Aberrant Methylation of the *HACE1* Gene is Frequently Detected in Advanced Colorectal Cancer

KENJI HIBI, MAKIKO SAKATA, KAZUMA SAKURABA, ATSUSHI SHIRAHATA, TETSUHIRO GOTO, HIROKI MIZUKAMI, MITSUO SAITO, KAZUYOSHI ISHIBASHI, GAKU KIGAWA, HIROSHI NEMOTO and YUTAKA SANADA

Department of Surgery, Showa University Fujigaoka Hospital, 1-30 Fujigaoka, Aoba-ku, Yokohama 227-8501, Japan

Abstract. *Background: It has been recently reported that HACE1, the E3 ubiquitin ligase, is epigenetically inactivated in human Wilms' tumors and HACE1 expression was also down-regulated in colon carcinomas. Materials and Methods: The methylation status of the HACE1 gene was examined in primary carcinomas and the corresponding normal tissues derived from 32 patients with colorectal cancer using quantitative methylation-specific PCR (qMSP) and the correlation between the methylation status and the clinicopathological findings was evaluated. Results: Aberrant methylation of the HACE1 gene was detected in 9 out of the 32 (28%) primary colon carcinomas, suggesting that the aberrant methylation of HACE1 was frequently observed in colorectal cancer. The clinicopathological data were then correlated with these results. A significant increase was observed in the maximal tumor size of the methylated HACE1 tumors (p=0.0304). Moreover, a trend was shown towards preferentially developing lymph node metastasis in the methylated HACE1 carcinomas (p=0.0612). Conclusion: HACE1 might act as a tumor suppressor in colorectal carcinomas and HACE1 methylation might present a malignant potential in colorectal cancer.*

There is now solid 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* (adenomatous polyposis coli) and *p53* genes, have been identified in colorectal cancer (1-3). In addition, we have also found several other genes to be related to the pathogenesis of colorectal cancer (4, 5). An investigation of genetic changes is important in clarifying the tumorigenic pathway of colorectal cancer (6).

Loss of heterozygosity (LOH) on chromosome 6q has been detected in various human malignancies, including breast, ovarian and prostatic cancer, leukemia, and lymphoma (7-11). Recently, it has been reported that *HACE1*, the E3 ubiquitin ligase, is epigenetically inactivated in human Wilms' tumors and maps to a region of chomosome 6q21 (12). Re-expression of *HACE1* in human tumor cells directly abrogates tumor growth, whereas down-regulation of *HACE1 via* siRNA allows non-tumorigenic human cells to form tumors, suggesting that *HACE1* is a tumor-suppressor gene in chromosome 6q. Additionally, *HACE1* expression was also down-regulated in colon cancer (12). These results prompted us to examine the methylation status of the *HACE1* gene in surgically-removed colorectal carcinomas.

In the present study, the methylation status of the *HACE1* gene was examined in primary carcinomas and the corresponding normal tissues derived from 32 patients with colorectal cancer, and the correlation between the methylation status and clinicopathological findings was evaluated.

Materials and Methods

Sample collection and DNA preparation. Thirty-two primary tumor and corresponding normal tissue specimens were collected consecutively at Showa University Fujigaoka Hospital, Yokohama, Japan, from colorectal cancer patients during colorectal surgery. All the tissue specimens were confirmed histologically. Written informed consent, as required by the Institutional Review Board, was obtained from all the patients. The samples were stored immediately at –80˚C until analysis. The DNA was prepared as described elsewhere (13). The clinicopathological profiles of the patients enrolled in the study are shown in Table I.

Abbreviations: qMSP, quantitative methylation-specific PCR.

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 45 971 7125, e-mail: kenjih-ngy@umin.ac.jp

Key Words: HACE1, quantitative methylation-specific PCR, colorectal cancer.

Table I. *Clinicopathological features and HACE1 methylation in colorectal cancer.*

Clinicopathological features	Variable	No. of cases	*HACE1* methylation		*p*-value
			+	–	
Gender	male	17	4	13	0.538[1]
	female	15	5	10	
Age	52-81		64.7±9.9[3]	65.7±8.2	0.766[2]
Maximal tumor size (mm)	15-120		65.3±34.7[3]	43.7±19.0	0.0304[2]
Extent of tumor	≤mt[4]	6	1	5	0.470[1]
	mt<	26	8	18	
Tumor site	C, A, T[5]	15	3	12	0.333[1]
	D, S, R[6]	17	6	11	
Histology	well[7]	25	7	18	0.976
	mod[8]	7	2	5	
Lymph node metastasis	+	13	6	7	0.0612[1]
	–	19	3	16	
Duke's stage	A	4	1	3	0.150[1]
	B	11	1	10	
	C	17	7	10	
Total		32	9	23	

[1]Chi-square test; [2]Student's *t*-test; [3]mean±S.D; [4]mt, muscular tunic; [5]C, cecum; A, ascending colon; T, transverse colon; [6]D, descending colon; S, sigmoid colon; R, rectum; [7]well-differentiated adenocarcinoma according to Japanese criteria; [8]moderately-differentiated adenocarcinoma according to Japanese criteria.

Sodium bisulfite modification. One μg of the genomic DNA extracted from the tumor and the corresponding normal colorectal tissue specimens was subjected to bisulfite treatment using an Epitect Bisulfite Kit (Qiagen, Hilden, Germany).

qMSP. The bisulfite-treated DNA was amplified with quantitative methylation-specific PCR (qMSP), that was conducted in a Thermal Cycler Dice® Real Time System TP800 (Takara Bio Inc., Otsu, Japan). Thermocycling was done in a final volume of 25 μL containing 1.0 μL of the DNA sample, 100 nM each of the *HACE1* or β-actin primers (forward and reverse), and 12.5 μL of SYBR Premix Ex Taq II (Takara Bio Inc., Otsu, Japan), which consists of Taq DNA polymerase, reaction buffer and deoxynucleotide triphosphate mixture. The qPCR primer sequences for *HACE1* has been described elsewhere (12) and were: *HACE1* MS (sense), 5'-GAATGGAAGGTTTAATTTCGC-3', and *HACE1* MAS (antisense), 5'-CTAAAACCCTACGTCAACCG-3'. The PCR amplification consisted of 40 cycles (95˚C for 5 sec and 60˚C for 30 sec) after an initial denaturation step (95˚C for 10 sec). The bisulfite-treated DNA obtained from L132 cells that was fully methylated by *SssI* methylase was used as a positive control. To correct for differences in both quality and quantity between samples, β-actin was used as an internal control. The targets were obtained from the same bisulfite-treated DNA.

HACE1 methylation scores. The relative amounts of *HACE1* methylated DNA in the colorectal carcinomas and the corresponding normal tissues that were normalized to the internal control β-actin were calculated. The *HACE1* methylation score in each tissue was defined as follows: relative amount of *HACE1* in tumor / relative amount of *HACE1* in corresponding normal tissue. *HACE1* methylation was positive when the methylation score was more than 1.5.

Statistical analysis. The associations between *HACE1* methylation and clinicopathological parameters were analyzed using Chi-square test or Student's *t*-test. A p-value <0.05 indicated statistical significance.

Results

Aberrant methylation of the *HACE1* gene was detected in 9 out of the 32 (28%) primary colon carcinomas, suggesting that the aberrant methylation of *HACE1* was frequently observed in colorectal carcinomas.

The clinicopathological data were correlated with the methylation results. No significant correlations were observed between the presentation of abnormal methylation in the colorectal carcinomas and patient gender or age, tumor extent, tumor site, histology or Dukes stage (Table I). A significant difference was observed in the maximal tumor size (p=0.0304) (Figure 1, Table I). Moreover, a trend was shown towards preferentially developing lymph node metastasis (p=0.0612), thus indicating that *HACE1* was more frequently methylated in advanced colorectal carcinomas.

Discussion

Colorectal cancer is one of the most aggressive malignancies and occurs at a high incidence in most countries (14). The treatment option for this fatal cancer is surgery and subsequent chemotherapy and radiotherapy. For this purpose, it is important to identify the occurrence of genetic alterations as a new parameter to estimate the malignancy of the cancer.

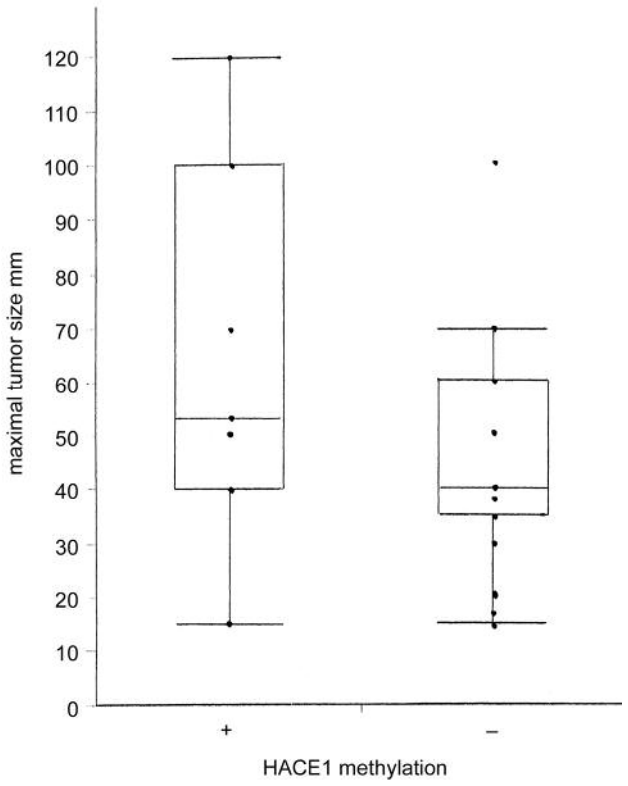

Figure 1. *Difference in maximal tumor size according to the methylation status of HACE1. Upper and lower limits of boxes, and line across boxes, indicate the 75th and 25th percentiles, and the median, respectively. Upper and lower horizontal bars indicate maximal and minimal scores, respectively. Outliers are illustrated as circles. Maximal tumor sizes were 65.3±34.7 in colorectal carcinomas with HACE1 methylation and 43.7±19.0 in those without HACE1 methylation (p=0.0306; Student's t-test).*

In the present study, the frequent methylation of *HACE1* was observed in colorectal cancer. A significant increase was observed in the maximal tumor size in the methylated *HACE1* tumors (p=0.0304). Moreover, a trend was shown toward the development of a lymph node metastasis in these carcinomas (p=0.0612). We have previously examined the methylation status of the *p16* and *p14* genes in 86 primary colorectal carcinomas using methylation-specific PCR (MSP) and found a significant difference in maximal tumor size (p=0.022) when patients with both *p16* and *p14* methylation were compared to other patients (15). We also investigated the methylation status of the *CDH13* gene and found a significant difference in histology (p=0.0053) compared the *CDH13* methylation of poorly-differentiated colorectal carcinomas was compared to that of differentiated ones (16). Finally, we examined the combined methylation status of *p16, p14, HLTF* (helicase-like transcription factor), *SOCS-1* (suppressor of cytokine signaling-1), *CDH13, RUNX3* (a member of the human runt-related transcription factor family), and *CHFR* (checkpoint with FHA and RING finger) in 58 resected primary colorectal carcinomas and correlated it with the clinicopathological features of the affected patients. A significant difference in histology (p=0.0041) was found when we compared the number of methylated genes of poorly-differentiated colorectal carcinomas was compared to that of differentiated ones (17). Taken together, all the results such as large tumor size and poor differentiation indicated that the methylated status of colorectal carcinomas was significantly correlated with malignant potential.

This study provides solid evidence that could be used in further studies of the molecular mechanism of *HACE1* in colorectal cancer and also suggests that *HACE1* may play a role in the carcinogenic pathway in some patients with colorectal cancer. These observations indicate the possibility that tumor formation in the colorectum may be controlled by inducing the expression of silenced *HACE1* using demethylating reagents.

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, Hedge P, Markham A, Krush AJ, Petersen G, Hamilton SR, Nilbert MC, Levy DB, Bryan TM, Preesinger AC, Smith KJ, Su LK, Kinzler KW and Vogelsteen B: 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 Hyytinen ER, Saadut R, Chen C, Paull L, Koivisto PA, Vessella RL, Frierson HF Jr and Dong JT: Defining the region(s) of deletion at 6q16-q22 in human prostate cancer. Genes Chromosomes Cancer *34*: 306-312, 2002.

8 Inoue M, Marx A, Zettl A, Ströbel P, Müller-Hermelink HK and Starostik P: Chromosome 6 suffers frequent and multiple aberrations in thymoma. Am J Pathol *161*: 1507-1513, 2002.

9 Orphanos V, McGown G, Hey Y, Thorncroft M, Santibanez-Koref M, Russell SE, Hickey I, Atkinson RJ and Boyle JM: Allelic imbalance of chromosome 6q in ovarian tumours. Br J Cancer *71*: 666-669, 1995.

10 Utada Y, Haga S, Kajiwara T, Kasumi F, Sakamoto G, Nakamura Y and Emi M: Mapping of target regions of allelic loss in primary breast cancers to 1-cM intervals on genomic contigs at 6q21 and 6q25.3. Jpn J Cancer Res *91*: 293-300, 2000.

11 Zhang Y, Matthiesen P, Harder S, Siebert R, Castoldi G, Calasanz MJ, Wong KF, Rosenwald A, Ott G, Atkin NB and Schlegelberger B: A 3-cM commonly deleted region in 6q21 in leukemias and lymphomas delineated by fluorescence *in situ* hybridization. Genes Chromosomes Cancer *27*: 52-58, 2000.

12 Zhang L, Anglesio MS, O'Sullivan M, Zhang F, Yang G, Sarao R, Mai PN, Cronin S, Hara H, Melnyk N, Li L, Wada T, Liu PP, Farrar J, Arceci RJ, Sorensen PH and Penninger JM: The E3 ligase HACE1 is a critical chromosome 6q21 tumor suppressor involved in multiple cancers. Nat Med *13*: 1060-1069, 2007.

13 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.

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

15 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.

16 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.

17 Hibi K and Nakao A: Highly-methylated colorectal cancers show poorly-differentiated phenotype. Anticancer Res *26*: 4263-4266, 2006.

Received December 31, 2007
Revised March 4, 2008
Accepted March 6, 2008