Co-ordinate activation of lipogenic enzymes in hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma is a very common neoplastic disease in countries where hepatitis viruses B and/or C are prevalent. Small hepatocellular carcinoma lesions detected by ultrasonography at an early stage are often hyperechoic because they are composed of well-differentiated cancer cells that are rich in triglyceride droplets. The triglyceride content of hepatocytes depends in part on the rate of lipogenesis. Key lipogenic enzymes, such as fatty acid synthase, are co-ordinately regulated at the transcriptional level. We therefore examined the mRNA expression of lipogenic enzymes in human hepatocellular carcinoma samples from 10 patients who had undergone surgical resection. All of the samples exhibited marked elevation of expression of mRNA for lipogenic enzymes, such as fatty acid synthase, acetyl-CoA carboxylase and ATP citrate lyase, compared with surrounding non-cancerous liver tissue. In contrast, the changes in mRNA expression of SREBP-1, a transcription factor that regulates a battery of lipogenic enzymes, did not show a consistent trend. In some cases where SREBP-1 was elevated, the main contributing isoform was SREBP-1c rather than SREBP-1a. Thus, lipogenic enzymes are markedly induced in hepatocellular carcinomas, and in some cases SREBP-1c is involved in this activation.

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1. Introduction

Hepatocellular carcinoma is a very common neoplastic disease in countries where hepatitis viruses B and/or C are prevalent. In Japan, 90% of hepatocellular carcinomas develop in patients with chronic liver diseases such as liver cirrhosis and chronic hepatitis [1]. Periodic imaging using ultrasonography in patients with chronic liver diseases has resulted in an increased rate of detection of small hepatocellular carcinomas of 2 cm diameter or less [2,3]. These are often hyperechoic lesions, which are composed of well-differentiated cancer cells rich in triglyceride droplets [4,5].

The triglyceride content of hepatocytes depends in part on the rate of fatty acid synthesis [6–8]. The fatty
zymes, is now elucidated in detail [9]. Among these enzymes, the following are of particular importance: fatty acid synthase (FAS), the main synthetic enzyme that catalyses the condensation of malonyl-CoA to produce the 16-carbon saturated fatty acid palmitate; acetyl-CoA carboxylase 1 (ACCL), which synthesises malonyl-CoA from acetyl-CoA; ATP citrate lyase (ACL), which synthesises acetyl-CoA from citrate provided through glycolysis. These enzymes are known to be co-ordinately induced when lipogenesis is needed, and this induction of enzymes is primarily at the transcriptional level [10]. It has therefore been presumed that these genes have a regulatory sequence in their promoter that interacts with common trans-acting factors such as sterol regulatory element-binding protein (SREBP)-1c [11,12].

SREBPs are members of the basic helix–loop–helix leucine zipper family of transcription factors that regulate fatty acid and cholesterol synthesis (reviewed in [13–15]). Unlike other members of the family, SREBPs are synthesised as precursors bound to the endoplasmic reticulum and nuclear envelope, and are released from the membrane into the nucleus as mature proteins by cleavage processes. To date, three isoforms of SREBP, -1a, -1c and -2, have been identified and characterised. The predominant SREBP-1 isoform in liver and adipose tissue is SREBP-1c. Whereas SREBP-2 plays a crucial role in regulation of cholesterol synthesis, SREBP-1c co-ordinately upregulates the transcription and expression of lipogenic enzymes such as FAS and ACL, leading to elevated triglyceride synthesis ([16–18]). It is remarkable that SREBP-1c controls not only the synthetic rate of triglycerides but also their content in the liver [7,8,19].

As well-differentiated hepatocellular carcinomas often have abundant triglyceride droplets and SREBP-1c is a determinant of triglyceride content in hepatocytes, we hypothesised that the activation of SREBP-1 and thereby lipogenic enzymes might be involved in the early stage of cancer development in hepatocytes. To test this hypothesis, we examined the expression levels of SREBP-1 and its downstream lipogenic enzymes in surgically resected hepatocellular carcinomas.

2. Materials and methods

2.1. Patients

Ten consecutive patients who had undergone surgical resection of hepatocellular carcinoma at Tokyo University Hospital from January to June 1999 were included in the study. Informed consent was obtained from the patients. All cases were diagnosed as hepatocellular carcinoma by pathohistological examination.

2.2. Northern blotting

Small pieces of hepatocellular carcinoma and non-cancerous liver tissue were snap frozen in liquid nitrogen immediately after surgical resection. Necrotic portions, identified macroscopically, were excluded. Total RNA from these samples was extracted using TRIzol reagent (Invitrogen), and 10 μg RNA was run on a 1% agarose gel containing formaldehyde and transferred to a nylon membrane. The cDNA probe for SREBP-1 was cloned as described previously [6]. The cDNA probes for FAS, ACC1, ACL, stearoyl-CoA desaturase (SCD) and malic enzyme (ME) were prepared by cloning RT-PCR products from human liver total RNA into pGEM-T easy vectors (Promega). The PCR primers used for FAS were 5’-TCGGAGAAGACTTCAGAGAGGTT-3’ and 5’-CCAGCCCTCAAAGACTGCAC-3’; for ACC1, 5’-CACATAAGTGCCATGTTCATCTG-3’ and 5’-CCAGGCAGGACATGACTAGT-3’; for ACL, 5’-TCTCTCTGTAGCCATGTTCG-3’ and 5’-GGAGATTTCTCTGTAGAC-3’; for SCD, 5’-TGCTGGCAGGCTATCTGCTAG-3’ and 5’-CCAGAGGACATGACTAGT-3’; for ME, 5’-CATCTGAACTGTGACTTGGACATG-3’ and 5’-CCGCAATGGAGATTGAGATGCATC-3’. The probes were labelled with [α-32P]dCTP using Mega-prime DNA Labeling System kit (Amersham Biosciences). The membranes were hybridised with the radiolabelled probe in Rapid-hyb Buffer (Amersham Biosciences) at 65°C. The membranes were washed in 0.1 × SSC, 0.1% SDS at 65°C. Blots were exposed to Kodak XAR-5 film and the BAS2000 Bio Imaging Analyzer (Fuji Photo Film, Tokyo, Japan).

2.3. Immunohistochemistry

The expression of FAS was immunohistochemically detected in formalin-fixed, paraffin-embedded sections as described previously [20]. As the primary antibody, rabbit polyclonal antibody to FAS (IBL, Fujioka, Japan) was used at 2 μg/ml IgG.

2.4. RNase protection assay

RNase protection assay was performed as described previously [21]. Briefly, after linearisation of the template plasmid DNA with HindIII, antisense RNA probes for SREBP-1a and -1c were transcribed with [α-32P]CTP using bacteriophage T7 RNA polymerase (MAXIscript in vitro transcription kit, Ambion) and purified by electrophoresis on 5% polyacrylamide gel. Then aliquots of probes and total RNA (10 μg) from each sample were mixed and subjected to the RNase protection assay using HybSpeed RPA kit (Ambion). After digestion with RNase A/T1, protected fragments were separated on 8 M urea/10% polyacrylamide gels.
The protected fragments for SREBP-1a and -1c contained the same number of cytidine phosphates (32 cytidines). The gels were dried and subjected to Kodak XAR-5 film and the BAS2000 Bio Imaging Analyzer (Fuji Photo Film, Tokyo, Japan).

3. Results

3.1. Induction of lipogenic enzymes in hepatocellular carcinomas

Table 1 shows the outline of patients included in the current study. Because we analysed 10 consecutive cases hospitalised at random, the aetiology of background chronic liver disease was diverse, but the majority patients had viral hepatitis, mostly due to the hepatitis C virus, in accordance with epidemiological data for Japan. We performed Northern blot analysis of surgically resected samples frozen during operation and compared the mRNA expressions of lipogenic enzymes and their upstream transcription factor SREBP-1 between the hepatocellular carcinoma tissue and surrounding non-cancerous liver tissue. As shown in Figs. 1 and 5(b), all carcinoma samples exhibited marked elevation of mRNA levels for FAS, ACC1, ACL, SCD and ME, representative enzymes in the fatty acid biosynthetic pathway, compared with corresponding adjacent non-cancerous liver tissue. The expression levels of lipogenic enzymes were not related to the grade of carcinoma cell differentiation or to the virus type. Meanwhile, the mRNA level of SREBP-1 in carcinoma did not show consistent differences from that in non-cancerous tissue. Fatty changes in carcinoma were observed in cases #4, #6 and #9 by histological examination (Table 1), but these were not inevitably accompanied by higher levels of expression of lipogenic enzymes.

Table 1
Clinical characteristics of cases included in the current study

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Virus</th>
<th>Histological findings</th>
<th>AFP (IU/ml)</th>
<th>PIVKA2 (IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
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<td></td>
<td>Tumour differentiation</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Liver cirrhosis</td>
<td>Fatty change</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>36</td>
<td>M</td>
<td>B</td>
<td>Moderate</td>
<td>Early</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>65</td>
<td>M</td>
<td>C</td>
<td>Moderate</td>
<td>Advanced</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>54</td>
<td>M</td>
<td>C</td>
<td>Moderate</td>
<td>Early</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>65</td>
<td>M</td>
<td>C</td>
<td>Moderate</td>
<td>Advanced</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>63</td>
<td>M</td>
<td>C</td>
<td>Moderate</td>
<td>None</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>35</td>
<td>F</td>
<td>B</td>
<td>Moderate–poor</td>
<td>Advanced</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>81</td>
<td>M</td>
<td>nBnC</td>
<td>Poor</td>
<td>None</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td>61</td>
<td>F</td>
<td>C</td>
<td>Moderate</td>
<td>Advanced</td>
<td>–</td>
</tr>
<tr>
<td>9</td>
<td>45</td>
<td>M</td>
<td>nBnC</td>
<td>Well</td>
<td>None</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>58</td>
<td>M</td>
<td>C</td>
<td>Moderate</td>
<td>Advanced</td>
<td>–</td>
</tr>
</tbody>
</table>

nBnC, not B nor C; AFP, alpha fetoprotein; PIVKA2, protein induced by vitamin K absence 2.
analysis were plotted in a scatter diagram (Fig. 2). There was significant correlation between the mRNA expression of FAS, ACC1, ACL and SCD, whose $P$-value by Fisher's formula was less than 0.001. In contrast, there was no significant correlation between the mRNA level for SREBP-1 and that for lipogenic enzymes such as FAS, ACC1 and ACL.

3.3. Immunohistochemical detection of FAS

To confirm the elevated expression of FAS protein in hepatocellular carcinoma, immunostaining was performed on formalin-fixed, paraffin-embedded sections using anti-FAS antibody. In all of the 10 cases, FAS protein was strongly stained in hepatocellular carcinoma specimens. Moreover, although FAS was also detected in non-cancerous liver tissues, there was a tendency for stronger staining in carcinoma samples (Fig. 3).

3.4. RNase protection assay reveals SREBP-1c dominance

For further analysis of SREBP-1 isoform, we selected four cases out of 10 covering each virus type (B, C, or non-B non-C), and conducted an RNase protection assay with probes that can discriminate SREBP-1a and -1c. Because the protected fragments for SREBP-1a and -1c contained the same number of cytidine phosphates (32 cytidines), the intensity of each band was proportional to the amount of each isoform in the samples.

![Fig. 2. Correlation among mRNA levels of sterol regulatory element-binding protein-1 (SREBP-1) and lipogenic enzymes. Correlation between (a) mRNA level of SREBP-1 and fatty acid synthase (FAS) and (b) FAS and acetyl-CoA carboxylase 1 (ACC1) or ATP citrate lyase (ACL). Northern blot analysis data presented in Fig. 1 were quantified and plotted. A regression line was plotted in (b), where significant correlations were detected. Con, control non-cancerous liver tissue; HCC, hepatocellular carcinoma.](image1)

![Fig. 3. Immunostaining of fatty acid synthase (FAS). Immunohistochemical expression of FAS is shown in two representative cases. Case #4 (upper panels) has the highest FAS mRNA expression and case #10 (lower panels) has the second lowest FAS mRNA. The carcinoma in case #4 shows clear cell change that indicates lipid accumulation. Control, surrounding non-cancerous liver tissue; HCC, hepatocellular carcinoma.](image2)
As shown in Fig. 4, the RNase protection assay did not exhibit a definite pattern of isoform change, and both in non-cancerous tissue and in hepatocellular carcinoma SREBP-1c was more abundant than SREBP-1a. In two cases (cases 7 and 9), where SREBP-1 mRNA was increased by malignant transformation as shown by Northern blot analysis (Fig. 1), the change in the expression of SREBP-1c was more prominent than that of SREBP-1a. These results were quantified and presented in a graph in Fig. 5 (a).

4. Discussion

Our present studies clearly demonstrate that lipogenic enzymes are induced in hepatocellular carcinomas compared with background liver tissue. It is tempting to speculate that this induction is associated with fatty changes often seen in well-differentiated hepatocellular carcinomas. Notably, however, there was no correlation between the increase in lipogenic enzyme expression and the degree of carcinoma differentiation.

In general, cancers show high levels of glycolysis and lipogenesis (reviewed in [22]). Accelerated fatty acid synthesis in tumour tissues was first reported in the 1950s [23]. Since then, elevated expression of FAS in malignant cells has been documented in various cancers including breast [24–28], prostate [29–31], ovarian [32], endometrial [33], colon [34], stomach [20], tongue [35] and lung carcinoma [36]. Our study demonstrates, for the first time, that hepatocellular carcinoma can be added to this list.

As an underlying mechanism for this upregulation, it has been assumed that there is a link between cellular proliferation and fatty acid synthesis, because fatty acid synthesis has been observed to be activated when cells enter into proliferative phase in several systems [37–40]. Our results are in accordance with these data and provide further evidence for the link between cellular proliferation and lipogenesis. Furthermore, it would be of therapeutic value if inhibitors of fatty acid synthesis such as cerulenin were found to be useful for the treatment of hepatocellular carcinoma.

It has been established that the lipogenic enzymes are in general regulated co-ordinately at mRNA level [10], and our current finding that lipogenic enzymes such as FAS, ACC1 and ACL are at the same time activated in carcinoma is in agreement with this. Although the precise molecular mechanism for this regulation is currently unclear, this co-ordinated control of gene expression is believed to be achieved by a regulatory sequence that interacts with common trans-acting factors [10]. As documented previously [11,12], SREBP-1 is one of the candidate transcription factors responsible for this regulation. In fact, the
potential involvement of SREBP-1 has been demonstrated in some cancers [41–43]. Hence, we examined the expression of SREBP-1 at the mRNA level, but, unexpectedly, there was no consistent trend in SREBP-1 mRNA elevation in hepatocellular carcinoma. It is possible, however, that SREBP-1 might be increased post-translationally, that is, at the processing level, and be involved in lipogenic gene induction. To address this issue, it is necessary to quantify the amount of nuclear SREBP-1 protein in the active form by nuclear protein extraction. This analysis could not be performed, however, due to the limited amounts of surgically resected human samples available.

The 1a isoform of SREBP-1 is known to be expressed relatively abundantly in organs where cells are rapidly turned over, such as in the spleen, thymus and small intestine as well as in whole embryos [21]. It has also been shown that transformed cell lines usually express SREBP-1a dominantly. We therefore expected that hepatocarcinomas would exhibit a 1a-dominant pattern of SREBP-1 isoform. This did not seem to be the case, however, in our study. In the samples we examined, 1c was more prominently induced, a phenomenon that may be related to the highly differentiated feature of this cancer.

In conclusion, we have shown that lipogenic enzymes are markedly induced in hepatocarcinomas, and that SREBP-1c rather than SREBP-1a might be partly involved in this activation.

Conflict of interest statement

None declared.

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