Methylation-Mediated Silencing of SOCS-1 Gene in Hepatocellular Carcinoma Derived from Cirrhosis

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ABSTRACT

Purpose: Suppressor of cytokine signaling-1 (SOCS-1) is a negative regulator of Janus kinase and signal transducer and activation of transcription pathway. Recently, it was demonstrated that SOCS-1 gene was silenced frequently by methylation of CpG island in human hepatocellular carcinoma (HCC). We examined the methylation-mediated silencing of SOCS-1 in tumors of HCC patients.

Experimental Design: Fifty patients with HCC were investigated in this study. We examined the methylation status of the SOCS-1 promoter region by methylation-specific PCR and then confirmed the methylation-mediated silencing of SOCS-1 by Northern blot analysis. Furthermore, this methylation status was compared with clinicopathological findings.

Results: Aberrant methylation of the SOCS-1 gene was detected in 30 of 50 (60%) HCC specimens. No corresponding nontumorous liver tissues showed SOCS-1 methylation. Subsequent Northern analysis proved that methylation of the SOCS-1 promoter inactivated translation and diminished expression of SOCS-1 mRNA. We then analyzed the correlation between the clinicopathological data and SOCS-1 aberrant methylation and found that HCC derived from liver cirrhosis had a significant relationship with SOCS-1 methylation (P = 0.0207).

Conclusions: SOCS-1 may be a novel tumor suppressor, and its aberrant methylation may be a key event for HCC transformation of cirrhotic nodules.

INTRODUCTION

HCC is a common malignant disease, and its incidence has recently increased around the world (1, 2). The main risk factors associated with HCC are infection with hepatitis C virus, infection with hepatitis B virus, and liver cirrhosis followed by these viral infections. According to etiological study, liver cirrhosis is considered to be a premalignant region because HCC occurs frequently in the background of liver cirrhosis.

Recent advances in genetics have proved that accumulated genetic alterations through the repeating destruction and regeneration of hepatocytes are supposed to result in carcinogenesis. A variety of oncogenes such as c-myc, cyclin D1, and β-catenin and tumor suppressor genes such as p16, p53, and Rb genes were reported to be associated with hepatocarcinogenesis (3–6). However, the specific genes that play a leading role have not been identified.

Recently, Yoshikawa et al. (7) demonstrated that the SOCS-1 gene is frequently silenced by methylation of the CpG island in human HCC. SOCS-1 is an intracellular protein that negatively regulates the JAK/STAT signaling pathway, which is a principal cytokine signaling transduction pathway. The JAK/STAT pathway has also been known to play an important role in the regeneration of hepatocytes (8, 9), and furthermore, additional studies have indicated that both JAKs and STATs are involved in the oncogenesis of several tumors (10–12).

These findings suggested a potential role of SOCS protein, a growth suppressor of HCC, through negative regulation of the JAK/STAT pathway.

To define the role of SOCS-1 in the tumorigenic pathway of the liver, we examined the methylation-mediated silencing of SOCS-1 in tumors of 50 HCC patients. The results obtained were then compared with clinicopathological aspects.

MATERIALS AND METHODS

Sample Collection and DNA Preparation. Fifty primary tumors and corresponding nontumorous liver tissues were collected at Nagoya University Hospital from HCC patients during liver resection surgery. All tissues were confirmed histologically. Collected samples were stored immediately at −80°C until analysis. DNA was prepared as described previously (13). Clinicopathological profiles of the patients enrolled in the study are shown in Table 1. All patients with liver cirrhosis belonged to class A according to Child-Pugh classification.

Sodium Bisulfite Modification. One µg of genomic DNA extracted from tumors and nontumorous tissues was subjected to bisulfite treatment as described previously (14). Briefly, alkali-denatured DNA was modified by 2.1 M sodium bisulfite/0.5 mM hydroquinone at pH 5.0. The bisulfite-reacted DNA was then treated with NaOH, purified using Wizard DNA Clean-Up System (Promega, Madison, WI), precipitated with ethanol, and resuspended in distilled water.

MSP. The bisulfite-treated DNA was amplified by MSP. The primers for MSP were described previously (7). The PCR was hot-started at 95°C for 5 min, and the protocol consisted of

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2 The abbreviations used are: HCC, hepatocellular carcinoma; JAK, Janus kinase; STAT, signal transducer and activation of transcription; MSP, methylation-specific PCR; TNF-α, tumor necrosis factor α; IL-6, interleukin 6.
35 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min and a final extension of 72°C for 5 min. DNA from Hep3B and HuH-7 (HCC cell lines) was used as a positive control for methylated and unmethylated alleles, respectively. Control reaction without DNA was performed for each set of PCR. Ten μl of each PCR product were loaded directly onto nondenaturing 8% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination.

**Northern Analysis.** Resected specimens were lysed in guanidine thiocyanate, and RNA was extracted using cesium chloride density gradient centrifugation. Northern blot hybridization was performed essentially as described previously (15). The SOCS-1 cDNA probe for hybridization was generated using the reverse transcription-PCR method. This probe was designed as nucleotide position 679-1030 of the SOCS-1 cDNA sequence. A human α-actin probe was used as an internal control.

**Statistical Analysis.** The association between SOCS-1 methylation status and clinicopathological findings was examined by the χ² test. Statistical significance was considered as P < 0.05.

<table>
<thead>
<tr>
<th>Clinicopathological findings</th>
<th>Variable</th>
<th>No. of cases</th>
<th>SOCS-1 methylation</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Male</td>
<td>42</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>8</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>Liver cirrhosis</td>
<td>−</td>
<td>15</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>35</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>Hepatitis virus infection</td>
<td>−</td>
<td>6</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>35</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>Tumor size</td>
<td>&lt;5 cm</td>
<td>32</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥5 cm</td>
<td>10</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>Distribution of tumors</td>
<td>Solitary</td>
<td>25</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Multiple</td>
<td>25</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>Tumor differentiation</td>
<td>Well diff.</td>
<td>6</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Moderately diff.</td>
<td>42</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Poorly diff.</td>
<td>2</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Vascular invasion</td>
<td>−</td>
<td>43</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>7</td>
<td>−</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; α-actin, α-actin.

**RESULTS**

We first examined the methylation status of the SOCS-1 promoter region in 50 HCC and 50 corresponding nontumorous liver tissue specimens using MSP (Fig. 1). Aberrant methylation of the SOCS-1 gene was detected in 30 of 50 (60%) HCC specimens. Most of the tumors also exhibited unmethylation, which might account for the contamination of nontumorous cells in tumor specimens. No corresponding nontumorous liver tissues showed SOCS-1 methylation.

To confirm inactivation of the SOCS-1 gene by methylation, we then examined SOCS-1 mRNA expression in primary tumors and corresponding liver tissues by Northern analysis (Fig. 2). Thirty-one paired RNA samples obtained from the 50 patients described above were available for this examination. Of 18 tumors that showed SOCS-1 methylation, 15 had lower expression of SOCS-1 mRNA than the corresponding nontumorous liver tissues. On the other hand, in the 13 tumors that showed no SOCS-1 methylation, we observed the same or higher level of SOCS-1 expression compared with the corre-
tumor, and vascular invasion were correlated with infection, tumor size, distribution of tumors, differentiation of

**DISCUSSION**

Cytokines are crucial secreted proteins that regulate cellular proliferation and differentiation. The stimuli of these mediators are mainly led to the transcriptional activation of cytokine-induced genes through the JAK/STAT signaling pathway (16). Recently, the potential role of the JAK/STAT pathway in oncogenesis has been proposed in many kinds of tumors (10–12, 17, 18). On the other hand, the SOCS family has been identified as a negative feedback protein of cytokine-induced signaling pathway (19–21). These proteins are activated by STATs and negatively regulate the JAK/STAT pathway by inhibiting the JAKs directly or blocking the access of the STATs. Although the mechanism by which SOCS proteins regulate cytokine signaling has been studied to an extent, their biological roles continue to be examined. In recent studies, it was reported that SOCS-1 expression was suppressed through aberrant methylation of the CpG island in several HCC cell lines and that restored SOCS-1 expression suppressed both the growth rate and anchorage-independent growth of cells (7, 22). Another study (23) has shown that GFI-1, a proto-oncogenetic protein derived from GFI-1 in T-cell lymphoma, repressed reporter activity of SOCS-1/SOCS-3 promoters. In the current study, we frequently observed aberrant methylation of the SOCS-1 promoter in the tumors of HCC patient, whereas the same methylation was not detected in the corresponding nontumorous liver tissues. Furthermore, we showed methylation-mediated silencing of SOCS-1 expression in the tumors using Northern analysis. These observations suggested that SOCS-1 might act as a tumor suppressor, at least in certain HCCs.

Subsequently, we compared the methylation status of SOCS-1 in the tumors of HCC patients with their clinicopathological features, and we demonstrated that SOCS-1 methylation was observed more frequently in HCCs derived from cirrhosis than in those that were not derived from cirrhosis. With regard to this result, it was supposed that inactivation of SOCS-1 might be an important factor for hepatocarcinogenesis, especially in patients with cirrhosis. Growth factors and cytokines are critical for maintaining liver volume and physiology (24), and the JAK/STAT pathway activated in response to these agents is associated with the proliferation of hepatocytes. In particular, TNF-α and IL-6 are important components of the signaling pathway that lead to liver regeneration (9). Plasma TNF-α and IL-6 levels were significantly higher than in patients with liver cirrhosis than in those without it, and the severity of liver cirrhosis was an important factor for the occurrence of increased IL-6 level (25, 26). This phenomenon was supposed to be caused by decreased cytokine clearance of the liver as well as enhanced endogeneous lipopolysaccharide levels. High levels of plasma TNF-α and IL-6 after liver cirrhosis may induce the activation of the JAK/STAT pathway. Both activated cytokine pathway and inactivated negative regulators such as SOCS-1 may result in unrestricted proliferation of hepatocytes.

Cirrhotic nodules have long been considered to be premalignant lesions followed by HCC (27). It was suggested that accumulated genetic alterations induced by continuous regeneration of hepatocytes might lead to HCC. Recently, it was reported that several cirrhotic nodules already had some chromosomal aberrations, and more allelic imbalances appeared in the progression to HCC (28). In this study, methylation of the SOCS-1 promoter was closely related to the pathogenesis of HCC patients with liver cirrhosis. Besides, SOCS-1 methylation was detected in early stages of HCC, whereas it was undetectable in the corresponding nontumorous liver tissues. These findings indicated that SOCS-1 might be a novel tumor suppressor and that its aberrant methylation might be a key event for HCC transformation of cirrhotic nodules.

This study provided solid evidence for additional studies on the molecular mechanism of SOCS-1 in HCC and also confirmed that SOCS-1 might play an important role in the carcinogenic pathway in the liver of patients with cirrhosis. These observations offered the possibility that tumor formation in liver cirrhosis might be controlled by inducing the expression of silenced SOCS-1 using demethylation reagents.

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**REFERENCES**


Silencing in Hepatocellular Carcinoma


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