The role of chemokine CC ligand 20 in patients with liver cirrhosis and hepatocellular carcinoma

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ABSTRACT

Background and aim: To evaluate the role of chemokine CC ligand 20 (CCL20) as a biomarker for hepatocellular carcinoma (HCC).

Patients and methods: Ninety patients in four groups were enrolled in this prospective cross-sectional study: 30 with HCC (group I), 30 with liver cirrhosis (group II), 15 with hepatitis C virus infection (group III), and 15 healthy blood donors as controls. Alpha fetoprotein (AFP), CCL20 and vascular endothelial growth factor (VEGF) were measured in all groups.

Results: Serum levels of CCL20 were significantly different among the study groups (F=230.979, p<0.001). The highest level was found in HCC patients (57.305 ± 6.386 pg/mL) followed by patients with cirrhosis (45.999 ± 5.165 pg/mL) compared with 22.781 ± 5.986 pg/mL and 18.585 ± 3.554 pg/mL in asymptomatic patients with HCV infection and controls, respectively. In HCC patients, CCL20 significantly correlated with VEGF (r=0.559, p=0.001), AFP (r=0.814, p=0.001), Child score (r=0.748, p<0.001), and tumor size (r=0.825, p<0.001). The cutoff value of CCL20 for the detection of HCC in HCV-infected patients was 54 pg/mL with 93.1% accuracy, 89.6% negative predictive value, 92.6% positive predictive value, 83.3% sensitivity, and 93.3% specificity. In patients with cirrhosis, CCL20 significantly correlated with VEGF (r=0.455, p=0.011), AFP (r=0.975, p<0.001), and Child score (r=0.977, p<0.001).

Conclusion: CCL20 may be used for the detection of HCC in HCV-infected patients with comparable specificity and higher sensitivity than AFP.

Key words: Hepatocellular carcinoma, Cirrhosis, Markers

Received: May 16, 2011; Accepted: October 10, 2011

INTRODUCTION

The incidence of hepatocellular carcinoma (HCC) in Egypt has doubled over the past 10 years and this is linked mainly to hepatitis C virus (HCV) infection and cirrhosis (1). As HCC is a highly vascular tumor, angiogenesis has been suggested to play a major role in its development and progression (2). The vascular endothelial growth factor (VEGF) is a well-known angiogenic factor that could be linked to vascular invasion of HCC (3).

Elevated serum levels of VEGF have been reported in many carcinomas including those of the lung (4), kidneys (5), pancreas (6), and colon-rectum (7). Chemokines belong to a superfamily of low-molecular-weight cytokines that can be classified into 4 main families (CC, CXC, CX3C, and C) based on the relative positions of the first 2 of the 4 conserved cysteine residues. The largest subgroups are the CXC and CC families (8). Both are involved in angiogenesis in acute and chronic inflammation and tumorigenesis (9).

The chemokine system contains approximately 50 ligands and 20 receptors, as the receptors can be stimulated by more than one ligand (9). Chemokine CC ligand 20 (CCL20), also known as liver activation-regulated chemokine (LARC) and macrophage inflammatory protein 3alpha (MIP3), is the only chemokine known to interact with the G-protein coupled 7-transmembrane receptor, CCR6 (10).

Expression of CCL20 has been confirmed in many tumors including prostate cancer (11), colorectal adenocarcinoma (12), oral squamous cell carcinoma (13), as well as HCC (14). Few studies reported the overexpression of CCL20 in liver biopsies from HCV-infected patients. Serum CCL20 was found to be elevated in patients with acute liver failure treated with molecular adsorbent recirculating system (MARS) (15). The aim of this study was to evaluate the serum level of CCL20 in different stages of HCV infection, and its potential role as a biomarker in HCC.
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PATIENTS AND METHODS

After the approval of the research ethics committee at Tanta Faculty of Medicine, 90 people divided into 4 groups were enrolled in this prospective cross-sectional study. The groups were as follows: group I: 30 patients with HCC associated with HCV diagnosed by spiral CT; group II: 30 patients with HCV and cirrhosis diagnosed by ultrasound and liver function tests; group III: 15 patients with asymptomatic HCV infection discovered accidentally during blood donation; and group IV: 15 healthy blood donors who served as control. The groups were matched for age and sex. For groups I and II, blood samples were collected from inpatient wards and outpatient clinics of the departments of Tropical Medicine and Infectious Disease, Internal Medicine, and Clinical Oncology. For groups III and IV samples were obtained from blood donors at the blood bank and the department of Clinical Pathology. Ten milliliters of blood was withdrawn after patient consent. Of each sample, 2 mL was added to an EDTA tube to obtain a complete blood picture and 2 mL was added to a sodium-citrate vacutainer tube to measure prothrombin time and activity. The remainder of the sample was transferred to a plain tube and serum was separated by centrifugation and aliquoted into 2 Eppendorf tubes. One was used to perform liver function tests including serum albumin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and bilirubin, the other was stored at -80°C until analysis for:

- alpha fetoprotein (AFP) by enzyme-linked immunosorbent assay (ELISA) as previously tested (16);
- CCL20 by ELISA using a commercially available kit (QuantiKine, human MIP3a, R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions (17);
- VEGF by ELISA using the Biotrak VEGF human ELISA system (Amersham Pharmacia, Buckinghamshire, UK) (18).

Statistical analysis

The serum levels of CCL20, VEGF, and AFP were summarized as mean ± SD and 1-way analysis of variance (ANOVA) was used to compare differences between the studied groups. Comparisons between groups were performed using the Kruskal-Wallis test. Coefficients of correlation (r) between serum CCL20 concentrations and other laboratory and clinical parameters were calculated using Spearman’s rank test. Statistical significance was defined as p<0.05 for all statistical tests. A receiver operating characteristic (ROC) curve was used to calculate the cutoff value for CCL20 in HCC patients. All statistical analyses were performed using a statistical software package (SPSS, version 15).

RESULTS

This study was performed on 75 HCV-infected individuals grouped into asymptomatic, cirrhotic and suffering from HCC; in addition, 15 healthy subjects served as the control group. All groups were matched for age and sex.

Serum levels of CCL20 were significantly higher in HCC patients (mean value; 57.305 ± 6.386 pg/mL) compared to the other groups; 45.999 ± 5.165, 22.781 ± 5.986, and 18.585 ± 3.554 pg/mL in cirrhotic, asymptomatic HCV-infected and control individuals, respectively. CCL20 was also significantly higher in patients with cirrhosis than in asymptomatic individuals with HCV infection and controls (p<0.001). There was no significant difference between the level of CCL20 in HCV-infected

| TABLE I - COMPARISON OF CCL20 SERUM LEVELS IN THE STUDY GROUPS |
|------------------|------------------|------------------|------------------|------------------|
|                  | CCL20 (pg/mL)    | ANOVA            |
|                  | Range            | Mean ± SD        | F                | P value          |
| Group I          | 42.00 - 65.417   | 57.305 ± 6.386   |                  |                  |
| Group II         | 36.723 - 55.475  | 45.999 ± 5.165   | 230.979          | <0.001*          |
| Group III        | 16.260 - 42.000  | 22.781 ± 5.986   |                  |                  |
| Group IV         | 10.700 - 23.000  | 18.585 ± 3.554   |                  |                  |
| I&II             |                  |                  | 0.000*           |                  |
| I&III            |                  |                  | 0.000*           |                  |
| I&IV             |                  |                  | 0.000*           |                  |
| II&III           |                  |                  | 0.000*           |                  |
| II&IV            |                  |                  | 0.000*           |                  |
| III&IV           |                  |                  | 0.169            |                  |

* = significant (p<0.05). CCL20, chemokine CC ligand 20
Group I, hepatocellular carcinoma; group II, liver cirrhosis; group III, hepatitis C virus infection; group IV, control
The concentration of VEGF was significantly different among the study groups (F=301.563, p=0.000). It was significantly increased in HCC patients, with a mean value of 370.165 ± 67.248 pg/mL versus 134.477 ± 44.226, 17.717 ± 4.550 and 8.086 ± 2.628 pg/mL in cirrhotic, asymptomatic HCV-infected and control individuals, respectively (p<0.001). VEGF was significantly increased in

**TABLE II - COMPARISON OF VEGF SERUM LEVELS IN THE STUDY GROUPS**

<table>
<thead>
<tr>
<th>Group</th>
<th>Range</th>
<th>Mean ± SD</th>
<th>F</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>193.800 - 451.729</td>
<td>370.165 ± 67.248</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group II</td>
<td>44.600 - 173.699</td>
<td>134.477 ± 44.226</td>
<td>301.563</td>
<td>0.000</td>
</tr>
<tr>
<td>Group III</td>
<td>9.678 - 23.000</td>
<td>17.717 ± 4.550</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group IV</td>
<td>3.200 - 11.500</td>
<td>8.086 ± 2.628</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

I&II I&III I&IV II&III II&IV III&IV
0.000* 0.000* 0.000* 0.000* 0.000* 0.943

*= significant (p<0.05). VEGF, vascular endothelial growth factor
Group I, hepatocellular carcinoma; group II, liver cirrhosis; group III, hepatitis C virus infection; group IV, control

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cirrhotic patients compared with the asymptomatic HCV infection and control groups (p<0.001), but there was no significant difference between HCV-infected blood donors and the control group (p=0.943) (Tab. II).

With regard to the serum levels of AFP, there were significant differences between groups (F=119.113, p<0.000), with the highest level being found in the HCC group. AFP was also significantly higher in patients with cirrhosis compared with the asymptomatic HCV infection and control groups (p<0.001). However, there was no significant difference between HCV-infected blood donors and controls (p=1.000) (Tab. III).

There was a significant positive correlation between CCL20 and VEGF, AFP, and Child score in the HCC and cirrhosis groups (p<0.001). In the HCC group there was a significant positive correlation between CCL20 and tumor size (p<0.001) (Tab. IV and Fig. 2).

Using the ROC curve, a CCL20 level of 54 pg/mL was calculated as the cutoff for the detection of HCC in HCV-infected patients with 93.1% accuracy, 89.6% negative predictive value, 92.6% positive predictive value, 83.3 sensitivity, and 93.3% specificity (Figs. 3 and 4).

DISCUSSION

The progression of hepatic injury through HCV infection is related to cytokine release from accumulated antigen-presenting cells, mainly dendritic cells, activated macrophages, and cytotoxic T lymphocytes (19, 20). Chemokines, and particularly CCL20, seem to play a major role in the recruitment of these cells to the liver as increased expression of CCL20 has been reported in HCV-infected hepatocytes (21).

In our study, patients with cirrhosis had significantly higher levels of CCL20 than healthy blood donors, which was in agreement with the results of Roth et al (15). The CCL20 level was correlated with Child score, as also reported by Yamauchi et al (17), who correlated the level of CCL20 with the degree of liver pathology in patients with active chronic hepatitis C.

We found that serum levels of CCL20 were significantly higher in HCC patients than in all other groups. This finding may reflect an active source of CCL20 production in HCC patients. Similarly, Rubie et al (14) suggested that CCL20 mRNA expression was upregulated in liver biopsies from HCC patients. Furthermore, they found that tissue ELISA for CCL20 concentration was 10-fold increased in HCC tissue compared with surrounding tissues.

### TABLE III - COMPARISON OF AFP SERUM LEVELS IN THE STUDY GROUPS

<table>
<thead>
<tr>
<th>Group</th>
<th>Range</th>
<th>Mean ± SD</th>
<th>F</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>520.000 - 2230.00</td>
<td>1154.36±427.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group II</td>
<td>105.910 - 300.000</td>
<td>219.820±57.882</td>
<td>119.113</td>
<td>0.000*</td>
</tr>
<tr>
<td>Group III</td>
<td>2.000 - 10.000</td>
<td>6.400±2.720</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group IV</td>
<td>0.000 - 5.000</td>
<td>2.733±1.580</td>
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<thead>
<tr>
<th>I&amp;II</th>
<th>I&amp;III</th>
<th>I&amp;IV</th>
<th>II&amp;III</th>
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<th>III&amp;IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.000</td>
<td>0.000*</td>
<td>0.000*</td>
<td>0.041*</td>
<td>0.037*</td>
<td>0.000*</td>
</tr>
</tbody>
</table>

*= significant (p<0.05). AFP, alpha fetoprotein

Group I, hepatocellular carcinoma; group II, liver cirrhosis; group III, hepatitis C virus infection; group IV, control
significant increase in the serum level of VEGF in HCV-infected blood donors compared to controls. By contrast, Kim et al (24) reported a significant difference between HCV-infected cases and controls. This discordance might be explained by the variable disease severity between the studies: the members of group III in our study were completely asymptomatic with normal liver function, whilst most of their cases were cirrhotic (Child B or C). An interesting finding of our study is the correlation between the serum level of CCL20 and VEGF in both cirrhotic and HCC patients, a finding that indicates a direct link between CCL20 and VEGF in those patients. To our knowledge this is the first report of such a correlation in liver diseases. However, Hosokawa et al reported CCL20 to induce VEGF production by human gingival fibroblasts when added to cell cultures (22). Similar mechanisms may occur in hepatic lesions.

Many explanations may be suggested for the significant positive correlation between CCL20 and VEGF in both cirrhotic and HCC patients, a finding that indicates a direct link between CCL20 and VEGF in those patients. To our knowledge this is the first report of such a correlation in liver diseases. However, Hosokawa et al reported CCL20 to induce VEGF production by human gingival fibroblasts when added to cell cultures (22). Similar mechanisms may occur in hepatic lesions.

Concerning VEGF, we found significantly higher levels in HCC and cirrhotic patients than in blood donors. Similarly, Gadelhak et al (23) reported significantly elevated expression of serum VEGF in patients with HCC or cirrhosis compared with healthy individuals. However, in that study there was no significant difference in VEGF level between HCC and cirrhotic patients, which differs from our results, where VEGF was significantly higher in patients with HCC than in those with cirrhosis, as was reported by others (18, 24). In our cohort there was no significant increase in the serum level of VEGF in HCV-infected blood donors compared to controls. By contrast, Kim et al (24) reported a significant difference between HCV-infected cases and controls. This discordance might be explained by the variable disease severity between the studies: the members of group III in our study were completely asymptomatic with normal liver function, whilst most of their cases were cirrhotic (Child B or C).

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which suggests that hepatoma cells are the source of the increased CCL20.

The CCL20 level was significantly higher in patients with cirrhosis than in asymptomatic HCV-infected and healthy blood donors. This might be explained by the activation of the cytokine cascade, particularly TNF-α, during the cirrhotic process. Hosokawa et al (22) reported the induction of CCL20 by TNF-α and IL-1β in other conditions. There was no significant difference between the asymptomatic HCV-infected and healthy blood donor groups, suggesting CCL20 to be related to disease progression rather than viral infection as such.

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increase in tumor size (22). Furthermore, there might be a direct effect of CCL20 on tumor growth, as Fujii et al (26) reported the CCL20-CCR6 axis to promote the growth of the hepatoma cell line Huh7 in vitro.

Although AFP is considered the gold standard for the diagnosis of HCC, its cutoff value varies substantially among ethnic groups. For instance, the cutoff value of AFP has been reported to be as low as 30 ng/mL (sensitivity 65%, specificity 89%) in the Sicilian population versus 200 ng/mL (sensitivity 70%, specificity 100%) in the Burmese population (27, 28). Both values were lower than the levels reported in our cirrhotic patients (mean 219.82 ± 57.882; range 105.910-300 ng/mL). This may be due to the ethnic variation of the patients included in these studies. Furthermore, in our cohort, patients with HCC and cirrhosis were both infected with HCV and the predictive value of AFP is known to be lower in HCC with viral etiology compared to non-viral HCC. Therefore, for AFP, many authors reported a cutoff value of 400 ng/mL for the diagnosis of HCC (27, 29, 30).

In our cohort there was no overlap between AFP levels among different groups (300 ng/mL for the upper range of the cirrhotic group and 520 ng/mL for the lower range of the HCC group). Therefore, we did not need to perform a ROC curve for the level of AFP. In this cohort, CCL20 was analyzed in 75 HCV-infected patients, 30 of whom had HCC. Using the ROC curve, a CCL20 level of 54 pg/mL was calculated as the cutoff for the detection of HCC in HCV-infected patients, with comparable specificity (93.3%) and higher sensitivity (83.3%) than previously reported for AFP. These results make serum CCL20 a promising biomarker for HCC diagnosis either alone or combined with AFP.

CONCLUSIONS

The serum chemokine CCL20 and VEGF are correlated with the clinicopathological progression of liver disease from HCV infection to HCC formation. CCL20 may be involved in HCC pathogenesis through induction of VEGF, which in turn increases angiogenesis, thereby facilitating tumor formation and progression. A CCL20 level of 54 pg/mL was estimated to be the cutoff value for detection of HCC in HCV-infected patients with comparable specificity and higher sensitivity than AFP, which make it a promising biomarker for the diagnosis of HCC.

Abbreviations

HCV, hepatitis C virus infection
HCC, hepatocellular carcinoma

Conflict of interest statement: None.

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