UNIFORM EXPRESSION OF HEPCIDIN MRNA IN TUMORS DIFFERING IN NUMBER, DEGREE OF DIFFERENTIATION, AND VESSEL INVASION, OF HEPATOCELLULAR CARCINOMA.

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ABSTRACT: The present study evaluated the expression of hepcidin mRNA in hepatocellular carcinoma (HCC). Samples of cancerous and non-cancerous liver tissue were taken from 40 patients with HCC who underwent hepatectomy. Expression of hepcidin mRNA was evaluated by real-time PCR, and compared in tumors differing in their degree of differentiation, number of tumors, and vessel invasion. Hepcidin mRNA expression is uniformly suppressed in HCC. Hepcidin mRNA expression in non-cancerous and cancerous tissues was 1991.8 (35.3–25187.4) and 62.6 (1.9–3185.8), respectively ($P < 0.0001$). There were no significant differences in hepcidin expression among tumors differing in their degree of differentiation, number of tumors, or vessel invasion.

Keywords: HCC, Hepcidin, mRNA expression, vessel invasion.
INTRODUCTION

Hepatocellular carcinoma (HCC) is a major cause of death worldwide [Ferlay J et al., 2001], and chronic inflammatory stress caused by hepatitis viruses B and C plays a major role in HCC carcinogenesis [Fattovich G et al., 2004]. Patient prognosis is generally dismal, and nutritional problems commonly worsen the outcome. Most concerns about malnutrition in HCC patients focus on protein-calories deficiency, but little is known about the disturbance of iron metabolism. Furthermore, studies have indicated that iron overload is a major risk factor for development of HCC [Deugnier et al., 1998]. Iron overload leads to the generation of reactive oxygen species (ROS), which cause chronic inflammation in the liver [Hentze et al., 2004]. Iron accumulation is associated not only with the genetic iron overload disorder, hemochromatosis, but also with acquired hemosiderosis after chronic viral hepatitis or in fatty liver [Di Bisceglie et al., 1992, Metwally et al., 2004, Yamamoto et al., 2007]. Hepcidin is a key molecule for maintenance of iron homeostasis [Nicoras et al., 2002]. Hepcidin is produced in hepatocytes, and binds to, internalizes, and degrades ferroportin-1 [Ganz T et al., 2006], resulting in a decrease of serum iron concentration and an increased intracellular iron content [Pietrangelo et al., 2004]. It rises with iron overload [Pigeon et al., 2001], inflammation and infection, and decreases with hypoxia and anemia [Nicolas et al. 2002]. Deficiency of hepcidin leads to hemochromatosis [Nicolas et al. 2001; Roetto et al. 2003]. Over expression of hepcidin results in severe iron refractory anemia, and is implicated in anemia of inflammation [Roy et al. 2007; Weinstein et al. 2002]. Anemia of inflammation induces over expression of hepcidin [Frazer et al., 2004, Nicolas G et al., 2002]. It has been reported that hepcidin mRNA is downregulated in alcoholic liver injury [Bridle et al. 2006. Ohtake et al. 2007] but not significantly affected by the presence of hepatitis B virus (HBV) or hepatitis C virus (HCV) infection [Aoki et al. 2005; Fujita et al. 2007] and cirrhosis (Bergmann et al. 2008). Hepcidin expression has been found reduced in rat model HCC (Holmstrom et al. 2006)

MATERIALS AND METHODS

Patient Selection
Forty patients who had been diagnosed as having HCC by histological examination and who had undergone hepatic resection at ESIC hospitals were included in the present study. The documented consent was obtained from each patient. The patients were men and their mean age was 54.5 years. HCC specimens and adjacent non tumor tissue specimens were collected during hepatic resection, and were freshly stored in liquid nitrogen. Based on the WHO grading system (Hirohashi et al., 2000), the numbers of patients with well, moderately, and poorly differentiated HCC were 4, 32, and 4, respectively.
Number of tumors 1 \( n = 29 \), 2 \( n = 5 \), 3 \( n = 4 \), 4- \( n = 2 \). Vessel invasion Negative \( n = 31 \), Positive \( n = 9 \). According to pTNM staging system of the American Joint Committee on Cancer (American Joint Committee on Cancer 1997), the numbers of patients in stage I, II, IIIA, IIIB, and IV were 14, 17, 6, 3, and 10, respectively. Tumor size was defined as the greatest diameter of each tumor.

**Real-time PCR**

For real-time PCR, samples of both non-cancerous and cancerous liver tissue were available for all 40 patients. Surgical samples weighing 500 mg were stored in liquid nitrogen immediately after the operation, and kept at -80°C until RNA extraction. Total RNA from each sample was isolated using a Total RNA Isolation Kit (Bioserve biotechnologies). Reverse transcription reactions were performed using a cDNA Synthesis Kit (Bangalore biogene). Briefly, 1 μg of total RNA, oligo dT-primer, and dNTPs were incubated at 65°C for 5 min, then 10 μL of a cDNA synthesis mixture was added and the mixture was incubated at 50°C for 50 min. The reaction was terminated by adding 1 μL of RNaseH and incubating the mixture at 37°C for 20 min. Real-time PCR was performed with a sequence detector. The PCR reaction was carried out in a final volume of 2 μL cDNA, 12.5 μL 2 × SYBR Green (Applied Biosystems), 0.5 μL of 25 nM sense and antisense primers, and H₂O up to 25 μL. The PCR conditions consisted of 40 cycles at 95°C for 15 s and 60°C for 60 s. The sequences of the primers were as follows:

**Table 1 Primer sequences of hepcidin genes and HPRT reference gene**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward 5'-3',</th>
<th>Reverse 5'-3'</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepcidin</td>
<td>GACCAGTGGCTCTGTTTCC</td>
<td>CTCCCTCGCCTCTGGAACAT</td>
<td>111</td>
</tr>
<tr>
<td>HPRT</td>
<td>GACCAGTCAACAGGGGACAT</td>
<td>CCTGACCAAGGAAAGCAAAG</td>
<td>132</td>
</tr>
</tbody>
</table>

The level of expression was calculated using the formula:

Relative expression \((t) = \frac{\text{Copy number of target molecule}}{\text{Copy number of HPRT}} \times 1000\) [Iso Y, et al., 2005]. Samples were assayed in triplicate. Means and standard deviations were calculated from the data obtained. For each sample, at least three assays were performed. The \(t\) value was calculated from the mean of three different assays.
RESULTS

Table 2: Hepcidin m RNA expression levels in tumors differing in degree of differentiation

<table>
<thead>
<tr>
<th>Type of tissue</th>
<th>Hepcidin m RNA expression levels</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non cancerous</td>
<td>1991.8 (35.3–25187.4)</td>
<td></td>
</tr>
<tr>
<td>Cancerous</td>
<td>62.6 (1.9–3185.8)</td>
<td>$P &lt; 0.0001$</td>
</tr>
<tr>
<td>Well differentiated</td>
<td>385.6</td>
<td></td>
</tr>
<tr>
<td>Moderately</td>
<td>70.8</td>
<td></td>
</tr>
<tr>
<td>Poorly</td>
<td>135.3</td>
<td>$P = 0.999$</td>
</tr>
</tbody>
</table>

Table 3: Hepcidin m RNA expression levels in patients differing in number of tumors

<table>
<thead>
<tr>
<th>Number of tumors</th>
<th>Hepcidin m RNA expression levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>81.4</td>
</tr>
<tr>
<td>2</td>
<td>14.6</td>
</tr>
<tr>
<td>3</td>
<td>80.2</td>
</tr>
<tr>
<td>4 &amp; more</td>
<td>124.0</td>
</tr>
</tbody>
</table>

($P = 0.512$)

Table 4: Hepcidin m RNA expression levels in patients with and without vessel invasion

<table>
<thead>
<tr>
<th>Vessel invasion</th>
<th>Hepcidin m RNA expression levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>72.4</td>
</tr>
<tr>
<td>Negative</td>
<td>66.6</td>
</tr>
</tbody>
</table>

($P = 0.883$).
Hepcidin mRNA expression is uniformly suppressed in hepatocellular Carcinoma. The median t values for hepcidin mRNA in non-cancerous and cancerous tissues were 1991.8 (35.3–25187.4) and 62.6 (1.9–3185.8), respectively ($P < 0.0001$) (Fig. 1). Expression of hepcidin mRNA was significantly inhibited in cancerous tissue. Median t values for hepcidin mRNA in well, moderately and poorly differentiated HCC were 385.6, 70.8, and 135.3, respectively ($P = 0.999$). Median t values for hepcidin mRNA in patients who had 1, 2, 3 and 4 or more tumors were 81.4, 14.6, 80.2, and 124.0, respectively ($P = 0.512$). Median t values for hepcidin mRNA in patients who were negative and positive for vessel invasion were 72.4 and 66.6 respectively ($P = 0.883$).

Hepcidin mRNA expression did not differ among well, moderately, and poorly differentiated carcinoma, patients with 1, 2, 3, and 4 or more tumors, and negative and positive for vessel invasion.

**DISCUSSION**

Hepcidin is a molecule playing a key role in iron homeostasis. It is produced by the liver, and inhibits intestinal iron absorption by enterocytes in the duodenum and also release of iron by macrophages and hepatocytes [Kuston MD et al., 2005]. Production of hepcidin is controlled by various stimuli and factors. Production of hepcidin is stimulated by iron overload, inflammation, and proinflammatory cytokines such as IL-6, whereas it is decreased by iron deficiency and erythropoiesis, leading to iron accumulation in the body [Nicelolas G et al., 2002]. It is well known that HCC develops in more than 40% of patients with hemochromatosis [Fargion S et al., 1992]. On the other hand, iron is an essential nutrient for cell growth, and cancer cells in particular require iron in order to proliferate [Le NT, 2002]. The present study clearly demonstrated that expression of hepcidin mRNA was suppressed in tumors differing in size, number and with and without vessel invasion HCC, irrespective of the degree of tumor differentiation.

Expression of hepcidin was maintained in non-cancerous liver tissue of patients with HCC. Although the mechanism responsible for suppression of hepcidin mRNA expression in HCC remains unclear, suppression of hepcidin transcription contradicts the previously proposed scheme for iron homeostasis in cancer cells, because cancer cells must retain iron in order to proliferate. Hepcidin is produced in patients with HCC, from non-cancerous liver tissue, even though production is inhibited in cancerous tissue.
CONCLUSION

Expression of hepcidin mRNA is constitutively suppressed in cancerous, but not in non-cancerous liver tissue of patients with HCC. Hepcidin mRNA expression did not differ among well, moderately, and poorly differentiated carcinoma, patients with 1, 2, 3, and 4 or more tumors, and negative and positive for vessel invasion. The precise mechanism responsible for the suppression of hepcidin in HCC should be investigated further, focusing on its role in the development and maintenance of this cancer.

Competing interests
The authors declare that they have no competing interests.

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