Evaluation of Hepatitis C Viral Load in Association with Serum TGF-
β1 and Retinol Binding Protein and Their Role in Hepatitis C Virus
Infection and hepatocellular Carcinoma

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Background and Objectives: Hepatitis C virus [HCV] infection is one of the most common infectious diseases leading to high morbidity and mortality due to the development of liver fibrosis/cirrhosis and hepatocellular carcinoma [HCC]. Transforming growth factor-beta 1 [TGF-β1] is the most relevant growth factor that plays a role in induction of hepatic fibrogenesis through stellate cell activation, with a decrease in vitamin A storage in these cells. However, the impact of TGF-β1 and vitamin A depletion on initiation of liver neoplasia is controversial. In this study, we intended to evaluate hepatitis C viremia in association with serum levels of TGF-β1 and retinol binding protein [RBP], which has been considered to be a good and sensitive index of vitamin A depletion, to assess if there is a potential link between serum titer of HCV and those markers and their importance as risk factors for the development of HCC. Methods: Serum levels of TGF-β1 and RBP were assayed using commercial ELISA and radial immunodiffusion kits in 30 patients (15 with HCV infection and 15 with HCV infection associated with HCC) along with 10 healthy controls. Quantification of circulating HCV RNA by Real-time polymerase chain reaction was done using TaqMan probe technology. Alpha fetoprotein [AFP] levels and serum aminotransferases' activities were also measured. Results: Serum levels of TGF-β1 were considerably higher in HCC and HCV groups compared to healthy controls (23.88±12.73 and 13.63±7.58 vs. 8.08±2.51, p<0.001, p<0.05 respectively). Furthermore, patients having HCV infection associated with HCC showed significantly higher values of TGF-β1 than HCV group (23.88±12.73 vs. 13.63±7.58, p<0.01). Serum RBP levels were 55.32±16.87, 51.30±20.10 and 35.11±16.21 in the controls, HCV and HCC patients respectively. There was a significant decrease in serum RBP levels in HCC group compared to HCV and control groups (p<0.05, p<0.01 respectively). A positive correlation was found between hepatitis C viremia and serum TGF-β1 levels in all patients (p<0.05). A negative correlation was observed between TGF-β1 and RBP, in particular, more evident in HCC group also between RBP and AFP but did not reach statistical significance. Neither Serum TGF-β1 nor RBP levels were correlated with aminotransferases' activities in HCV and HCC patients. Conclusions: The progressive increase in serum TGF-β1 associated with progressive reduction in serum RBP levels from controls to HCC patients suggest their tumor-promoting effect through facilitating TGF-β1-mediated liver fibrogenesis with progressive loss of vitamin A storage giving the chance for the tumor to grow. Correlation of hepatitis C viremia with serum TGF-β1 levels signifies that HCV proteins could induce expression of TGF-β1 and may contribute to liver carcinogenesis. Therefore, increased serum TGF-β1 associated with reduced RBP levels could be considered as risk factors for the evolution of HCC in HCV-infected patients.

INTRODUCTION

Hepatitis C virus (HCV) is a major human pathogen that infects more than 100 million people worldwide. Epidemiological studies have revealed that more than 80% of acutely HCV-infected patients fail to eradicate the virus and they subsequently develop chronic hepatitis. A hallmark of HCV is its ability to establish persistent infections reflecting the evasion from the immunological defense mechanisms of the host. However, once HCV persistency develops, HCV utilizes multifaceted arms to subvert various immune effectors. Hepatic fibrosis is the main complication of chronic HCV infection. Fibrosis is characterized by an increase in extracellular matrix constituents that collectively form hepatic scars. However, extensive scarring (Liver cirrhosis) representative for hepatic fibrosis is clinically very important as high risk conditions being a crucial precondition for the development of hepatocellular carcinoma. The hepatic stellate cell is a key component in the pathogenesis of hepatic fibrosis. During hepatic fibrosis, these vitamin A rich stellate cells (contain about 90% of the total vitamin A reserves of the body) undergo transition into myofibroblast-like cells and lose their intracellular droplets of retinyl esters, the storage form of vitamin A. These activated cells proliferate rapidly and are responsible for increased production.
of extracellular matrix proteins. The most relevant growth factor that plays a key role in hepatic stellate cell activation is the transforming growth factor-beta 1 (TGF-β1). (5,6,7)

Retinol-binding protein (RBP) is a circulating carrier protein for serum and cellular retinol. It mediates the paracrine transfer of retinol from hepatocytes to perisinusoidal stellate cells in the liver. (8–10) RBP is synthesized primarily in the liver as a single polypeptide chain with a molecular weight of about 21000 Daltons and has a single binding site for retinol, then released into the blood stream where it plays an important role in transport of vitamin A to various target cells to be stored. Vitamin A regulates the rates of synthesis and secretion of RBP by the liver. Actually, on vitamin A depletion, the RBP secretion is specifically blocked, resulting in reduced serum levels of the later. (10–12) RBP could serve as a useful surrogate marker for serum retinol in estimating the vitamin A status because of the approximate 1:1 (molar) correlation between retinol and RBP in the serum. Moreover, RBP as a serum protein, it may be more stable than retinol, and the conditions under which specimens are collected, processed, and transported for analysis may not need to be stringent. (13,14)

In HCV infected patients, fibrogenesis is initially beneficial but becomes pathologic upon viral persistence. (14) Since monitoring fibrosis process by liver biopsy is greatly limited. Therefore, the search for predictive factors which could be easily accessible markers of the ongoing intrahepatic process of fibrogenesis and the associated dismal outcome is essential for the management of these patients. (15) It is well-established that TGF-β1 drives fibrosis. However, its impact on the initiation or progression of neoplasia is controversial. (16) Furthermore, the increasing evidence for a role of vitamin A in controlling proliferation and differentiation of a variety of human cells suggests that RBP may be important in mediation of such anti-tumor effects. (17) Therefore, this study was designed to evaluate hepatitis C viremia in association with serum levels of TGF-β1 and RBP, to assess if there is a potential link between hepatitis C viral load and those markers and their importance as risk factors for the development of HCC.

SUBJECTS AND METHODS

We studied fifteen patients with HCV infection, fifteen with HCC associated with HCV infection along with ten healthy controls. Samples were tested for HBsAg by enzyme linked immunosorbent assay [ELISA] kit [Murex HBsAg Version 3, Abbott-Murex, Murex Biotech Limited. Central Road, Dartford, UK]. All patients with hepatitis B surface antigen seropositivity were excluded from our study. HCV diagnosis was based on anti-HCV seropositivity. Quantification of circulating HCV RNA by Real-time polymerase chain reaction [Mx3000P™ Real-Time PCR System, stratagene] was done using TaqMan probe technology [Roche-Applied biosystems] after HCV RNA extraction using QIAamp® viral RNA mini kit [Qiagen GmbH, Germany], according to manufacturer's instructions. HCV RNA was amplified according to the following program: 1 cycle each of 48ºC for 30 min and 95ºC for 10 min, followed by 40 cycles each of 95ºC for 15 sec and 60 ºC for 1 min. Serum levels of TGF-β1 and RBP were measured by ELISA [Human TGF-β1 ELISA BMS249/2, Bender MedSystems GmbH, Vienna, Austria, Europe] and radial immunodiffusion [Human Retinol binding protein NANORID™. The Binding Site Ltd, Birmingham, B29 6AT, UK], respectively. Both Kits were applied according to manufacturer's instructions. Correlations of the assessed parameters [TGF-β1 and RBP] to alpha fetoprotein (AFP) levels and serum aminotransferases' [ALT and AST] activities were evaluated.

Statistical analysis

All analyses were completed using SPSS program (Version 10). Data were expressed as mean value ± SD. Statistical analysis was performed using Student t-test and correlation coefficient. P values ≤0.05 were considered statistically significant.

RESULTS

Serum levels of TGF-β1 were considerably higher in HCC and HCV groups compared to healthy controls (23.88±12.73 and 13.63±7.58 vs. 8.08±2.51, p<0.001, p<0.05, respectively). Furthermore, patients having HCV infection associated with HCC showed significantly higher values of TGF-β1 than HCV group (23.88±12.73 vs.
13.63±7.58, p<0.01) [Fig.1] [table1].

Regarding serum RBP levels, HCV infected patients associated with HCC had significantly lower RBP serum levels than those HCV infected patients and control groups (35.11±16.21 vs. 51.30±20.10 and 55.32±16.87, p<0.05, p<0.01, respectively), while no significant difference was found between HCV infected group of patients and controls [Fig 2] [Table1].

A significant positive moderate correlation was found between hepatitis C viral load and serum TGF-β1 levels in all patients (r=0.491, p<0.05), it was evident in HCC group but did not reach statistical significance (r=0.513, p>0.05). On the other hand, a negative moderate correlation was observed between TGF-β1 and RBP in all patients, particularly, more evident in HCC group but did not reach statistical significance (r=-0.493, p>0.05) [fig 3]. A week negative correlation between RBP and AFP(r=-0.298), and positive correlation between TGF-β1 and AFP(r= 0.354) were observed in all patients, both of them did not reach statistical significance. A significant positive correlation was observed between hepatitis C viral load and AST activities in all patients (r=0.460, p<0.05). Neither Serum TGF-β1 nor RBP levels were correlated with aminotransferases' activities in HCV and HCC patients.

### Table 1. Serum concentrations of TGF-β1 and retinol binding protein within the investigated groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>N</th>
<th>Mean± SD</th>
<th>P Values</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TGF-β1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>8.08 ± 2.51</td>
<td>0.036*(C*HCV)</td>
</tr>
<tr>
<td>HCV</td>
<td>15</td>
<td>13.63 ± 7.58</td>
<td>0.001*(C*HCC)</td>
</tr>
<tr>
<td>HCC</td>
<td>15</td>
<td>23.88 ± 12.73</td>
<td>0.012*(HCV*HCC)</td>
</tr>
<tr>
<td><strong>RBP</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10</td>
<td>55.32 ± 16.80</td>
<td>NS (C*HCV)</td>
</tr>
<tr>
<td>HCV</td>
<td>15</td>
<td>51.30 ± 20.10</td>
<td>0.006*(C*HCC)</td>
</tr>
<tr>
<td>HCC</td>
<td>15</td>
<td>35.11 ± 16.21</td>
<td>0.022*(HCV*HCC)</td>
</tr>
</tbody>
</table>

![Fig 1. TGF-beta 1 levels [ng/ml]](image)
DISCUSSION

Chronic HCV infection is one of the most common infectious diseases leading to high morbidity and mortality due to the development of liver fibrosis/cirrhosis and hepatocellular carcinoma. TGF-β1 plays a pivotal role in the pathogenesis of post-inflammatory liver scarring contributing to the increased synthesis and deposition of a broad spectrum of extracellular matrix molecules. \(^{(18,19)}\)

In the current study, serum levels of TGF-β1 were considerably higher in HCV group compared to healthy controls. Our results were in agreement with previous studies\(^{(15,20,21)}\) which showed that TGF-β1 serum levels were significantly increased in chronic hepatitis C patients. Also with Tsushima et al.\(^{(22)}\) who reported that plasma TGF-β1 levels in HCV infected patients before therapy were significantly higher than in controls. Furthermore, patients having HCV infection associated with HCC showed significantly higher values of TGF-β1 than HCV and control groups. Our results were in accordance with that of Abou-Shady et al.\(^{(23)}\) and Matsuzaki et al.\(^{(24)}\) who found that in HCC, expression of TGF-β1 appears to be increased suggesting a tumor promoting effect, also with Kim et al.\(^{(25)}\) who found that
serum TGF-β1 levels were higher in HCC than in chronic hepatitis.

The elevated TGF-β1 serum levels in HCV group reflect the activation of TGF-β1 system in patients with chronic HCV infection. Furthermore, the progressive increase in serum TGF-β1 from HCV infected patients to HCC patients predicts that TGF-β1 could represent a potentially important link between fibrosis and neoplasia in the liver. TGF-β1 is an important factor in the regulation of liver growth. However, its overexpression may increase hepatocytes' turnover that predisposes to hepatocarcinogenesis.(26) Therefore, TGF-β1 has been suggested to play a role in HCC development, growth and progression.

Hepatic vitamin A depletion plays a key role in hepatic fibrosis.(27,28) Since serum RBP concentrations are highly correlated with serum retinol and can be used as a simple surrogate measure for vitamin A concentrations, RBP has been considered to be a good and sensitive index of vitamin A depletion. (13) Accordingly, serum level of RBP appeared to be a valid index of the functional status of the liver. (17,29)

In the present study, there was a progressive reduction in serum RBP levels from controls to patients with HCV infection. Moreover, patients with HCV infection associated with HCC had significantly lower values than patients with HCV infection alone. Our findings were in agreement with those of Newsome et al. (30) Decreased RBP serum concentrations may be due to impaired hepatic synthesis and/or release of this vitamin A transport protein. In view of the fact that vitamin A depletion may be associated with the development of tumors, (17) therefore, decreased serum RBP levels in patients with HCV infection associated with HCC may be considered as a risk factor for the development of HCC.

In our work, the negative correlation between TGF-β1 and RBP in all patients, being more evident in HCC group, points to their tumor-promoting effect through enhancing fibrogenesis. Recent evidence (31-33) suggested that loss of retinyl esters is associated with increased retinoic acid formation, which in turn facilitates TGF-β1 mediated liver fibrogenesis. As a role for vitamin A in cancer prevention has been proposed, therefore, progressive loss of vitamin A storage, associated with blocked RBP secretion give the chance for the tumor to grow.

The mechanism by which hepatitis C virus induces liver fibrosis remains largely obscure. Our results revealed that, hepatitis C viral load was positively correlated with serum TGF-β1 levels in all patients; this correlation was more evident in HCC group. Although data of previous studies deals with the correlation of hepatitis C viral load to serum TGF-β1 levels were controversial, (6,20,25,34) this positive correlation signifies that HCV proteins may contribute to hepatic fibrogenesis via up-regulation of TGF-β1. (35,36) Through this bystander effect, HCV infection may contribute to pre-early stages of cancer development, beginning in the early stages of chronic hepatitis.

The positive correlation observed between hepatitis C viral load and AST activities in all patients suggests that liver injury induced by persistent HCV infection plays an important role in HCC development. Persistent hepatocyte injury and the resulting regeneration in the liver accelerate the development of HCC due to increased misreplication, chromosomal instabilities and gene mutations but it is not a sufficient single factor for HCC development. (37)

In the present work, a week negative correlation between RBP and AFP and a positive correlation between TGF-β1 and AFP were observed in all patients, although they did not reach statistical significance, but they may be useful serological markers in predicting HCC. Neither Serum TGF-β1 nor RBP levels were correlated with aminotransferases' activities in HCV and HCC patients, as they were mediators for hepatic fibrogenesis than for hepatocellular injury.

In conclusions, the progressive increase in serum TGF-β1 associated with progressive reduction in serum RBP levels from controls to HCC patients suggests their tumor-promoting effect through facilitating TGF-β1-mediated liver fibrogenesis with progressive loss of vitamin A storage giving the chance for the tumor to grow. Correlation of hepatitis C viral load with serum TGF-β1 levels signifies that HCV proteins could induce expression of TGF-β1 and may contribute to liver carcinogenesis. Also, correlation of hepatitis C viral load with AST activities
suggests that chronic hepatocyte injury plays an important role in HCC development. Extracellular matrix remodeling mainly occurs as a consequence of chronic hepatocyte injury. Accordingly, both events, chronic hepatocyte injury and extracellular matrix remodeling are important for HCC development. Therefore, persistent hepatitis C viremia in association with increased TGF-β1 and reduced RBP serum levels could be considered as risk factors for the evolution of HCC in HCV-infected patients.

REFERENCES


17. Leo M A, Lieber CS. Alcohol, vitamin A, and b-carotene: adverse interactions, including hepatotoxicity and


