Highly-methylated Colorectal Cancers Show Poorly-differentiated Phenotype

KENJI HIBI[1,2] and AKIMASA NAKAO[2]

[1]*Department of Surgery, Showa University Fujigaoka Hospital, 1-30 Fujigaoka, Aoba-ku, Yokohama 227-8501;*
[2]*Gastroenterological Surgery, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8560, Japan*

Abstract. *The combined methylation status of p16, p14, HLTF, SOCS-1, CDH13, RUNX3 and CHFR was examined in 58 resected primary colorectal cancers using methylation-specific PCR, and the methylation status was correlated with the clinicopathological features of the affected patients. A significant difference in histology (p=0.0041) was observed when the number of methylated genes of poorly-differentiated colorectal cancers was compared to that of other differentiated ones. Poorly-differentiated colorectal cancers preferentially exhibited gene methylation.*

There is now strong evidence that a series of genetic alterations in both dominant oncogenes and tumor suppressor genes are involved in the pathogenesis of human colorectal cancer. The activation of oncogenes, such as the *ras* gene, and the inactivation of tumor suppressor genes, such as the *APC* and *p53* genes, have been identified (1-3). In addition, several other genes were found to be related to the pathogenesis of colorectal cancer (4, 5) and investigation of genetic changes is important in clarifying its tumorigenic pathway (6).

Several tumor suppressor genes contain CpG islands in their promoters, prompting many investigations into the role of methylation in silencing these genes. Many tumor suppressor genes show evidence of methylation silencing, providing a new potential pathway for the deactivation of tumor suppressor genes. At first, a tumor suppressor gene, *p16*, was found to harbor promoter hypermethylation associated with the loss of protein expression in cancer cells (7). Though homozygous deletions of the *p16* locus were not present (8), *p16* promoter methylation was detected in colorectal cancer (9). Subsequently, it has been found that human *p14* was also silenced by promoter hypermethylation in colorectal cancer (10). *p14* interacts with the MDM2 protein and neutralizes the MDM2-mediated degradation of *p53*. Thus, *p14* acts as a tumor suppressor gene *via* inhibition of *p53* degradation. These studies indicated that *p16* and *p14* inactivation due to promoter methylation was important for colorectal tumorigenesis. We previously examined the methylation status of these genes in 86 primary colorectal cancers using methylation-specific PCR (MSP) (11). Aberrant promoter methylation of *p16* and *p14* genes was detected in 43 out of 86 (50 %) and 25 out of 86 (29%) colorectal cancer specimens, respectively.

The loss of expression of a helicase-like transcription factor (HLTF), a SWI/SNF family member gene, accompanied by HLTF promoter methylation was reported in colon cancers (12). In this report, all colon cancer cell lines that lacked the HLTF gene expression demonstrated methylation of CpG sites within the putative HLTF promoter, while methylation was not detected in the HLTF-expressing cell lines. Moreover, HLTF methylation was detected in 25 out of 76 primary colon cancers, suggesting that HLTF is a common target for methylation and epigenetic gene silencing in colon cancer and qualifies as a potential colon cancer suppressor gene (13).

Yoshikawa *et al.* demonstrated that suppressor of cytokine signaling-1 (*SOCS-1*) was silenced frequently by methylation of the CpG island in human HCC (14). *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 (15, 16) and additional studies indicated that both JAKs and STATs were involved in the oncogenesis of several tumors (17, 18). These findings suggested a potential role of the SOCS protein, a growth suppressor in hapatocarcinogenesis through negative regulation of the JAK/STAT pathway. The aberrant methylation of the *SOCS-1* CpG island in 6 out of 74 (8%) colorectal cancer specimens was also detected in our laboratory (19). The correlation between the clinicopathological features

Correspondence to: Kenji Hibi, Department of Surgery, Showa University Fujigaoka Hospital, 1-30 Fujigaoka, Aoba-ku, Yokohama 227-8501, Japan. Tel: +81-45-971-1151, Fax: +81-45-973-1019, e-mail: kenjih-ngy@umin.ac.jp

Key Words: Methylation, colorectal cancer, *p16, p14, HLTF, SOCS-1, CDH13, RUNX3, CHFR.*

and *SOCS-1* aberrant methylation was subsequently analyzed and younger age was found to be significantly related to *SOCS-1* methylation ($p=0.048$).

Subsequently, it also recently became clear that *CDH13* (H-cadherin, T-cadherin) expression is frequently silenced by aberrant methylation in colorectal cancers and adenomas (20, 21). *CDH13* encodes a protein belonging to the cadherin family of cell surface glycoproteins responsible for selective cell recognition and adhesion (22). Ubiquitous methylation of *CDH13* in colorectal cancers and adenomas indicated that such methylation occurs at an early stage in the multistage process of oncogenesis.

The loss of expression of *RUNX3*, a transcription factor containing a highly conserved domain, designated as a 'runt domain', accompanied by *RUNX3* gene methylation was reported in gastric cancers (23). In this report, between 45% and 60% of human gastric cancer cells did not significantly express RUNX3 due to hemizygous deletion and hypermethylation of the *RUNX3* gene. The tumorigenecity of human gastric cancer cell lines in nude mice was inversely related to their level of *RUNX3* expression, suggesting that *RUNX3* is a common target for methylation and epigenetic gene silencing in gastric cancer and qualifies as a potential gastric cancer suppressor gene. Aberrant promoter methylation of the *RUNX3* gene was detected in 31 out of 92 (34%) colorectal cancers (24).

In recent years, the *CHFR* (checkpoint with the FHA and RING finger) gene was reported as a new mitotic checkpoint gene. *CHFR* protein delayed chromosome condensation and entry into metaphase in response to the mitotic stress induced by a microtubule inhibitor (25). However, cancer cell lines lacking *CHFR* entered into metaphase without any delay after treatment with a microtubular inhibitor, thus, suggesting the function of *CHFR* as a mitotic checkpoint. Recently, the aberrant methylation of the *CHFR* gene associated with gene silencing was reported in several primary tumors (26-30). The aberrant methylation of the *CHFR* gene was also detected in 25 out of 98 (26%) primary colon cancers. This finding suggested that an aberrant methylation of the *CHFR* gene occurs frequently in colorectal cancers.

In this study, the combined methylation status of *p16, p14, HLTF, SOCS-1, CDH13, RUNX3* and *CHFR* was examined in 58 resected primary colorectal cancers using methylation-specific PCR (MSP). To investigate whether the methylation status could be a marker for the malignancy of colorectal cancers, the methylation status was correlated with the clinicopathological features of affected patients.

Materials and Methods

Sample collection and DNA preparation. Fifty-eight primary tumors and corresponding normal tissues were collected consecutively at Nagoya University Hospital from colorectal cancer patients. All tissues were confirmed histologically. The collected samples were stored immediately at –80˚C until analysis. DNA was prepared as described previously (31).

Sodium bisulfite modification. One μg of genomic DNA extracted from tumors and corresponding normal colorectal tissues was subjected to bisulfite treatment, as described previously (32). 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 by 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 with MSP. The primers and PCR conditions were described previously (7, 10, 12, 14, 20, 23). Ten μl of each PCR product were loaded directly onto nondenaturing 8% polyacrylamide gels, stained with ethidium bromide and visualized under UV illumination.

Statistical analysis. The possible association between the number of methylated genes and clinicopathological features was examined using the Chi-squared test or Student's *t*-test. The computed *p*-values were two-tailed. Statistical significance was considered as $p<0.05$.

Results

The methylation status of *p16, p14, HLTF, SOCS-1, CDH13, RUNX3* and *CHFR* genes was examined in colorectal cancer and corresponding normal tissue specimens using MSP, as described previously (11, 13, 19, 21, 24). The methylation status of *p16, p14, HLTF, SOCS-1, CDH13, RUNX3* and *CHFR* genes were originally examined in 86, 86, 76, 74, 84, 92, and 98 colorectal cancer specimens, respectively. Fifty-eight specimens were tested for the methylation status of all 7 genes. Aberrant methylation of *p16, p14, HLTF, SOCS-1, CDH13, RUNX3* and *CHFR* genes was detected in 34 (58%), 20 (34%), 18 (31%), 6 (8%), 22 (37%), 22 (37%) and 25 (26%) of 58 colorectal cancer specimens, respectively (Figure 1). All tumors also exhibited unmethylation of each gene, which might be the result of the contamination of non-tumorous cells in the tumor specimens. Otherwise, it might be possible that these gene expressions had not been completely inhibited in these cancer specimens.

The correlation between the methylation status and clinicopathological data was subsequently analyzed (Table I). Colorectal cancers with more than 4 methylated genes were included in the highly-methylated group (HMG), while colorectal cancers with less than 3 methylated genes made the low-methylated group (LMG). A significant difference in histology ($p=0.0041$) was found when the number of methylated genes of poorly-differentiated colorectal cancers was compared to that of other differentiated ones (Table I), suggesting that poorly-differentiated colorectal cancers preferentially exhibited gene methylation. No other factors such as gender, age, tumor size, extent of tumor, tumor site, or lymph node metastasis were correlated with the number of methylated genes.

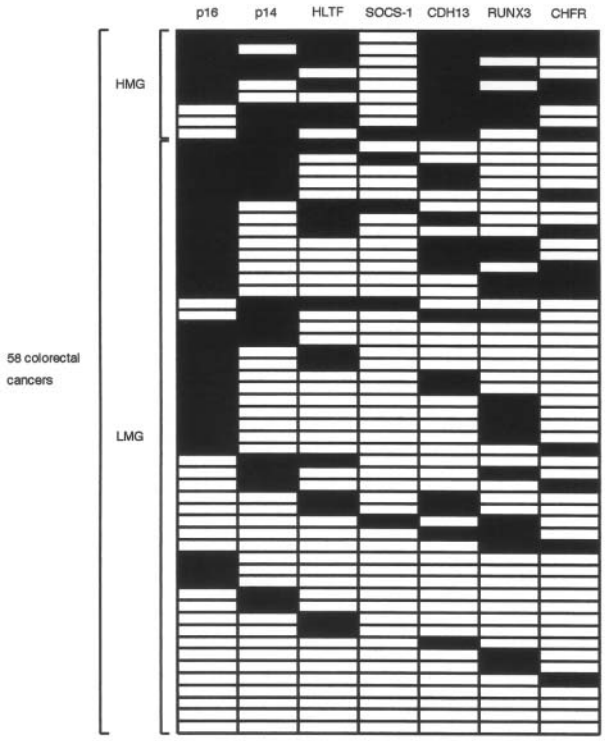

Figure 1. *Methylation status of p16, p14, HLTF, SOCS-1, CDH13, RUNX3 and CHFR genes in 58 colorectal cancers. The methylated gene is shown as a closed box. Colorectal cancers with more than 4 methylated genes were included in the highly-methylated group (HMG) while colorectal cancers with less than 3 methylated genes in the low-methylated group (LMG).*

Table I. *Clinicopathologic features and methylation status in colorectal cancer patients.*

Clinicopathological features	Variables	No. of cases	Methylation status		*p*-value
			HMG	LMG	
Gender	Male	32	6	26	0.451[a]
	Female	26	3	23	
Age	22-83	58	64±10[c]	61±10	0.230[b]
Maximum tumor size	15-100 mm	58	38±11	47±18	0.906[b]
Extent of tumor	≤pm[d]	17	3	14	0.773[a]
	pm<	41	6	35	
Histology	Well[e]	53	6	47	0.0041[a]
	Poor[f]	5	3	2	
Tumor site	C, A, T[g]	18	3	15	0.871[a]
	D, S, R[h]	40	6	34	
Lymph node	–	34	5	29	0.839[a]
metastasis	+	24	4	20	
Total		58	9	49	

[a]chi-squared test; [b]Student's t-test; [c]mean±S.D.; [d]proper muscle; [e]well- or moderately-differentiated adenocarcinoma; [f]poorly-differentiated or mucinous adenocarcinoma; [g]C, cecum; A, ascending colon; T, transverse colon; [h]D, descending colon; S, sigmoid colon; R, rectum.

Discussion

Our results indicated that poorly-differentiated colorectal cancer specimens preferentially exhibited a higher number of methylated genes including *p16, p14, HLTF, SOCS-1, CDH13, RUNX3* and *CHFR*. Therefore, the methylation of these genes is a high malignancy factor, as it is for lymph node metastasis. Shannon *et al.* also investigated the methylation status of *hMLH1, p16* and *MDR1* genes in 58 colorectal cancers (33) and found methylation of the *hMLH1, p16* and *MDR1* genes in 23, 29, and 28% of colorectal cancers, respectively. As a result, these 3 genes were significantly methylated in MSI+ colorectal cancers compared to MSI– cancers. Additionally, these genes were significantly associated with poor histological differentiation. This result supports our notion that poorly-differentiated colorectal cancers preferentially exhibit gene methylation.

Colorectal cancers are classified histopathologically as either differentiated carcinomas forming tubular or papillary structures, or poorly-differentiated carcinomas including mucinous adenocarcinoma, in which such structures are inconspicuous. Poorly-differentiated colorectal carcinomas are quite rare, comprising only 3-5% of all colorectal carcinomas. It is well-known that mucinous carcinoma is frequently observed in colorectal cancer with genetic instability, but the difference in genetic pathways between these histological types is mostly unknown because of the very small number of cases (34).

Colorectal cancer, one of the most aggressive cancers, occurs at a high incidence rate in most countries (35). To eliminate this fatal cancer, surgery and subsequent chemotherapy and radiotherapy are generally performed. For this purpose, it is important to seek new genetic and epigenetic alteration parameters to estimate cancer malignancy.

References

1 Bos JL, Fearon ER, Hamilton SR, Verlaan-de Vries M, van Boom JH, van der Eb AJ and Vogelstein B: Prevalence of ras gene mutations in human colorectal cancers. Nature *327*: 293-297, 1987.

2 Nishisho I, Nakamura Y, Miyoshi Y, Miki Y, Ando H, Horii A, Koyama K, Utsunomiya J, Baba S and Hedge P: Mutations of chromosome 5q21 genes in FAP and colorectal cancer patients. Science *253*: 665-669, 1991.

3 Baker SJ, Markowitz S, Fearon ER, Willson JK and Vogelstein B: Suppression of human colorectal carcinoma cell growth by wild-type p53. Science *249*: 912-915, 1990.

4 Hibi K, Nakamura H, Hirai A, Fujikake Y, Kasai Y, Akiyama S, Ito K and Takagi H: Loss of H19 imprinting in esophageal cancer. Cancer Res *56*: 480-482, 1996.

5 Hibi K, Taguchi M, Nakamura H, Hirai A, Fujikake Y, Matsui T, Kasai Y, Akiyama S, Ito K and Takagi H: Alternative splicing of the FHIT gene in colorectal cancer. Jpn J Cancer Res *88*: 385-388, 1997.

6 Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AM and Bos JL: Genetic alterations during colorectal-tumor development. N Engl J Med *319*: 525-532, 1988.

7 Herman JG, Graff JR, Myohanen S, Nelkin BD and Baylin SB: Methylation-specific PCR: A novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA *93*: 9821-9826, 1996.

8 Cairns P, Polascik TJ, Eby Y, Tokino K, Califano J, Merlo A, Mao L, Herath J, Jenkins R, Westra W, Rutter JL, Buckler A, Gavielson E, Tockman M, Cho KR, Hedrick L, Bova GS, Issacs W, Schwab D and Sidransky D: Frequency of homozygous deletion at p16/CDKN2 in primary human tumours. Nat Genet *11*: 210-212, 1995.

9 Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB and Issa J-P: CpG island methylator phenotype in colorectal cancer. Proc Natl Acad Sci USA *96*: 8681-8686, 1999.

10 Esteller M, Tortola S, Toyota M, Capella G, Peinado MA, Baylin SB and Herman JG: Hypermethylation-associated inactivation of $p14^{ARF}$ is independent of $p16^{INK4a}$ methylation and p53 mutational status. Cancer Res *60*: 129-133, 2000.

11 Hibi K, Nakayama H, Koike M, Kasai Y, Ito K, Akiyama S and Nakao A: Colorectal cancers with both *p16* and *p14* methylation show invasive characteristics. Jpn J Cancer Res *93*: 883-887, 2002.

12 Moinova HR, Chen W-D, Shen L, Smiraglia D, Olechnowicz J, Ravi L, Kasturi L, Myeroff L, Plass C, Parsons R, Minna J, Willson JK and Green SB: HLTF gene silencing in human colon cancer. Proc Natl Acad Sci USA *99*: 4562-4567, 2002.

13 Hibi K, Nakayama H, Kanyama Y, Kodera Y, Ito K, Akiyama S and Nakao A: Methylation pattern of HLTF gene in digestive tract cancers. Int J Cancer *104*: 433-436, 2003.

14 Yoshikawa H, Matsubara K, Qian GS, Jackson P, Groopman JD, Manning JE, Harris CC and Herman JG: 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 *28*: 29-35, 2001.

15 Cressman DE, Diamond RH and Taub R: Rapid activation of the Stat3 transcription complex in liver regeneration. Hepatology *21*: 1443-1449, 1995.

16 Cressman DE, Greenbaum LE, DeAngelis RA, Ciliberto G, Furth EE, Poli V and Taub R: Liver failure and defective hepatocyte regeneration in interleukin-6-deficient mice. Science *274*: 1379-1383, 1996.

17 Lacronique V, Boureux A, Valle VD, Poirel H, Quang CT, Mauchauffe M, Berthou C, Lessard M, Berger R, Ghysdael J and Bernard OA: A TEL-JAK2 fusion protein with constitutive kinase activity in human leukemia. Science *278*: 1309-1312, 1997.

18 Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C and Darnell JE Jr: Stat3 as an oncogene. Cell *98*: 295-303, 1999.

19 Fujitake S, Hibi K, Okochi O, Kodera Y, Ito K, Akiyama S and Nakao A: Aberrant methylation of SOCS-1 was observed in younger colorectal cancer patients. J Gastroenterol *39*: 120-124, 2004.

20 Toyooka S, Toyooka KO, Harada K, Miyajima K, Makarla P, Sathyanarayama UG, Yin J, Sato F, Shivapurkar N, Meltzer SJ and Gazdar AF: Aberrant methylation of the CDH13 (H-cadherin) promoter region in colorectal cancers and adenomas. Cancer Res *62*: 3382-3386, 2002.

21 Hibi K, Nakayama H, Kodera Y, Ito K, Akiyama S and Nakao A: CDH13 promoter region is specifically methylated in poorly differentiated colorectal cancer. Br J Cancer *90*: 1030-1033, 2004.

22 Takeichi M: Cadherin cell adhesion receptors as a morphogenetic regulator. Science *251*: 1451-1455, 1991.

23 Li QL, Ito K, Sakakura C, Fukamachi H, Inoue K, Chi XZ, Lee KY, Nomura S, Lee CW, Han SB, Kim HM, Kim WJ, Yamamoto H, Yamashita N, Yano T, Ikeda T, Itohara S, Inazawa J, Abe T, Hagiwara A, Yamagishi H, Ooe A, Kaneda A, Sugimura T, Ushijima T, Bae SC and Ito Y: Causal relationship between the loss of RUNX3 expression and gastric cancer. Cell *109*: 113-124, 2002.

24 Imamura Y, Hibi K, Koike M, Fujiwara M, Kodera Y, Ito K and Nakao A: RUNX3 promoter region is specifically methylated in poorly-differentiated colorectal cancer. Anticancer Res *25*: 2627-2630, 2005.

25 Scolnick DM and Halazonetis TD: *CHFR* defines a mitotic stress checkpoint that delays entry into metaphase. Nature *406*: 430-435, 2000.

26 Toyota M, Sasaki Y, Satoh A, Ogi K, Kikuchi T, Suzuki H, Mita H, Tanaka N, Itoh F, Issa JP, Jair KW, Schuebel KE, Imai K and Tokino T: Epigenetic inactivation of *CHFR* in human tumors. Proc Natl Acad Sci USA *100*: 7818-7823, 2003.

27 Satoh A, Toyota M, Itoh F, Sasaki Y, Suzuki H, Ogi K, Kikuchi T, Mita H, Yamashita T, Kojima T, Kusano M, Fujita M, Hosokawa M, Endo T, Tokino T and Imai K: Epigenetic inactivation of CHFR and sensitivity to microtubule inhibitors in gastric cancer. Cancer Res *63*: 8606-8613, 2003.

28 Mizuno K, Osada H, Konishi H, Tatematsu Y, Yatabe Y, Mitsudomi T, Fujii Y and Takahashi T: Aberrant hypermethylation of the CHFR prophase checkpoint gene in human lung cancers. Oncogene *21*: 2328-2333, 2002.

29 Shibata Y, Haruki N, Kuwabara Y, Ishiguro H, Shinoda N, Sato A, Kimura M, Koyama H, Toyama T, Nishiwaki T, Kudo J, Terashita Y, Konishi S, Sugiura H and Fujii Y: *CHFR* expression is down-regulated by CpG island hypermethylation in esophageal cancer. Carcinogenesis *23*: 1695-1699, 2002.

30 Corn PG, Summers MK, Fogt F, Virmani AK, Gazdar AF, Halazonetis TD and El-Deiry WS: Frequent hypermethylation of the 5' CpG island of the mitotic stress checkpoint gene *CHFR* in colorectal and non-small cell lung cancer. Carcinogenesis *24*: 47-51, 2003.

31 Hibi K, Takahashi T, Yamakawa K, Ueda R, Sekido Y, Ariyoshi Y, Suyama M, Takagi H and Nakamura Y: Three distinct regions involved in 3p deletion in human lung cancer. Oncogene *7*: 445-449, 1992.

32 Hibi K, Taguchi M, Nakayama H, Takase T, Kasai Y, Ito K, Akiyama S and Nakao A: Molecular detection of p16 promoter methylation in the serum of patients with esophageal squamous cell carcinoma. Clin Cancer Res *7*: 3135-3138, 2001.

33 Shannon BA and Iacopetta BJ: Methylation of the hMLH1, p16, and MDR1 genes in colorectal carcinoma: associations with clinicopathological features. Cancer Lett *167*: 91-97, 2001.

34 Risio M, Reato G, di Celle PF, Fizzotti M, Rossini FP and Foa R: Microsatellite instability is associated with the histological features of the tumor in nonfamilial colorectal cancer. Cancer Res *56*: 5470-5474, 1996.

35 Greenlee RT, Murray T, Bolden S and Wingo PA: Cancer statistics, 2000. CA Cancer J Clin *50*: 7-33, 2000.

Received September 18, 2006
Accepted October 12, 2006