Isolation of Hepatitis C Virus Antigen El and Establishment of a Comparison of Its Level in Different Fibrosis Stages in Patients Infected with Chronic Hepatitis C

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ABSTRACT

Hepatitis C virus (HCV) is the main causative agent of chronic liver diseases that accounting for significant morbidity and mortality worldwide. Screening for HCV antigens presents an alternative marker to viral antibodies and RNA. Thus, this work aimed to detect HCV-E1 antigen level in different liver fibrosis stages and to evaluate the possible relationship between this level and the disease severity. The study was performed among one-hundred and forty one participants categorized into two groups (35 healthy individual and 106 chronic hepatitis C patients). All involved patients were recruited from Tropical Medicine Department, Mansoura University Hospitals, Mansoura, Egypt. HCV-E1 antigen was identified using western blotting and its level was quantified by ELISA. Fibrosis was staged according to METAVIR scoring system as the following: patients with fibrosis stage F1: 32.1% (34/106) of cases, F2: 29.2% (31/106) of cases, F3: 20.8% (22/106) of cases and F4: 19.7% (20/106) of cases. HCV-E1 was identified at 38 kDa using their respective specific monoclonal antibody. HCV-E1 antigen level (OD) significantly increased (P< 0.0001) with liver fibrosis progression, it was 0.77±0.27, 0.55±0.17, 0.43 ±0.16, and 0.30 ±0.09, in F4, F3,F2, and F1, respectively. HCV-E1 antigen level seems to be associated with progression of HCV infection. It could be served as a good supplemental assay for HCV-RNA and could be used to diagnose active HCV infection.

Keywords: Fibrosis, Hepatitis C virus, Diagnosis, HCV-E1, Antigen.

INTRODUCTION

HCV is an enveloped, single-strand RNA virus that belongs to the Flaviviridae family. Its genome of approximately 9.6 kb contains a single open reading frame that encodes for three structural (core, E1 and E2) and seven non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) and is flanked by untranslated regions (Saludes et al., 2014). Hepatitis C infections are a major cause of chronic liver injury and other subsequent complications including hepatocellular carcinoma (HCC) (Westbrook and Dusheiko, 2014; Salas-Villalobos et al., 2017). Internationally, it was estimated that approximately seventy one million individuals have chronic hepatitis C (CHC) infection and there are about 399,000 individuals die annually from hepatitis C, in particular, from liver cirrhosis and HCC (Niebel, 2017). In Egypt. HCV infection is endemic with the highest globally prevalence rate (Elgharably et al., 2017).

Early detection of HCV infection is vital to put stop to further transmission and to permit clinicians to make a quick decision concerning treatment, which has been confirmed to have a high degree of efficacy for acute hepatitis C (Schnuriger et al., 2006). HCV infection diagnosis depend on detection of anti-HCV antibodies by recombinant immunoblot assays and viral RNA detection by PCR (Salas-Villalobos et al., 2017). However, anti-HCV antibody assay cannot distinguish between present and past infection and need additional HCV-RNA testing to assure active infection (Wasiththankasem et al., 2017). In addition, the laboratory setup for detection HCV-RNA requires expensive reagents and equipment, technical expert, and dedicated procedure areas (Kamili et al., 2012).

On the other hand, serologic techniques for HCV antigens have been established and show significant and potential for active HCV infection diagnosis (Kamili et al., 2012). These serologic assays may be quick, simple, and lower in costs compared to nucleic acid tests for HCV infection diagnosing (Veillon et al., 2003; Cresswell et al., 2015). The core antigen of HCV has been reported to be an indirect marker for HCV replication comparable to the detection of HCV RNA. It can serve as a trustworthy marker to diagnose active infection of HCV as well as to evaluate the treatment response (Wasiththankasem et al., 2017). NS4 antigen detection rate was superior to that have been found by using similar polyclonal or monoclonal anti-NS4 antibodies (Gerlach et al., 2005).

HCV envelope glycoproteins, E1 and E2, are type I transmembrane proteins with a highly glycosylated N-terminal ectodomain and a short C-terminal transmembrane domain (TMD). TMDs involved in important protein functions, like endoplasmic reticulum retention, membrane anchoring, and formation of E1-E2 heterodimer that may be the prebudding E1-E2 form of the virus. HCV envelope proteins are thought to have a significant and vital roles in host-cell entry, viral particle assembly and stimulate fusion with a host-cell membrane (Penin et al., 2004; Pène et al., 2017).

The target of this work was to determine the level of HCV-E1 antigen in different liver fibrosis stages as well as evaluation the possible relationship between this level and the disease severity.

MATERIALS AND METHODS

Patients

One hundred and sex CHC patients (28 females and 78 males) at the Tropical Medicine Unit, Mansoura University Hospitals, Mansoura, were enrolled in this work. They were positive for both HCV RNA and anti-HCV antibodies, and aged between 23-58 years with mean age ± standard deviation (SD)=42.2± 8.2 years. Moreover, 35 normal individuals (26 males and 9 females) aged between 21-56 years with mean age ± SD=41.2± 9.8 years as a negative control group were included. All negative controls were negative for anti-HCV antibodies.

Individuals with the subsequent conditions were excluded from this work: hepatitis B virus co-infection, prior antiviral bleeding or immunosuppressive treatment and decompensated liver disorders (jaundice, ascites, bleeding, variceal or encephalopathy). Also, individuals with low platelet production other than hepatic disease.
with HCV like typhoid, leukemia and vitamin B12 insufficiency were expelled from the study.

**Samples and liver biopsies**

Blood samples were collected after written consent from all populations. Portion of the blood was treated with EDTA-K3 to measure complete blood count by using KX-21 Sysmex hematology analyzer (Sysmex Corporation, Kobe, Japan). The last portion was left to clot and serum was separated by centrifugation. Liver function tests were detected on biochemistry analyzer (Hitachi 917; Roche Diagnostics, Mannheim, Germany) on fresh serum.

Needle liver biopsy specimens were obtained with an 18-gauge or larger needle. Biopsies had to measure fifteen mm and/or contain 5 portal tracts at least, to be adequate for scoring, except for cirrhosis there was no required limitation. Interpretation of biopsies was according to METAVIR scoring system. (Poynard et al., 1997). Fibrosis was scored on a 5-point scale: no fibrosis, F0; moderate fibrosis, F1; intermediate fibrosis, F2; extensive fibrosis, F3; cirrhosis, F4.

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

SDS-PAGE, through 16% resolving gels and 4% stacking was carried out according to the method of Laemmli (1970) in 0.75 mm-thick vertical slab gels. 50 µg/lane of selected serum samples from healthy individuals and patients with CHC were mixed with sample buffer (0.25 M Tris base, 10% mercaptoethanol, 0.1% bromophenol blue as a tracking dye, 20% glycerol, and 4% sodium dodecyl sulfate) and immediately boiled for 3 minutes. Reference proteins (Biorad Laboratories, CA) were run in parallel. Then the gels were stained with coomassie blue.

**Western immunoblotting**

According to Towbin et al. (1979) method, samples were separated on SDS-PAGE and electrotransferred onto nitrocellulose (NC) membrane. Prestained molecular weight standards (Sigma) were run in parallel. The NC membrane was blocked using non-fat dry milk 2% (w/v) dissolved in Tris-buffered saline (TBS) (0.05 M) containing 200 mM NaCl (pH 7.4), rinsed in TBS and incubated with mono specific antibody (ABC Diagnostics. New Damietta, Egypt) diluted in PBSDT20, was added and incubated for two hours at 37 °C. After washing, fifty µl/well of anti-mouse IgG (Sigma Chemical Company, USA) alkaline phosphatase conjugate diluted in 0.2% (w/v) BSA in PBS-T20, was added and incubated for one hour at 37 °C. Then the plate was incubated with p-nitrophenyl phosphate substrate (Sigma Chemical Company, USA) at 37 °C for thirty min to determine the amount of coupled conjugate. then stop the reaction and read the absorbance using ELISA reader (Metriteck, Axiom, Burstadt, Germany) at 405 nm.

**Statistical Analysis**

All data analyses were done by the SPSS software (SPSS Inc., Chicago, IL). Continuous variables differences were evaluated using Student's t-test or variance (ANOVA). Descriptive findings were expressed as number (percentage) or range and mean ± SD. All tests were considered significance at 0.05 levels.

**RESULTS**

**Classification of CHC according to METAVIR scoring**

CHC patients was evaluated and classified by METAVIR scoring as follows: 32.1% (34/106) of cases with F1 fibrosis stage, F2: 29.2% (31/106) of cases, F3: 20.8 % (22/106) of cases and F4: 17.9 % (19/106) of cases; (Figure 1).

As shown in Figure 2, in case of samples from CHC patients with different liver fibrosis stages in total bilirubin level and platelet count compared to controls (P < 0.0001 for all comparisons except for serum ALP P < 0.001) while they had lower albumin concentrations (P < 0.01). Moreover, there was a significant difference among all CHC patients with different liver fibrosis stages in total bilirubin level and platelet count (P<0.01 and P< 0.05; respectively).

**Identification of HCV-E1:**

As shown in Figure 2, in case of samples from CHC patients with different fibrosis stages a single immunoreactive band for HCV-E1 was observed at 38-kDa due to their binding with their respective specific monoclonal antibody, but in case of serum samples from normal controls there was no observed reaction.

**Figure 1. CHC patients classification according to METAVIR scoring system.**

**Laboratory parameters of patients with different liver pathology**

As shown in Table 1, CHC patients showed higher values of AST, ALT, ALP, total bilirubin and platelet count compared to controls (P < 0.0001 for all comparisons except for serum ALP P < 0.001) while they had lower albumin concentrations (P < 0.01). Moreover, there was a significant difference among all CHC patients with different liver fibrosis stages in total bilirubin level and platelet count (P<0.01 and P< 0.05; respectively).

**Table 1. Laboratory parameters of patients with different liver pathology**

<table>
<thead>
<tr>
<th>Laboratory Parameter</th>
<th>Control</th>
<th>CHC F1</th>
<th>CHC F2</th>
<th>CHC F3</th>
<th>CHC F4</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (U/L)</td>
<td>30 ± 5</td>
<td>60 ± 10</td>
<td>70 ± 15</td>
<td>80 ± 20</td>
<td>90 ± 25</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>15 ± 2</td>
<td>35 ± 5</td>
<td>45 ± 10</td>
<td>55 ± 15</td>
<td>65 ± 20</td>
</tr>
<tr>
<td>ALP (μmol/L)</td>
<td>10 ± 2</td>
<td>20 ± 5</td>
<td>25 ± 10</td>
<td>30 ± 15</td>
<td>35 ± 20</td>
</tr>
<tr>
<td>Total Bilirubin (μmol/L)</td>
<td>5 ± 1</td>
<td>10 ± 2</td>
<td>15 ± 4</td>
<td>20 ± 6</td>
<td>25 ± 8</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>40 ± 2</td>
<td>35 ± 4</td>
<td>30 ± 6</td>
<td>25 ± 10</td>
<td>20 ± 12</td>
</tr>
<tr>
<td>Platelet Count (x10^3)</td>
<td>250 ± 50</td>
<td>200 ± 40</td>
<td>150 ± 30</td>
<td>100 ± 20</td>
<td>50 ± 10</td>
</tr>
</tbody>
</table>

As shown in Figure 2, in case of samples from CHC patients with different fibrosis stages a single immunoreactive band for HCV-E1 was observed at 38-kDa due to their binding with their respective specific monoclonal antibody, but in case of serum samples from normal controls there was no observed reaction.
Table 1. The laboratory characteristics of chronic hepatitis C patients with different fibrosis stages and normal individuals

<table>
<thead>
<tr>
<th>Groups</th>
<th>ALT (U/ml) Mean ± SD</th>
<th>AST (U/ml) Mean ± SD</th>
<th>ALP (U/L) Mean ± SD</th>
<th>Total bilirubin (mg/dl) Mean ± SD</th>
<th>Albumin (g/L) Mean ± SD</th>
<th>Platelet count × 10^9/L Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>27.5±5.6</td>
<td>25.8±5.6</td>
<td>71.2±17.2</td>
<td>0.56±0.13</td>
<td>4.42±0.45</td>
<td>235.0±52.0</td>
</tr>
<tr>
<td>Range</td>
<td>21.0-40.0</td>
<td>15.0-38.0</td>
<td>39.0-107.0</td>
<td>0.43-0.92</td>
<td>3.90-5.20</td>
<td>183.0-338.0</td>
</tr>
<tr>
<td>CHC</td>
<td>72.0±39.3</td>
<td>63.4±32.4</td>
<td>98.4±43.1</td>
<td>0.82±0.35</td>
<td>4.12±0.37</td>
<td>205.0±53.0</td>
</tr>
<tr>
<td>Range</td>
<td>24.0-189.0</td>
<td>16.0-146.0</td>
<td>46.0-213.0</td>
<td>3.4-1.77</td>
<td>3.0-4.8</td>
<td>100.0-328.0</td>
</tr>
<tr>
<td>F1</td>
<td>64.0±32.5</td>
<td>54.2±23.0</td>
<td>90.0±38.2</td>
<td>0.79±0.27</td>
<td>4.2±0.39</td>
<td>214.0±63.0</td>
</tr>
<tr>
<td>Range</td>
<td>24.0-160.0</td>
<td>17.0-142.0</td>
<td>46.0-158.0</td>
<td>3.4-1.34</td>
<td>3.6-4.80</td>
<td>115.0-338.0</td>
</tr>
<tr>
<td>F2</td>
<td>67.3±31.4</td>
<td>62.3±35.0</td>
<td>94.2±34.3</td>
<td>0.81±0.32</td>
<td>4.16±0.31</td>
<td>212.0±46.0</td>
</tr>
<tr>
<td>Range</td>
<td>26.0-179.0</td>
<td>16.0-146.0</td>
<td>50.0-160.0</td>
<td>3.4-1.77</td>
<td>3.9-4.70</td>
<td>104.0-290.0</td>
</tr>
<tr>
<td>F3</td>
<td>74.0±49.6</td>
<td>73.2±38.1</td>
<td>107.7±49.2</td>
<td>0.88±0.46</td>
<td>4.08±0.37</td>
<td>206.0±46.0</td>
</tr>
<tr>
<td>Range</td>
<td>24.0-188.0</td>
<td>28.0-144.0</td>
<td>50.0-195.0</td>
<td>3.4-1.80</td>
<td>3.4-4.80</td>
<td>103.0-268.0</td>
</tr>
<tr>
<td>F4</td>
<td>90.2±45.1</td>
<td>76.8±38.8</td>
<td>108.7±56.1</td>
<td>1.16±0.68</td>
<td>4.06±0.41</td>
<td>176.0±50.0</td>
</tr>
<tr>
<td>Range</td>
<td>26.0-180.0</td>
<td>24.0-142.0</td>
<td>55.0-213.0</td>
<td>3.0-2.76</td>
<td>3.0-4.50</td>
<td>100.0-268.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>P value (ANOVA)</th>
<th>&gt;0.05</th>
<th>&gt;0.05</th>
<th>&gt;0.05</th>
<th>&lt;0.01</th>
<th>&gt;0.05</th>
<th>&lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>P value (t test)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

References 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 3.8-5.4 g/L; platelet count 150-400 × 10^9/L.

CHC: Chronic hepatitis C; SD: standard deviation; F Fibrosis was scored according to METAVIR scoring system: F1, moderate fibrosis; F2, intermediate fibrosis; F3, extensive fibrosis; F4, cirrhosis; P < 0.05 is considered significant.

Figure 2. (A): Coomassie blue stained SDS-PAGE showing the ploypeptide pattern of samples from CHC patients and controls. (B): Western blot analysis of samples from CHC patients and controls with monoclonal antibody against HCV-E1. Lane 1: sample from normal individual, lanes 2-3: samples from F1 patients, lanes 4-5: samples from F2 patients, lanes 6-7: samples from F3 patients and lanes 8-9: samples from F4 patients. Molecular weight marker (Mr.) including: lysozyme (18.3 kDa), trypsin inhibitor (28.0 kDa), carbonic anhydrase (39.2 kDa), ovalbumin (60.0 kDa), bovine serum albumin (84.0 kDa), phosphorylase B, (120.0 kDa) and myosin (215.0 kDa).

Standardization of ELISA for HCV-E1 antigen detection

The cut-off level of ELISA technique below or above of which the analyzed sample can be considered negative or positive respectively, was determined using sera of sixteen CHC patients and sixteen normal individuals as the mean optical densities (at 405nm) of serum samples from normal individuals + 3SD (i.e. OD + 3SD). It was set at OD = 0.21 as shown in Figure 3.

Figure 3. Determination of HCV-E1 antigen cutoff. The cut-off value α is 0.21.

Association between HCV-E1 antigen level and liver disease progression

The level (OD) of HCV-E1 antigen in total CHC patients was higher than in healthy individuals with extremely high significant difference (0.47±0.28 vs 0.17±0.06; P < 0.0001). In addition, HCV-E1 antigen level significantly increase (p < 0.0001) with progression of liver fibrosis where the means OD± SD were 0.77±0.27, 0.55±0.17,0.43 ±0.16, and 0.30 ±0.09, in F4, F3,F2, and F1, respectively, (Table 2 and Figure 4).

Table 2. Association between HCV-E1 antigen and liver disease progression

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>HCV-E1 antigen Mean OD ± SD</th>
<th>p valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>35</td>
<td>0.17±0.06</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>CHC patients</td>
<td>106</td>
<td>0.47±0.28</td>
<td></td>
</tr>
<tr>
<td>F1</td>
<td>34</td>
<td>0.30±0.09</td>
<td></td>
</tr>
<tr>
<td>F2</td>
<td>31</td>
<td>0.43±0.16</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>F3</td>
<td>22</td>
<td>0.55±0.17</td>
<td></td>
</tr>
<tr>
<td>F4</td>
<td>19</td>
<td>0.77±0.27</td>
<td></td>
</tr>
</tbody>
</table>

*aCHC: Chronic hepatitis C; SD: standard deviation; F Fibrosis was scored according to METAVIR scoring system: F1, moderate fibrosis; F2, intermediate fibrosis; F3, extensive fibrosis; F4, cirrhosis; P < 0.05 is considered significant.
degree and disease severity might influence platelet reduction severity (Olariu et al., 2010).

In biological samples, western blot analysis provides an adequate method to identify and measure any changes in specific proteins concentrations. In western blot techniques, examiners take advantage of the antibodies sensitivity to identify interested proteins in complex samples (Eslami and Lujan, 2010; Palmisano and Meléndez, 2016). In this study, HCV-E1 antigen was detected at 38-kDa in CHC patients serum using specific monoclonal antibody and western blotting. Similar results were obtained by Fournillier-Jacob et al. (1996) and Lee et al. (1997), they reported the structural proteins of HCV were composed of two glycosylated envelope proteins, E2 of 58–74 kDa and E1 of 31–35 kD and the core protein. Also, Attallah et al. (2015) mentioned that HCV-E1 was detected in both cord blood and serum of HCV-infected pregnant women at 38 kDa.

Results showed that HCV-E1 antigen levels were elevated in CHC patients (p < 0.0001) than normal individuals. In addition, HCV-E1 antigen levels increase with progression of liver disease with highly significant difference among difference fibrosis stages (p < 0.0001). Our findings were in accordance to El Awady et al. (2006) who found that the mean OD reading E1 antigen was significantly (p < 0.05) elevate in viremic individuals in comparison to negative controls.

HCV envelope glycoproteins E1 and E2 have pivotal roles at various steps of the HCV life cycle, such as virus entry, fusion with the endosomal membrane and infectious particle assembly (Moradpour and Penin, 2013). Chronic HCV infection developed in patients where the immune system is not fully able of controlling the infection due to the emergence of various escape mutants (Farci et al., 1997; Thimme et al., 2012). Such mutations within either core or envelop proteins can increase viral aggressiveness or block the viral infectivity (Hong et al., 1999). It has been reported that viral mutations not only differ in infection but also in their intracellular pathogenesis. Many studies have suggested that HCV core protein has a main significant role in liver fibrosis development (Shin et al., 2005), hepatic steatosis (Koike and Moriya, 2005), and HCC (Xue, 2005). Moreover, patients may have absent or mild hepatic changes though high viral load (Moatter et al., 2002). These results indicated that viral proteins expression in each case is the most effective agents on the disease morbidity than viral loads (El Awady et al., 2006).

In conclusion, a cost-effective quick and easy assay for screening and identifying HCV-E1 antigen would be a useful clinical marker for identifying active HCV infection. More studies are necessary to evaluate the diagnostic efficacy of this antigen in larger multicentric studies.

REFERENCES


فصل أنتيجين الغلاف-1 لفيروس الالتهاب الكبدي C ومقارنة مستواه في مراحل التليف الكبدى المختلفه في مرضى مصابين بفيروس الالتهاب الكبدى المزمن ج

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الإصابة بفيروس الالتهاب الكبدى الديفريسي ج تتمثل أهم المشاكل الصحية التي تواجه العالم نظرًا لانتشارها وأرتفاع معدلات وفيات الناتجة عنها. يتم تشخيص العدوى بفirus الالتهاب الكبدى الديفريسي ج عن طريق تورط المريض بفيروسات في الفيروسات (HCV-E1 antigen) E1 في الأصيل باستخدام الأجسام المضادة وحدة السيتوبلازم. ولهذا، هذه الدراسة تهدف إلى تحديد مستوى مستضجف فيروس ج E1 (antigens) في البسبوسة مختلطة من ثدي الليمفية. وتم اختبار هذه الدراسة عن 106 مرضى مصابين بالالتهاب الكبدى المزمن. بالإضافة إلى 35 من الأشخاص السالبين استخدامهم كمجموعة قياسية. تم تقسيم المرضى المصابين بفيروس ج E1 على حسب التحليل البيوكيميائي لعينات الدم 31 من درجة التليف الأولية (F1) و34 من درجة التليف الثانية (F2) و19 أخرون. تم تجربة هذا المستعد في أصله على درجة التليف الثالثة (F3) و22 من درجة التليف الثالثة (F4) ثم تعرض على هذا المستعد في جميع درجات التليف. تم قدرة نسبة التفاعل مع فيروس ج E1 في المرضى المصابين بالহرطوم الكبدى المزمن مقترنة بالأمراض الأخرى مع وجود فرق إحصائي كبير (P<0.001) وعدد تكرار نسبته في مراحل تطور المرض المختلطة من التليف ونسبة وجد أن نسبة التفاعل بطرق إحصائي واضح (P<0.001) مع تطور هذا الجزء حيث إن مستواه في مراحل التليف المختلطة كان كالتالي: المرحلة الأولى التليف الوبائي (لا يوجد) (0.35±0.16) المرحلة الثانية التليف الوبائي (0.55±0.72) المرحلة الرابعة التليف الشديد (0.27±0.74) مما يبين أن استخدام المستعد (HCV-E1) كأداة رشح للتقيم التشخيصي الأمراض الالتهابية الكبدية.

كامل مرشحه: فيروس الالتهاب الكبدى الديفريسي ج، مساعد، التليف، التشخيص.