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Epigenetic alteration of the *SOCS1* gene in hepatocellular carcinoma

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Summary

Principles: It has been postulated that the induced suppressor of cytokine signalling (SOCS) protein inhibits the signalling pathway through the association with a variety of tyrosine kinase proteins, and decelerates or inhibits the progression of cirrhosis and hepatocellular carcinoma (HCC). The purpose of this study was to investigate the expression of *SOCS1* gene in HCC and explore the potential molecular mechanisms.

Methods: We investigated CpG island methylation status at the promoter region and the expression of the *SOCS1* gene in 46 HCC tumour and paired non-tumour samples.

Results: This immuno-histochemical study demonstrated strong homogeneous or heterogeneous staining in the non-tumour liver tissue compared to a marked decreased heterogeneous staining in the HCC (p <0.001). Real-time quantitative (qRT)-PCR showed that *SOCS1* mRNA was also down-regulated in tumour cells of HCC. The methylation analysis of CpG sites at the promoter region of *SOCS1* disclosed hypermethylation in 39% of HCC samples and 41% of non-tumour tissue. Promoter methylation of *SOCS1* was well correlated with HCC derived from liver cirrhosis (p = 0.044) and tumour size (p = 0.038).

Conclusions: Our findings suggest a tumour suppressor-like role of *SOCS1* in the hepatocarcinogenesis of human HCC.

Key words: *SOCS1; hepatocellular carcinoma; methylation; carcinogenesis*

Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent and lethal types of cancer in Asia, and an increased incidence has been found in many countries worldwide [1]. In many cases in South-eastern Asia, HCC develops from chronic liver diseases and cirrhosis by infection with either HBV or HCV [2]. According to clinicopathological studies, liver cirrhosis is considered to be a premalignant state because HCC occurs frequently in the background of liver cirrhosis [3]. The molecular mechanisms of HCC are not yet clearly understood.

The SOCS proteins are a family of negative regulators of cytokine and growth factor signalling [4–6]. These proteins are relatively small molecules containing a central src homology 2 (SH2) domains and a C-terminal SOCS box [7]. It is now clear that the eight SOCS family members, including SOCS1-7 and CIS (cytokine inducible SH2-containing protein), contribute significantly to the negative regulation of several cytokine pathways causing the inhibition of the Janus kinases (JAKs), tyrosine phosphorylation and nuclear translocation of the signal transducers and activators of transcription (STATs) proteins, interferon gamma signalling in T cell differentiation, and the suppression of steel factor-dependent proliferation [4].

The *SOCS1* gene is located on chromosome 16p12-p13.1 [8]. This gene contains two exons and a transcribed 1215-nt mRNA which encodes a protein of 211 amino acid residues. The *SOCS1* gene has been demonstrated to be frequently silenced by methylation of the CpG islands in human HCC [9], and can be activated by various kinds of cytokines, hormones, and growth factors. SOCS1 is an intracellular protein with critical roles in several kinds

of cytokine signalling, such as interferon gamma, interleukin-2 and interleukin-4 [10–12]. SOCS-1 also negatively regulates the JAK/STAT signalling pathway, which is a principal cytokine signalling transduction pathway [12]. Several studies have indicated that dysregulation of the JAK/STAT pathway is involved in the malignant transformation for several commonly-encountered human cancers, such as HCC [9], non-small-cell lung cancer [13–14], and head and neck squamous cell carcinoma [15]. These findings suggest a potential role of the SOCS protein as a growth suppressor through negative regulation of the JAK/STAT pathway.

To investigate the role of the *SOCS1* gene in the carcinogenesis of the liver, we performed immunohistochemical staining, real-time quantitative PCR (qRT-PCR), and methylation-specific PCR to demonstrate *SOCS1* expression and methylation in HCC versus surrounding non-tumour tissue. The relationship between *SOCS1* promoter methylation and clinicopathological aspects, such as tumour size, tumour differentiation, presence of liver cirrhosis, hepatitis infection, vascular invasion, and recurrence was examined in this study to further understand the pathogenesis of HCC.

Patients and methods

Samples

Resected human primary HCC tissues and nearby non-tumour parts, before radiation or chemotherapy, were obtained from forty-six patients at Changhua Christian Hospital, Taiwan. All tissue samples were collected from July, 1997 to December, 2001. The clinicopathological data included the presence of liver cirrhosis, hepatitis infection, tumour size and differentiation, vascular invasion and recurrence of tumour. Specimens were frozen immediately after surgical resection and pathological examination, and stored in liquid nitrogen until extraction of DNA. DNA extraction was performed as previously described [16].

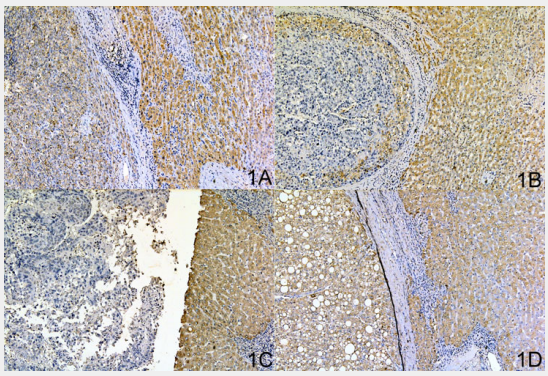

Figure 1

Representative SOCS1 staining patterns of HCC specimens. (A)(B) Non-tumour part at the right side of figures shows homogeneously strong positive staining by the SOCS1 antibody. Tumour part at the left side shows heterogeneously weak staining. (C) Non-tumour part at the right side shows homogeneously strong positive staining. Tumour part at the left side shows negative staining. (D) Non-tumour part at the right side shows heterogeneously strong positive staining and tumour part at the left side shows homogenously strong positive staining.

Methylation-specific PCR analysis and bisulphite direct sequencing

Methylation-specific PCR analysis and bisulphite direct sequencing were performed as described previously [17]. Briefly, 4 µg of DNA was incubated with 10 µl of 1 M NaOH at 37 °C for 10 min, followed by 30 µl of 10 mM hydroquinone and 520 µl of 1.5 M sodium bisulphite (pH 5.0) at 50 °C for 16 hours. DNA samples were then purified using Wizard DNA Purification System (Promega, Madison, WI, USA). Modified DNA were amplified by PCR (35 cycles consisting of 94 °C for 1 min, 65 °C for methylation primers and 60 °C for unmethylation primers for 1 min, 72 °C for 1 min, followed by a final 5 min extension at 72 °C). The primers used for methylation-specific (*SOCS1*-M) and unmethylated PCR (*SOCS1*-U) of the *SOCS1* promoter were described as follows: *SOCS1*-M (forward) 5'-TTGTTCGGAGGTCGGATTT-3'(nt -291 to -272 to the transcriptional start site) and *SOCS1*-M(reverse) 5'-ACTAAAACGCTACGAAACCG-3'(nt -93 to -74); *SOCS1*-U 5'-TTTTTTGGTGTTGTTTGGAGGTTGGATTTT-3'(nt -301 to -272) and SOCS1-U (reverse) 5'-AAAACAAAACAATAAACTAAAACACTACAAAACCA-3' (nt -108 to -74). The amplification products of *SOCS1*-M and *SOCS1*-U were 218 bp and 243 bp, respectively. CpG methylase (SssI)-treated genomic DNA was used as the positive control for methylation-specific primers (*SOCS1*-M). DNA extracted from blood samples from healthy individuals was used as the positive control for the unmethylation primers (*SOCS1*-U). To ensure the specificity of *SOCS1* methylation, unmodified genomic DNA preparations from tumour and non-tumour samples from HCC patients also served as negative controls. Direct sequencing of *SOCS1*-M and *SOCS1*-U PCR products using both forward and reverse primers were then carried out.

Analysis of protein expression by Immunohistochemistry

Four-micrometer-thick paraffin-embedded tissue sections on poly-L-lysine coated slides were de-paraffinised. After treatment with 3% H_2O_2 in methanol for quenching endogenous peroxidase, the sections were hydrated with gradient alcohol and PBS. Polyclonal antibody for the SOCS1 (Santa Cruz Biotechnology, CA, USA) was then incubated

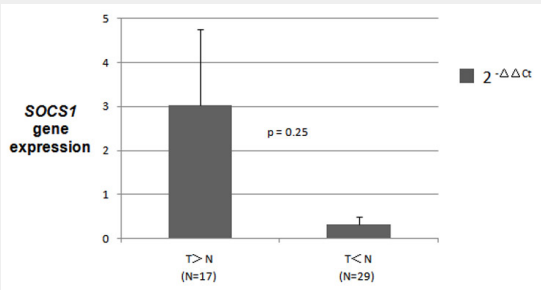

Figure 2

SOCS1 mRNA level in HCC and paired non-tumour tissues measured by qRT-PCR. Ratio of concentration of *SOCS1* mRNA of tumour/normal (T/N) is shown. Expression of *SOCS1* mRNA in paired tumours and normal samples was analysed by qRT–PCR and normalised to *HMBS*-expression.

with 10 mM citrate buffer and heated at 100 °C for 20 minutes in PBS. After being exposed to 50-fold-diluted antibody (1:50 dilution with PBS) for 30 minutes at room temperature, slides were incubated with a HRP/Fab polymer conjugate (Zymed, PicTure Polymer Kit, South San Francisco, CA, USA). The sections were thoroughly washed three times with PBS. The sites of peroxidase were visualised using 3,3'-diaminobenzidine tetrahydrochloride as substrates. Hematoxylin was used for counterstaining.

Real-time quantitative PCR (qRT-PCR) analysis.

SOCS1-specific forward and reverse primers and probes were designed with the aid of the Primer Express Software (Roche, USA). Sequences of the forward and reverse primers were as follows: *SOCS1*-L 5'-CCCCTGGTTGTTGTAGCAG -3'; *SOCS1*-R 5'-GTAGGAGGTGCGAGTTCAGG -3'. We used the *HMBS* gene as internal RNA control for RT-PCR. The expression levels of *SOCS1* genes were normalised to the endogenous *HMBS* reference to obtain the relative threshold cycle (ΔCt) which was in turn related to the ΔCt of the paired non-cancerous tissue to obtain the relative expression level ($2^{-\Delta\Delta Ct}$) of *SOCS1* gene. Reverse transcription was performed in a final volume of 25 µL containing 2 µg RNA, 0.5 µg random primers (10 mers), 2 mM dNTPs, 25 U RNasin (Promega, Madison, WI, USA), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$ and 200 U Moloney Murine Leukemia Virus reverse transcriptase (Promega). The reaction was first denatured for 5 min at 70 °C and incubated at 37 °C for 60 min, then stopped by heat inactivation at 95 °C for 5 min. Real-time quantitative PCR was performed as previously described[18]. $2^{-\Delta\Delta Ct}$ indicates the ratio of concentration of *SOCS1* mRNA of tumour/normal.

Statistics

Comparisons between the clinicopathological factors including gender, liver cirrhosis, tumour size, HBV antigen, anti-HCV, tumour recurrence, tumour differentiation, vascular invasion, *SOCS1* expression and methylation in the tumour and non-tumour part were analysed using the Fisher's and Chi square test with SPSS for Windows Release 9.0 (SPSS, Chicago, IL, USA). A *p* value of less than 0.05 was considered statistically significant.

Results

Immunohistochemical analyses of SOCS1 expression in HCC

In all cases, hepatocytes of the non-tumour parts were strongly and homogeneously or heterogeneously, stained by the SOCS1 antibody (fig. 1A, 1B, 1D). In contrast, negative or heterogeneous staining was noted in most tumour cells of the HCC (fig. 1A, 1B, 1C). The results of the immunohistochemical staining are listed in table 1. Expression of SOCS1 protein was significantly decreased in tumour tissues in comparison to non-tumour tissues.

Quantification of *SOCS1* mRNA by qRT-PCR

We used qRT-PCR to measure the mRNA transcript of *SOCS1*. The results showed that in 63% of all cases (29/46), there was a significant reduction of *SOCS1* mRNA in HCC cancerous cells in comparison with nearby non-cancerous cells (T<N). This was, in general, compatible with the results of immunohistochemical staining although we were unable to establish a statistical significant correlation ($p = 0.25$) (fig. 2).

Promoter methylation of *SOCS1* gene

Methylation specific PCR was carried out in this study to determine whether the hypermethylation of SOCS1 gene was correlated to the expression of the SOCS1 protein in HCC and paired non-tumour liver tissues. Promoter hypermethylation was noted in 39% (18/46) of HCC and 41% (19/46) of non-tumour parts (table 2). Homogeneous staining was noted in 85% (23/27) of unmethylated non-tumour tissues. Heterogeneous staining was present in 89% (17/19) of methylated non-tumour liver tissues. The results indicated the expression pattern of SOCS1 protein was significantly correlated with promoter methylation of *SOCS1* gene in non-tumour liver tissues ($p < 0.001$). However, the expression pattern of SOCS1 protein was not correlated with methylation of *SOCS1* in tumour cells of HCC. The CpG islands of the promoter region were examined by bisulphate sequencing analysis to confirm the finding (fig. 2).

Correlation between the clinicopathological parameters of HCC patients and promoter methylation of *SOCS1* gene

We also analysed the correlation between the clinicopathological features and *SOCS1* methylation and found that promoter methylation of *SOCS1* gene was closely associated with HCC derived from liver cirrhosis ($p = 0.044$) and tumour size ($p = 0.038$). No other clinicopathological parameter such as gender, hepatitis infection, differentiation of tumour, vascular invasion and recurrence of tumour was found to be correlated with *SOCS1* methylation status (table 3).

Discussion

Cytokines are crucial secreted proteins that regulate cellular proliferation and differentiation. The stimuli of these

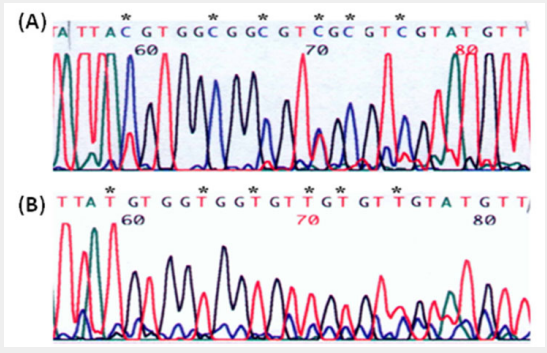

Figure 3

Representative methylation analysis of CpG islands at the promoter region of the *SOCS1* gene. (A) Modified DNA of a methylated HCC case by bisulphite with all cytosines in CpG dinucleotides remaining as cytosines (asterisk). (B) Bisulphite-modified DNA of an unmethylated CpG dinucleotides with all the cytosines converting into thymines (asterisk).

mediators lead to the transcriptional activation of cytokine-induced genes through the JAK/STAT signalling pathway. Recently, the potential role of the JAK/STAT pathway in oncogenesis has been proposed in many kinds of tumours [19]. The SOCS family has been identified as a negative feedback protein of cytokine-induced signalling pathway [20]. 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 signalling has been studied to an extent, their biological roles continue to be examined. *SOCS1* expression was suppressed through aberrant methylation of the CpG islands in several HCC studies [21–22].

In the present study, methylation status and mRNA level of *SOCS1*, and SOCS1 protein expression in HCC patients were examined to explore its role in HCC. The immunohistochemical staining showed a strong homogeneous staining in the non-tumour liver cells compared to a marked decrease of staining intensity in HCC (p <0.001). Although not statistically significant, qRT-PCR results also indicated that *SOCS1* mRNA was down-regulated in the majority of HCC patients. The methylation of CpG sites at the promoter area of the *SOCS1* gene was further analysed using methylation-specific PCR and direct sequencing. Silencing of CpG islands of the SOCS1 gene promoter by the hypermethylation was observed in both HCC and non-tumour liver samples. The immunohistochemical staining patterns of SOCS1 protein was statistically associated with *SOCS1* gene methylation pattern at the promoter region only in non-tumour liver tissues and not tumour cells of HCC. The seemingly conflicted results reflect the complexity of hepatocarcinogenesis of HCC cells. The observations that promoter methylation of *SOCS1* is well correlated with HCC derived from liver cirrhosis (p = 0.044) and tumour size (p = 0.038) suggested a tumour suppressor-like role of *SOCS1* in the hepatocarcinogenesis of human HCC.

Promoter methylation in the *SOCS1* CpG islands was identified in primary HCC. At least three previous studies have reported similar findings [22–24]. We compared the

Table 1
Analysis of SOCS1 protein expression of tissues from HCC and non-tumour part of liver by immunohistochemical staining.

IHC staining of the SOCS-1	Tumour	Non-tumour	p[a]
Homogeneous[b]	1	25	<0.001
Heterogenous[c]	45	21	

[a] Chi-square and Fisher's test.
[b] Homogeneous means all cells are stained homogeneously strong positive by the SOCS1 antibody
[c] Heterogeneous means cells are stained heterogeneously (strong or weak) positive by the SOCS1 antibody

Table 2
SOCS1 gene methylation pattern and immunohistochemical stain of SOCS1 protein expression in tumour and non-tumour part of HCC patients.

IHC staining of the SOCS-1	*SOCS-1* methylation Tumour part		p[a]	*SOCS-1* methylation Non-tumour part		
	+	–		+	–	p
Homogeneous[b]	0	1	1.000	2	23	<0.001
Heterogeneous[c]	18	27		17	4	

[a] Fisher's test.
[b] Homogeneous means all cells are stained homogeneously strong positive by the SOCS1 antibody
[c] Heterogeneous means cells are stained heterogeneously (strong or weak) positive by the SOCS1 antibody

Table 3
The clinicopathological features and *SOCS1* gene methylation patterns in HCC patients.

Clinicopathological findings	Variable	No. of cases	*SOCS-1* methylation					
			Tumour part		p[a]	Non-tumour part		p
			+	–		+	-	
Gender	Male	38	15	23	1.000	15	23	0.7
	Female	8	3	5		4	4	
Liver cirrhosis	–	31	9	22	**0.044**	14	17	0.533
	+	15	9	6		5	10	
Hepatitis virus infection	–	15	5	10	0.207	7	8	0.101
	HBV[b]	21	8	13		9	12	
	HCV	5	4	1		0	5	
	Both viruses	5	1	4		3	2	
Tumour size	<5 cm	21	6	15	0.232	5	16	**0.038**
	≥5 cm	25	12	13		14	11	
Tumour differentiation	Well diff.	4	1	3	0.391	1	3	0.763
	Moderately diff.	25	12	13		11	14	
	Poorly diff.	17	5	12		7	10	
Vascular invasion	–	37	16	21	0.448	16	21	0.716
	+	9	2	7		3	6	
Recurrence	–	23	10	13	0.736	11	12	0.550
	+	23	8	15		8	15	

[a] Chi-square and Fisher's test.
[b] HBV, hepatitis B virus; HCV, hepatitis C virus; diff, differentiated.

methylation status of *SOCS1* in the tumours of HCC patients with their clinicopathological features, and it was demonstrated that *SOCS1* methylation was observed more frequently in HCCs derived from cirrhosis than in those which were not derived from cirrhosis. This observation suggests that inactivation of SOCS1 might be an important factor in carcinogenesis of HCC, especially in patients with cirrhosis. Growth factors and cytokines are critical for maintaining liver volume and physiology, and the JAK/STAT pathway activated in response to these agents is associated with the proliferation of hepatocytes. In particular, TNF-α [25] and interleukin-6 (IL-6) [26] are important components of the signalling pathway that lead to liver regeneration. Plasma TNF-α and IL-6 levels were significantly higher 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 [27]. This phenomenon can be the result of decreased cytokine clearance of the liver as well as enhanced endogenous 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 SOCS1, may result in unrestricted proliferation of hepatocytes.

Cirrhotic nodules have long been considered to be premalignant lesions followed by HCC [28]. It has been 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 [29]. Methylation of the *SOCS1* promoter was closely related to the pathogenesis of HCC patients with liver cirrhosis and might be a key event for HCC transformation of cirrhotic nodules. In conclusion, our observations suggest the possibility that tumour formation in liver cirrhosis might be controlled by inducing the expression of the silenced *SOCS1* using demethylating reagents.

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