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Aberrant methylation of *SOCS-1* was observed in younger colorectal cancer patients

Shinichi Fujitake*, Kenji Hibi*, Osamu Okochi, Yasuhiro Kodera, Katsuki Ito, Seiji Akiyama, and Akimasa Nakao

Gastroenterological Surgery, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8560, Japan

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Background. Recently, it was demonstrated that suppressor of cytokine signaling-1 (*SOCS-1*) was frequently silenced by methylation of its CpG island in human hepatocellular carcinoma (HCC). To define the role of *SOCS-1* in the tumorigenic pathway of the colorectum, we examined the methylation of *SOCS-1* in tumors of colorectal cancer patients. ***Methods.*** We examined 74 colorectal cancer patients, using a methylation-specific polymerase chain reaction (PCR; MSP) for *SOCS-1* CpG island in primary tumors. ***Results.*** Aberrant methylation of the *SOCS-1* CpG island was detected in 6 of the 74 (8%) colorectal cancer specimens. No corresponding normal colorectal tissues showed *SOCS-1* methylation. We then analyzed the correlation between the clinicopathological features and *SOCS-1* aberrant methylation and found that younger age was significantly related to *SOCS-1* methylation ($P = 0.048$). ***Conclusions.*** These findings suggested that *SOCS-1* may act as a tumor suppressor in at least some colorectal cancers and that *SOCS-1* methylation may be a particular phenomenon related to an early onset of colorectal cancer.

Key words: *SOCS-1*, methylation, colorectal cancer

Introduction

There is now good evidence that a series of genetic alterations in both dominant oncogenes and tumor suppressor genes is involved in the pathogenesis of human colorectal cancer. Activation of oncogenes, such as the *ras* gene, and inactivation of tumor suppressor genes, such as the *APC* and *p53* genes, have been identified in colorectal cancer.[1–3] In addition, we have found that several other genes are related to the pathogenesis of colorectal cancer.[4,5] Investigation of genetic changes is important to clarify the tumorigenic pathway of colorectal cancer.[6]

Recently, Yoshikawa et al.[7] demonstrated that suppressor of cytokine signaling-1 (*SOCS-1*) was frequently silenced by methylation of the CpG island in human hepatocellular carcinoma (HCC). SOCS-1 is an intracellular protein that negatively regulates the Janus kinase (JAK) and the signal transducer and activation of transcription (STAT) signaling pathway, a principal cytokine signaling transduction pathway. The JAK/STAT pathway is also known to play an important role in the regeneration of hepatocytes,[8,9] and additional studies have indicated that both JAK and STAT pathways are involved in the oncogenesis of several tumors.[10,11] These findings suggested a potential role of the SOCS protein, a growth suppressor in hepatocarcinogenesis through negative regulation of the JAK/STAT pathway.

These results prompted us to examine *SOCS-1* status in colorectal cancer, because there are, as yet no such reports. In this study, we examined the methylation status of *SOCS-1* in 74 resected primary colorectal cancers, using a methylation-specific polymerase chain reaction (PCR; MSP), and we correlated the results with the clinicopathological features of affected patients.

Patients, materials, and methods

Sample collection and DNA preparation

Seventy-four primary tumors and corresponding normal tissues were collected consecutively at Nagoya University Hospital from colorectal cancer patients during colorectal surgery performed from 2000 to 2002. All tissues were confirmed histologically. Collected samples

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Reprint requests to: K. Hibi
* These authors contributed equally to this work

Table 1. Clinicopathological features and the methylation status of *SOCS-1* CpG island in colorectal cancer patients

Clinicopathological features	Variable	No. of cases	*SOCS-1* methylation		*P* value
			+	−	
Sex	Male	43	2	41	0.230[b]
	Female	31	4	27	
Age (years)	22–83	74	52.0 ± 15.2[c]	61.6 ± 10.8[c]	0.048[a]
Maximal tumor size	15–100 mm	74	52.8 ± 15.4[c]	48.1 ± 18.6[c]	0.546[a]
Histology	Mod[e]	67	6	61	>0.999[b]
	Others	7	0	7	
Extent of tumor	≦pm[d]	19	3	16	0.17[b]
	>pm	55	3	52	
Dukes' stage	A, B	45	3	42	0.679[b]
	C, D	29	3	26	
Total		74	6	68	

[a] Student's *t*-test
[b] Fisher's exact test
[c] Mean ± SD
[d] Proper muscle
[e] Mederately differentiated adenocarcinoma

were stored immediately at −80°C until analysis. DNA was prepared as described previously.[12] The clinicopathological profiles of patients enrolled in the study are shown in Table 1.

Sodium bisulfite modification

One microgram of genomic DNA extracted from tumors and corresponding normal colorectal tissues was subjected to bisulfite treatment, as described previously.[13] 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 the Wizard DNA Clean-Up System (Promega, Madison, WI, USA), precipitated with ethanol, and resuspended in distilled water.

MSP

The bisulfite-treated DNA was amplified by MSP. The primers for the unmethylated reaction were: SOCS UM S (sense), 5′-TTATGAGTATTTGTGTGTATTTTT AGGTTGGTT and SOCS UM AS (antisense), 5′-CACTAACAACACAACTCCTACAACAACCA. The primers for the methylated reaction were: SOCS M S (sense), 5′-TTCGCGTGTATTTTTAGGTCGGTC and SOCS M AS (antisense), 5′-CGACACAACTCCT ACAACGACCG. The PCR reaction was hot-started at 95°C for 5 min, and followed a protocol consisting of 35 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min, and 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. The methylation status of these cell line DNAs was confirmed by a previous study.[7] A control reaction without DNA was performed for each set of PCRs. Ten microliters of each PCR product was 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.[14] The *SOCS-1* probe for hybridization was designed as nucleotide position 679–1030 of the *SOCS-1* cDNA sequence. A human β-actin probe was used as an internal control.

Reverse transcription (RT)-PCR

First-strand cDNA was generated from RNA. The PCR amplification consisted of 30 cycles (95°C for 30 s, 55°C for 1 min, and 72°C for 1 min) after the initial denaturation step (95°C for 2 min). The primers used were: SOCS-1 S (sense), 5′-ACGCAGCATTAACTGGG ATG, and SOCS-1 AS (antisense), 5′-AAATAGGA TTCTGCACAGCAG. The predicted size of the PCR product was 352 bp. The housekeeping gene, β-actin, was used as an internal control to confirm the success of the RT reaction.

Statistical analysis

The association between *SOCS-1* methylation status and clinicopathological findings was examined using

Student's *t*-test or Fisher's exact test. *P* Values computed were two-tailed. Statistical significance was considered as $P < 0.05$.

Results

To confirm that the *SOCS-1* gene was inactivated by methylation, we first examined the *SOCS-1* methylation status and mRNA expression in Hep3B and HuH-7 cell lines, because the methylation status of these cell line DNAs was described in a previous report.[7] As described previously, HuH-7 with unmethylated DNA demonstrated substantial *SOCS-1* expression, while Hep3B with methylated DNA did not express *SOCS-1* (Fig. 1). We also examined the methylation and expression status of *SOCS-1* in colorectal cancer cell lines using MSP and RT-PCR. Col_3Jck with unmethylated DNA demonstrated substantial *SOCS-1* expression, while SW480 and SW1083 with methylated DNA did not express *SOCS-1* (Fig. 2). As described previously, 5-aza-dC treatment reactivated *SOCS-1* expression.[15] This result indicated that *SOCS-1* methylation was not a secondary event caused by gene repression due to loss of transcription factor.

Subsequently, we examined the methylation status of *SOCS-1* CpG island in 74 colorectal cancer and corresponding normal tissue specimens, using MSP. Aberrant methylation of the *SOCS-1* gene was detected in 6 of the 74 (8%) colorectal cancer specimens (Fig. 3). All tumors also exhibited unmethylation of the *SOCS-1* gene, which may account for the contamination of non-tumorous cells in tumor specimens. Otherwise, it is possible that *SOCS-1* gene expression was not completely inhibited in these cancers. No corresponding normal colorectal tissues showed *SOCS-1* methylation.

We then analyzed the correlations between the clinicopathological data and *SOCS-1* aberrant methylation (Table 1). We found that age was significantly related to *SOCS-1* methylation ($P = 0.048$). No other factors, such as sex, histology, tumor size, extent of tumor, or Dukes' stage were correlated with the *SOCS-1* methylation status.

Discussion

Colorectal cancer is one of the most aggressive cancers, and it occurs at a high incidence in most countries.[16] To eliminate this fatal cancer, we perform surgical operations and subsequent chemotherapy and radiotherapy. For this purpose, it is important to seek genetic alterations as a new parameter for estimation of the malignancy of the cancer.

To date, little is known about the role of *SOCS-1* in colorectal cancer. Recently, a potential role of the JAK/

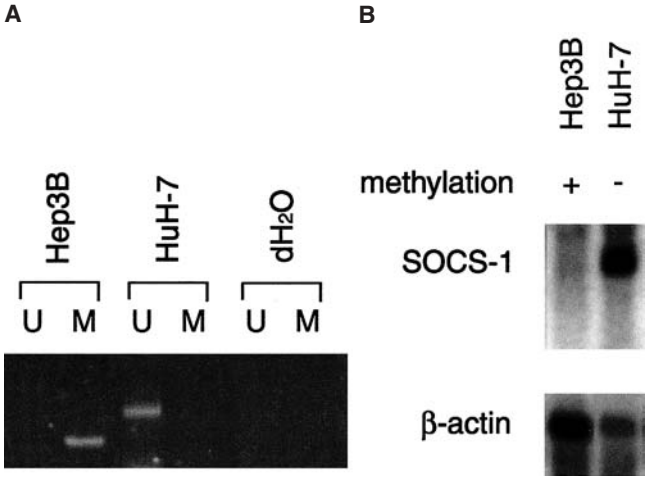

Fig. 1A,B. Methylation-specific polymerase chain reaction (PCR; MSP) and Northern analyses of Hep3B and HuH-7 cell lines. **A** MSP. Unmethylated PCR products are seen as a 175-bp band in lane *U*, and methylated PCR products as a 160-bp band in lane *M*. **B** Northern analysis. A 1.3-kb band was detected with the *SOCS-1*-specific probe

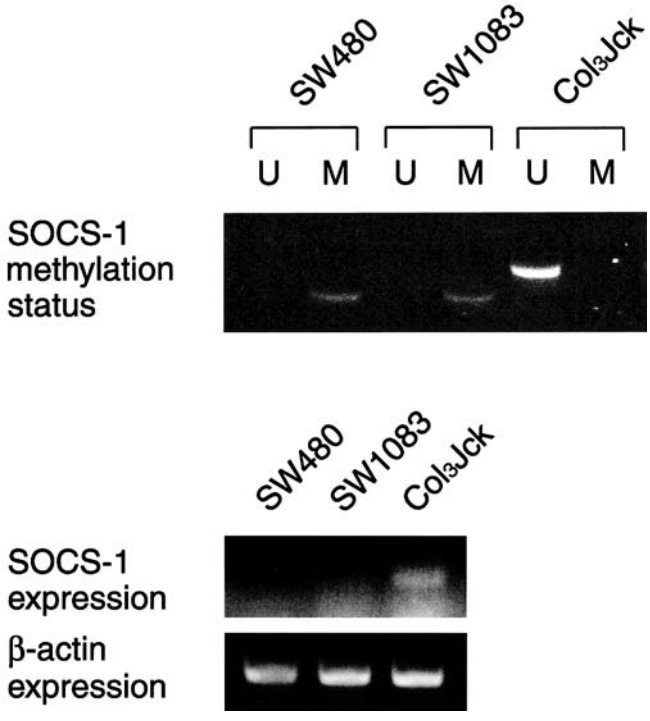

Fig. 2. MSP and reverse transcription (RT)-PCR of SW480, SW1083, and Col_3Jck colorectal cancer cell lines. SW480 and SW1083, with methylated DNA, lacked *SOCS-1* gene expression, while *SOCS-1* was expressed in Col_3Jck, with unmethylated DNA

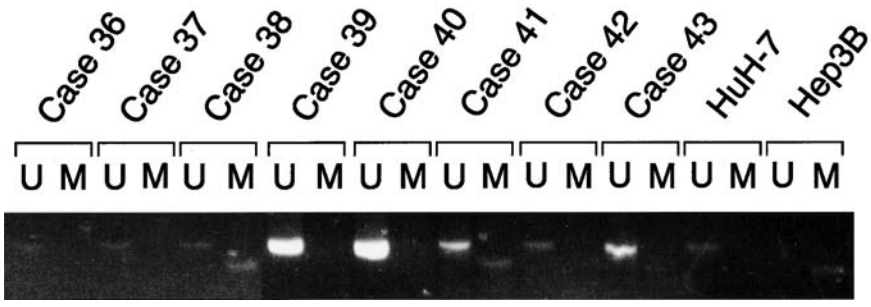

Fig. 3. Representative MSP analysis of *SOCS-1* in colorectal cancers. *SOCS-1* methylation was present in cases 38 and 41. DNA from Hep3B and HuH-7 cell lines was used as a positive control for methylated and unmethylated alleles, respectively

STAT pathway has been proposed for oncogenesis in many other kinds of tumors.[10,11,17,18] On the other hand, the SOCS family has been identified as consisting of negative feedback proteins of the cytokine-induced signaling pathway.[19–21] These proteins are activated by STATs and negatively regulate the JAK/STAT pathway be inhibiting the JAKs directly or blocking the access of the STATs. Although the mechanism of SOCS proteins in regulating cytokine signaling has been studied to some extent, the biological roles of these proteins continue to be examined. From recent studies, it was reported that *SOCS-1* expression was suppressed through the 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] On the other hand, *SOCS-1* was also frequently silenced by methylation in multiple myeloma.[15] Another study showed that GFI-1B, a protooncogenic protein derived from GFI-1 in T-cell lymphoma, repressed the reporter activity of *SOCS-1/SOCS-3* promoters.[23] In the present study, we observed the aberrant methylation of *SOCS-1* CpG islands in colorectal cancers for the first time, whereas the same methylation was not detected in corresponding normal tissues. This observation suggested that *SOCS-1* may act as a tumor suppressor in at least some colorectal cancers.

We also compared the methylation status of *SOCS-1* in colorectal cancer patients with their clinicopathological features, and demonstrated that patients with *SOCS-1* methylation were younger than those with no *SOCS-1* methylation. Thus, it was supposed that inactivation of *SOCS-1* may be an important factor for colorectal carcinogenesis in younger patients. The mechanism accounting for CpG island methylation with age is unclear at the present time. In cell culture, many unexpressed nonessential genes appear to acquire de-novo methylation with time,[24] and it is possible that the same process is responsible for age-related hypermethylation.[25] Our result was somehow opposed to these reports, suggesting that *SOCS-1* methylation may be a particular phenomenon related to an early onset of colorectal cancer.

This study provides solid evidence that can be used in further studies on the molecular mechanism of *SOCS-1* in colorectal cancers, and also suggests that *SOCS-1* may play an important role in the carcinogenic pathway in some patients with colorectal cancers. These observations indicate the possibility that tumor formation in the colorectum may be controlled by inducing the expression of silenced *SOCS-1*, using demethylating reagents.

References

1. Bos JL, Fearon ER, Hamilton SR, Verlaan-de Vries M, van Boom JH, van der Eb AJ, et al. Prevalence of *ras* gene mutations in human colorectal cancers. Nature 1987;327:293–7.
2. Nishisho I, Nakamura Y, Miyoshi Y, Miki Y, Ando H, Horii A, et al. Mutations of chromosome 5q21 genes in FAP and colorectal cancer patients. Science 1991;253:665–9.
3. Baker SJ, Markowitz S, Fearon ER, Willson JK, Vogelstein B. Suppression of human colorectal carcinoma cell growth by wild-type p53. Science 1990;249:912–5.
4. Hibi K, Nakamura H, Hirai A, Fujikake Y, Kasai Y, Akiyama S, et al. Loss of H19 imprinting in esophasgeal cancer. Cancer Res 1996;56:480–2.
5. Hibi K, Taguchi M, Nakamura H, Hirai A, Fujikake Y, Matsui T, et al. Alternative splicing of the *FHIT* gene in colorectal cancer. Jpn J Cancer Res 1997;88:385–8.
6. Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, et al. Genetic alterations during colorectal-tumor development. N Engl J Med 1988;319:525–32.
7. Yoshikawa H, Matsubara K, Qian GS, Jackson P, Groopman JD, Manning JE, et al. SOCS-1, a negative regulator of the JAK/STAT pathway, is silenced by methylation in human hepatocellular carcinoma and shows growth-suppression activity. Nat Genet 2001;28:29–35.
8. Cressman DE, Diamond RH, Taub R. Rapid activation of the Stat3 transcription complex in liver regeneration. Hepatology 1995;21:1443–9.
9. Cressman DE, Greenbaum LE, DeAngelis RA, Ciliberto G, Furth EE, Poli V, et al. Liver failure and defective hepatocyte regeneration in interleukin-6-deficient mice. Science 1996;274:1379–83.
10. Lacronique V, Boureux A, Valle VD, Poirel H, Quang CT, Mauchauffe M, et al. A TEL-JAK2 fusion protein with constitutive kinase activity in human leukemia. Science 1997;278:1309–12.
11. Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, et al. Stat3 as an oncogene. Cell 1999;98:295–303.
12. Hibi K, Nakayama H, Koike M, Kasai Y, Ito K, Akiyama S, et al. Colorectal cancers with both *p16* and *p14* methylation show invasive characteristics. Jpn J Cancer Res 2002;93:883–7.

13. Hibi K, Taguchi M, Nakayama H, Takase T, Kasai Y, Ito K, et al. Molecular detection of p16 promoter methylation in the serum of patients with esophageal squamous cell carcinoma. Clin Cancer Res 2001;7:3135–8.
14. Hibi K, Liu Q, Beaudry GA, Madden SL, Westra WH, Wehage SL, et al. Serial analysis of gene expression in non-small cell lung cancer. Cancer Res 1998;58:5690–4.
15. Galm O, Yoshikawa H, Esteller M, Osieka R, Herman JG. SOCS-1, a negative regulator of cytokine signaling, is frequently silenced by methylation in multiple myeloma. Blood 2003;101:2784–8.
16. Greenlee RT, Murray T, Bolden S, Wingo PA. Cancer statistics, 2000. CA Cancer J Clin 2000;50:7–33.
17. Migone TS, Lin JX, Cereseto A, Mulloy JC, O'Shea JJ, Franchini G, et al. Constitutively activated Jak-STAT pathway in T cells transformed with HTLV-1. Science 1995;269:79–81.
18. Danial NN, Pernis A, Rothman PB. Jak-STAT signaling induced by the *v-abl* oncogene. Science 1995;269:1875–7.
19. Starr R, Willson TA, Viney EM, Murray LJL, Rayner JR, Jenkins BJ, et al. A family of cytokine-inducible inhibitors of signaling. Science 1997;387:917–21.
20. Endo TA, Masuhara M, Yokouchi M, Suzuki R, Sakamoto H, Mitsui K, et al. A new protein containing an SH2 domain that inhibits JAK kinases. Science 1997;387:921–4.
21. Naka T, Narazaki M, Hirata M, Matsumoto T, Minamoto S, Aono A, et al. Structure and function of a new STAT-induced STAT inhibitor. Science 1997;387:924–9.
22. Nagai H, Kim YS, Lee KT, Chu MY, Konishi N, Fujimoto J, et al. Inactivation of SSI-1, a JAK/STAT inhibitor, in human hepatocellular carcinomas, as revealed by two-dimensional electrophoresis. J Hepatol 2001;34:416–21.
23. Jegalian AG, Wu H. Regulation of Socs gene expression by the protooncoprotein GFI-1B: two routes for STAT5 target gene induction by erythropoietin. J Biol Chem 2002;277:2345–52.
24. Antequera F, Boyes J, Bird A. High levels of de novo methylation and altered chromatin structure at CpG islands in cell lines. Cell 1990;62:503–4.
25. Issa J-PJ, Ottaviano YL, Celano P, Hamilton SR, Davidson NE, Baylin SB. Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon. Nat Genet 1994;7:536–40.