2009) showed that scores developed for non-transplanted hepatitis C patients were able to detect significant fibrosis after orthotrophic liver transplantation independent of the patients HCV status. Non-invasive markers of liver fibrosis have been widely evaluated in non-transplanted patients, mainly those who are HCV-Positive.

Serum fibrosis indices are fairly well correlated with the inflammation grade of the liver, fibrosis staging and the degree of chronic hepatitis. However, as diagnostic markers, they should be considered in combination with clinical manifestations liver function test and, ultrasonography (Gressner et al., 1986; Lebensztejn,
The diagnostic accuracy of serum LA as an index of liver fibrosis was studied by several investigators in patients with chronic viral hepatitis C (Gabrielli et al., 1997; Cai et al., 2003; El-Masry et al., 2000). To the best of our knowledge this study is the first report that addressed the pattern of laminin in viral hepatitis C patients in a high prevalence setting of these agents in Egypt. Several immunodiagnostic assays based on anti-LA monoclonal antibody for detection of serum LA in liver fibrotic patients have been described (Liang et al., 2002). These assays can not be easily applied in screening programs where they need a long time for completion. Moreover, special and highly expensive equipment is used. Therefore, there is a strong demand for a rapid, simple and reliable test for detection of LA. The results showed that laminin and INR had decrement scores than other parameters and the AUR were 0.833 (SE=0.048), 0.742 (SE=0.061) respectively while GGT was 0.658 (SE=0.063). ALT and platelet count did not differ significantly from 0.5 so that we used laminin and INR in calculation of the Discriminate Scores (DS=0.03 laminin + 4.2 INR–7.0). The sensitivity of this equation was 90% with 77% specificity and the overall accuracy of this equation was 86.1% in the diagnosis of fibrosis in patients who suffering from hepatitis C.

Applying the DS on our cases and comparing the results with the result of liver biopsy, the data showed a positive correlation between them so any increase in the DS value showed an increase in the degree of fibrosis obtained by liver biopsy. In conclusion, DS could be considered as an easy method for the diagnosis of fibrosis in the patients suffering from hepatitis C. However, this study is considered as a preliminary one and it is recommended that investigations on large scale are carried out.

References


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