RESEARCH ARTICLE

Hypermethylation of Suppressor of Cytokine Signaling 1 in Hepatocellular Carcinoma Patients

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Abstract

Hepatocellular carcinoma (HCC), the most common primary hepatic tumor, is highly prevalent in the Asia-Pacific region, including Thailand. Many genetic and epigenetic alterations in HCC have been elucidated. The aim of this study was to determine whether aberrant methylation of the suppressor of cytokine signaling 1 gene (SOCS1) occurs in HCCs. Methylation specific-PCR assays were performed to identify the methylation status of SOCS1 in 29 tumors and their corresponding normal liver tissues. An abnormal methylation status was detected in 17 (59%), with a higher prevalence of aberrant SOCS1 methylation significantly correlating with HCC treated without chemotherapy (OR=0.04, 95%CI=0.01-0.31; P=0.001). This study suggests that epigenetic aberrant SOCS1 methylation may be a predictive marker for HCC patients.

Keywords: Hepatocellular carcinoma - DNA methylation - SOCS1 - methylation-specific PCR

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Introduction

Hepatocellular carcinoma (HCC) is the most common primary hepatic tumor and the fifth most common cancer in the world (McClune and Tong, 2010). It is highly prevalent in the Asia-Pacific region and Africa, and is increasing in Western countries (Arii et al., 2010). Pathogenic mechanisms in HCC include cirrhosis caused by alcoholic liver disease, metabolic influences, chronic hepatitis infection, and mutations occurring in single or multiple oncogenes or tumor suppressor genes (Whittaker et al., 2010), including epigenetic alterations. Epigenetic alterations, such as DNA methylation of CpG islands, associated with the transcriptional silencing of many genes, including tumor-suppressor genes, DNA repair genes, and metastatic inhibitor genes (Wong et al., 2001).

The suppressor of cytokine signaling 1 gene (SOCS1), located on chromosome 16p13.13, is a negative regulator of the JAK/STAT (Janus kinase/signal transducer and activator of transcription) signaling pathway, encoding a JAK-binding protein that regulates the signal transduction pathway of JAK/STAT that is important in the transmission of cytokine signals from the cell surface to the nucleus (Ward et al., 2000; Koelsche et al., 2009). SOCS1 suppresses signaling by a wide variety of cytokines, including IL-1, IL-3, IL-4, IL-6, erythropoietin, granulocyte-macrophage colony-stimulating factor, and γ-interferon (Endo et al., 1997; Naka et al., 1997; Starr et al., 1997; Brysha et al., 2001; Sporri et al., 2001).

In the present study, we investigated SOCS1 methylation status among patients with HCC by methylation specific-polymerase chain reaction (MS-PCR). Moreover, to determine whether aberrant methylation correlates with loss of transcription, we investigated the expression of SOCS1 mRNA in HCC tumors by using real-time reverse transcription-PCR (RT-PCR). The correlation between methylation status, SOCS1 expression level, and clinico-pathological features of the patients, were also analyzed.

Materials and Methods

Tissue samples

Twenty-nine paired samples of HCC and normal liver tissues were collected from Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Thailand. The study was approved by the Khon Kaen University Institutional Review Board (HE471214). No therapeutic treatment (chemotherapy or radiation) was instituted before the operation. All tissues samples were frozen in liquid nitrogen at -80^{0}C for the extraction of DNA and RNA. Hematoxylin & eosin-stained samples from each tumor block were examined microscopically. The samples were graded differentially, according to the method of Edmonson and Steiner (1954), with classification into 3 groups-well differentiated (grade I), moderately differentiated (grades

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II-III), and poorly differentiated (grade IV). Tissues with >80% tumor cells and corresponding normal liver tissues from the same patients were used in this study.

DNA preparation and bisulfite modification

Genomic DNA was isolated by proteinase K digestion and salting-out method (Miller et al., 1988). DNA was loaded in agarose gel electrophoresis and stained with ethidium bromide to check purity. DNA was treated with sodium bisulfite, converting all the unmethylated, but not methylated, cytosines into uracil. Bisulfite conversion was carried out using the reagents provided with an EZ DNA Methylation Gold kit (Zymo Research, Orange, CA). One μg of DNA was treated with sodium bisulfite following the manufacturer's recommendations. The modified DNA was eluted in a total volume of 25 μl and stored at -20^{0}C until used.

RNA isolation and cDNA synthesis

Total RNA was extracted from 22 HCC and their corresponding normal liver tissues using Trizol reagent, according to the instruction manual (Invitrogen, Carlsbad, CA, USA). mRNA was isolated by Oligotex mRNA purification kit (Qiagen, Gmbh, Germany). Reverse transcription reactions were conducted according to the manufacturer's instructions, using the SuperScript III First-Strand Synthesis System for reverse transcription-polymerase chain reaction (Invitrogen, Carlsbad, CA, USA).

Methylation-specific PCR

The methylation status of the SOCS1 gene in 29 tumors, and their corresponding normal liver tissues, were analyzed by MS-PCR on DNA treated with sodium-bisulfite. The primers for the methylated sequences were FM-SOCS1 (5'-GGA TGG TAG TCG CGA GAG TTT C-3') and RM-SOCS1 (5'-ACG CGA CGC TAA CGC AAC G-3'), respectively. The unmethylated sequences were FU-SOCS1 (5'-TTT GGA TGG TAG TTG TGA GAG TTT T-3') and RU-SOCS1 (5'-CCA CAC ACA ACA CTA ACA CAA CA-3'), respectively (Brakensiek et al., 2005). The reactions were amplified in a total volume of 25 μl, containing 100 ng treated DNA, 1X PCR buffer, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 0.4 μM of each primer, 0.5X GC-rich solution, and 1 unit of FastStart TaqDNA Polymerase (Roche Diagnostics, Mannheim, Germany). Cycling conditions were hot-started at 95^{0}C for 5 min. Amplification was performed in a Mastercycler gradient (Eppendorf) for 32 cycles (1 min at 95^{0}C, 30 sec at 62^{0}C (methylated allele) and 65 ^{0}C (unmethylated allele) and 30 sec at 72^{0}C), followed by a final extension of 5 min at 72^{0}C. Twenty-five microliters of PCR product were electrophoresed in 1.5% agarose gel stained with ethidium bromide, and photographed under UV light. Normal lymphocyte DNA, treated with SssI methylase according to the manufacturer's instructions (New England Biolabs, Beverly, MA), was used as a positive control, modified with sodium bisulfite, and a negative control derived from normal lymphocyte DNA, modified with sodium bisulfite (Figure 1).

Quantitative real-time reverse transcription-PCR

The gene expression level of SOCS1 was further analyzed by iQ5 Real Time PCR Detection System (BioRad, Hercules, CA), with primers designed using the Primer 3 program. The primer sequences were F-SOCS1 (5'-GCC CCT TCT GTA GGA TGG TA-3') and R-SOCS1 (5'-GAG GAG GAG GAA GAG GAG GA-3'), respectively. β-globin (GB) gene was used as an endogenous reference to obtain relative expression values. The primer sequences were F-GB (5'-ACA CAA CTG TGT TCA CTA GC-3') and R-GB (5'-CAA CTT CAT CCA CGT TCA CC-3'), respectively. The reaction mixture was carried out using 20 ng of template cDNA, 1XFastStart SYBR Master (Roche Diagnostics, Mannheim, Germany), and 0.5 μM forward and reverse primers in a final volume of 25 μl. The PCR was then started at 95 ^{0}C for 5 min (to activate FastStartTaq), followed by 50-cycle amplification (95^{0}C for 10 s, 62^{0}C for 30 sec, and 72^{0}C for 30s). After PCR, each amplification reaction was analyzed using a dissociation curve. All real-time assays were done in duplicate.

The relative level of gene expression was determined as previously described Livak (2001). SOCS1 ranged from 0.07-14.01 (median 1.04, mean 2.90, SD 3.88). In this study, cutoff values for gene expression were adopted from median expression levels. Tumor gene expression < 1.0-fold was assigned as under-expression for SOCS1.

Statistical analysis

The correlation between the methylation status of SOCS1, SOCS1 mRNA expression, and clinico-pathological characteristics--age, gender, tumor

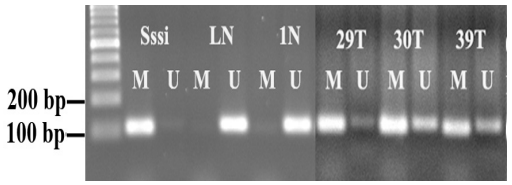

Figure 1. Representative Results of the MS-PCR Analysis of HCC Patients. Positive Control; Sssi, Negative Control; Lymphocyte (LN), N = Normal DNA, T = Tumor DNA, M = Methylated Sequence (101 bp), U = Unmethylated Sequence (108 bp); bp = Base Pair

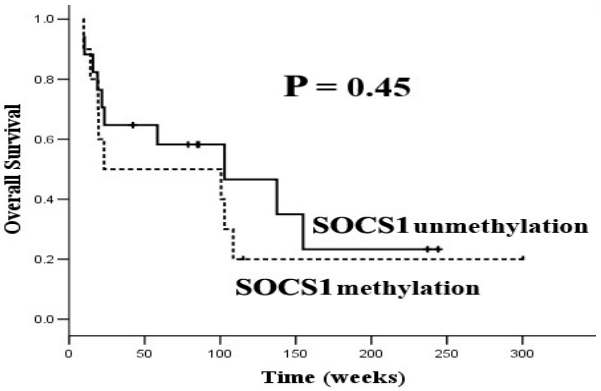

Figure 2. Overall Survival of HCC Patients According to Methylated SOCS1 (Dotted Line) and Unmethylated SOCS1 (Bold Line). The median survival time for methylated SOCS1 tumor patients was longer than for unmethylated SOCS1 patients (P=0.45)

Table 1. Association Between SOCS1 Methylation Status, SOCS1 Under-Expression and Clinico-Pathological Data

Parameter	No.	SOCS1 methylation		Odds ratio,	P
		M n (%)	U n (%)	95%CI	
Data in 29 HCCs:					
Methylation Status and Clinico-Pathological					
Gender				2.36, 0.21-25.91	0.62
Male	25	14 (56)	11 (44)		
Female	4	3 (75)	1 (25)		
Age				2.00, 0.45-8.96	0.46
<50	14	7 (50)	7 (50)		
≥50	15	10 (67)	5 (33)		
Histological type				1.25, 0.06-26.87	1.00
MD	9	4 (44)	5 (56)		
PD	2	1 (50)	1 (50)		
Unknown	18	12 (67)	6 (33)		
Tumor size (cm)				0.18, 0.04-0.94	0.06
≤6	14	11 (79)	3 (21)		
>6	15	6 (40)	9 (60)		
Metastasis				0.63, 0.14-2.82	0.71
Negative	14	9 (64)	5 (36)		
Positive	15	8 (53)	7 (47)		
Treatment				0.04, 0.01-0.31	0.001*
CMT	13	3 (23)	10 (77)		
No CMT	16	14 (88)	2 (12)		
HBsAg				5.25, 0.87-31.55	0.10
Positive	13	9 (69)	4 (31)		
Negative	10	3 (30)	7 (70)		
Unknown	6	5 (83)	1 (17)		
Anti-HCV				0.67, 0.05-9.19	1.00
Positive	3	1 (33)	2 (67)		
Negative	14	6 (43)	8 (57)		
Unknown	12	10 (83)	2 (17)		
SOCS1 expression				4.00,0.59-27.25	0.20
Positive	14	6 (43)	8 (57)		
Reduced	8	6 (75)	2 (25)		
Unknown	7	5 (71)	2 (29)		
Data among 22 HCCs:					
Under-Expression and Clinico-Pathological					
Gender				2.00, 0.22-17.89	0.60
Male	18	6 (33)	12 (67)		
Female	4	2 (50)	2 (50)		
Age				1.67, 0.28-9.82	0.67
<50	10	3 (30)	7 (70)		
≥50	12	5 (42)	7 (58)		
Histological type				-	1.00
MD	5	2 (40)	3 (60)		
PD	1	1 (100)	0		
Unknown	16	5 (31)	11 (69)		
Tumor size (cm)				1.25-0.21-7.41	1.00
≤6	9	3 (33)	6 (67)		
>6	13	5 (38)	8 (62)		
Metastasis				0.40, 0.66-2.44	0.39
Negative	14	4 (29)	10 (71)		
Positive	8	4 (50)	4 (50)		
Treatment				0.33, 0.05-2.02	0.38
CMT	12	3 (25)	9 (75)		
No CMT	10	5 (50)	5 (50)		
HBsAg				-	0.23
Negative	10	4 (40)	6 (60)		
Positive	6	0	6 (100)		
Unknown	6	4 (67)	2 (33)		
anti-HCV				3.33, 0.16-10.91	0.48
Negative	13	3 (23)	10 (77)		
Positive	2	1 (50)	1 (50)		
Unknown	7	4 (57)	3 (43)		

* 'CI=confidence interval; MD=moderate differentiation; PD, poor differentiation; CMT, chemotherapeutic treatment; HBsAg, hepatitis B surface antigen; anti-HCV, antibody to hepatitis C virus; M, methylated sequence; U, unmethylated sequence; *statistically significant association (P<0.05)

Table 2. Multivariate Analysis of Prognostic Factors for Survival of HCC Patients by Cox Regression Analysis

Variable	Relative risk	95%CI	P
Tumor size; <5 vs ≥5	5.35	0.78-36.8	0.09
Metastasis; positive vs negative	0.87	0.21-3.58	0.84
Treatment; No CMT vs CMT	0.38	0.08-1.79	0.22
SOCS1 hypermethylation status; + vs -	1.75	0.39-7.82	0.46
SOCS1 underexpression, + vs -	0.26	0.05-1.27	0.10

differentiation, tumor size, metastasis, treatment, serum HBsAg and anti-HCV-were examined by Fisher's exact tests. Survival was analyzed for patients followed up for at least 200 weeks, or until death, after surgery. One patient who died within four weeks, and one patient who was lost to follow-up, were excluded. Thus, only 27 patients were available for survival analysis. The Kaplan-Meier analysis and log-rank tests were used to analyze overall survival. Cox regression analysis was utilized to estimate the prognostic effect for aberrant SOCS1 gene methylation on HCC patient survival. A significant correlation was considered where P value <0.05.

Results

SOCS1 gene methylation and expression were detected in 29 pairs of tumor, and 22 corresponding normal, liver tissues. Aberrant SOCS1methylation was not found in any normal liver tissues. SOCS1 hypermethylation was detected in 17 of 29 cases (59%) tumor samples, while reduced gene expression was determined in 8 of 22 cases (36%) tumors, of which 6 cases also showed SOCS1 hypermethylation. However, no correlation was found between hypermethylation and SOCS1 expression (P=0.20) (Table 1.). In addition, a higher prevalence of SOCS1 methylation was significantly correlated with HCC treatment without chemotherapeutic drugs (OR=0.04, 95%CI =0.01-0.31; P=0.001), as shown in Table 1. No association was found between SOCS1 methylation and other clinicopathological parameters, nor any relation between gene expression and clinicopathological data (Table 1).

Kaplan-Meier analysis showed that patients with methylated SOCS1 had a tendency towards longer survival than those without (median survival time: 102.86 weeks vs 23.14 weeks), but without significant difference (P=0.45), as shown in Figure 2. Multivariate Cox regression analysis showed no significant correlation between overall survival and SOCS1 hypermethylation (P=0.46, see Table 2).

Discussion

This study used MS-PCR to examine DNA methylation in HCC, since it is sensitive and specific for detecting methylation of CpG sites in a CpG island, and can detect as few as 1 methylated allele in 1000 unmethylated alleles. In addition, sensitive MS-PCR can detect 0.1% cancer cell DNA from a heterogeneous cell population (Herman et al., 1994; Herman et al., 1996).

Recent studies have reported that aberrant SOCS1 gene methylation is frequently inactivated in HCC, such that

SOCS1 hypermethylation might be a key event for HCC transformation of cirrhotic nodules (Okochi et al., 2003) methylation of SOCS1 was more frequently observed in liver fibrosis, and HCV related-HCCs (Yang et al., 2003; Yoshida et al., 2004; Ko et al., 2008). A previous study demonstrated that SOCS1 gene methylation was correlated with advanced tumor stage and lymph node metastasis in gastric carcinoma (Oshimo et al., 2004). However, in this study, we found no significant correlation between gene methylation and prognostic parameter. Only 29 HCC were used in this study. Therefore, a larger sample size is needed to confirm this finding. We demonstrated that a higher prevalence of SOCS1 hypermethylation was significantly correlated with HCC treatment without chemotherapeutic drugs, and SOCS1 had a tendency to improve survival compared with those without methylated SOCS1 (median survival time: 102.86 weeks vs 23.14 weeks). Thus, our findings suggested that epigenetic aberrant SOCS1 methylation may be useful as a predictive marker for HCC.

In previous study, the loss of gene expression in HCCs, often related with hypermethylation of SOCS1. This study found reduced SOCS1 gene expression in 8 of 22 cases (36%), of which 6 cases were also hypermethylated. The reduced expression in another 2 tumors showing non-hypermethylation might be related to other gene-altering mechanisms. Likewise, the study by Lehmann et al. (2007) suggested that expression loss in the absence of hypermethylation could be due to genomic alterations (deletion or mutation), or other regulatory mechanisms. Six of 14 cases showed hypermethylation correlated with expression. This finding may result from one allele having escaped inactivation or partial methylation (Kuroki et al., 2003; Komazaki et al., 2004). However, our results showed that SOCS1 expression was not significantly correlated with SOCS1 hypermethylation among HCC patients. Confirmation of this finding requires further study with a larger sample size.

The study also found that patients with methylated SOCS1 tended to survive longer than patients without. Our results concur with other reports, i.e., that median survival time in recurrent HCC was longer for those with SOCS1 than for those without (Ko et al., 2008). SOCS1 hypermethylation also correlates with a good prognosis in colorectal cancer (Lee et al., 2008). Recently, several hypermethylated genes that correlated with improved survival among cancer patients have been reported, e.g., comethylation of E-cadherin and H-cadherin, 14-3-3sigma and RASSF1A hypermethylation, were related to significantly longer overall survival among patients with non-small-cell lung cancer (Ramirez et al., 2005; Fischer et al., 2007; Kim et al., 2007). Aberrant promoter CpG island hypermethylation of the APC gene served as a good prognostic factor in squamous cell carcinoma of the esophagus (Kim et al., 2009), while p16 and MGMT comethylation showed an association with longer survival among patients with colorectal carcinoma (Krtolica et al., 2009).

In conclusions, we evaluated the abnormal methylation status of SOCS1 by MS-PCR. SOCS1 hypermethylation was correlated with the non-chemotherapeutic treatment HCC patients group. Our data suggested that epigenetic abnormal SOCS1 methylation may be a useful predictive marker for HCC.

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