# Methylation of *HACE1* in Gastric Carcinoma

MAKIKO SAKATA, YO-HEI KITAMURA, KAZUMA SAKURABA, TETSUHIRO GOTO, HIROKI MIZUKAMI, MITSUO SAITO, KAZUYOSHI ISHIBASHI, GAKU KIGAWA, HIROSHI NEMOTO, YUTAKA SANADA and KENJI HIBI

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

**Abstract.** *Background: We recently examined the methylation status of the HACE1 gene in primary carcinomas derived from 32 patients with colorectal cancer. A significant increase was observed in the maximal tumor size of the tumors with methylated HACE1 (p=0.0304). Moreover, a trend was shown toward preferentially developing lymph node metastasis in the carcinomas with methylated HACE1 (p=0.0612), suggesting that HACE1 might present a malignant potential in colorectal cancer. These results prompted us to examine the methylation status of the HACE1 gene in gastric carcinomas. Materials and Methods: The methylation status of the HACE1 gene was examined in primary carcinomas and the corresponding normal tissues derived from 34 patients with gastric carcinoma using quantitative methylation-specific PCR (qMSP) and the correlation between the methylation status and the clinicopathological findings was evaluated. Results: An aberrant methylation of the HACE1 gene was detected in 9 out of 34 (26%) primary gastric carcinomas. Subsequently, clinicopathological data were tested for correlation with the methylation score. A significant difference was observed in patient gender (p=0.0429). Conclusion: HACE1 was frequently methylated in gastric carcinoma derived from male patients.*

Accumulating evidence indicates that gastric cancer is the result of various genetic and epigenetic alterations of oncogenes, tumor suppressor genes, DNA repair genes, cell-cycle regulators, and cell adhesion molecules (1). Aberrant methylation of CpG-rich sequences (CpG islands) is an epigenetic change that is common in human cancer (2). In gastric cancer, the inactivation of *hMLH1 (human mutL homolog 1), MGMT (O-6-methylguanine-DNA methyl-transferase), TIMP-3 (tissue inhibitor of metalloproteinase 3)* and *p16 (cyclin-dependent kinase inhibitor p16)* by promoter hypermethylation has been demonstrated (3-6). There has been substantial interest in attempting to adapt such cancer-associated aberrant gene methylation for clinical use.

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*, coding for the E3 ubiquitin ligase, is epigenetically inactivated in human Wilm's 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). We recently examined the methylation status of the *HACE1* gene in primary carcinomas and the corresponding normal tissues derived from 32 patients with colorectal cancer using quantitative methylation-specific PCR (qMSP) and evaluated the correlation between the methylation status and the clinicopathological findings (13). Aberrant methylation of the *HACE1* gene was detected in 9 out of the 32 (28%) primary colon carcinomas. A significant increase was observed in the maximal tumor size of the tumors with methylated *HACE1* ($p$=0.0304). Moreover, a trend was shown toward preferentially developing lymph node metastasis in patients with the methylated *HACE1* carcinomas ($p$=0.0612), suggesting that *HACE1* might act as a tumor suppressor in colorectal carcinomas and *HACE1* methylation might present a malignant potential in colorectal cancer. These results prompted us to examine the methylation status of the *HACE1* gene in the gastric carcinomas we surgically removed.

In the present study, we examined the methylation status of the *HACE1* gene in primary tumors derived from 34 patients with gastric carcinoma and evaluated the correlation between the methylation status and the clinicopathological findings.

*Correspondence to:* Kenji Hibi, Department of Surgery, Showa University Fujigaoka Hospital, 1-30 Fujigaoka, Aoba-ku, Yokohama 227-8501, Japan. Tel: +81 459711151, Fax: +81 459717125, e-mail: kenjih-ngy@umin.ac.jp

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Table I. *Clinicopathological features and HACE1 methylation in gastric carcinoma.*

| Clinicopathological feature | Variable | No. of cases | *HACE1 methylation* | | *p*-Value |
|---|---|---|---|---|---|
| | | | + | − | |
| Gender | Male | 28 | 9 | 19 | 0.0402* |
| | Female | 6 | 0 | 6 | |
| Age (years, mean±S.D.) | 52-81 | 34 | 71.6±8.8 | 68.0±9.9 | 0.337** |
| Maximal tumor size (mm, mean±S.D.) | 15-150 | 34 | 68.8±33.4 | 64.6±29.9 | 0.722** |
| Extent of tumor | ≤SE[3] | 23 | 7 | 16 | 0.438* |
| | >SE | 11 | 2 | 9 | |
| Histology | Well | 11 | 2 | 9 | 0.438* |
| | Other | 23 | 7 | 16 | |
| Lymph node metastasis | + | 23 | 7 | 16 | 0.438* |
| | − | 11 | 2 | 9 | |
| TNM stage | I, II, IIIA | 15 | 4 | 11 | 0.981* |
| | IIIB, IV | 19 | 5 | 14 | |
| Total | | 34 | 9 | 25 | |

*Chi-square test; **Student's *t*-test; SE, serosa; Well, well-differentiated adenocarcinoma.

## Materials and Methods

*Sample collection and DNA preparation.* Thirty-four primary tumor and corresponding normal tissue specimens were collected consecutively at Showa University Fujigaoka Hospital 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 (14). The clinicopathological profiles of the patients enrolled in the study are shown in Table I.

*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).

*Quantitative methylation-specific PCR (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 carried out 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 12.5μl 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* have 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 s and 60˚C for 30 s) after an initial denaturation step (95˚C for 10 s). 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 gastric 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 considered 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 tests or Student's *t*-tests. A *p*-value <0.05 indicated statistical significance.

## Results

The methylation status of *HACE1* in primary gastric carcinoma samples was examined using qMSP. An aberrant methylation of the *HACE1* gene was detected in 9 out of 34 (26%) primary gastric carcinomas.

Subsequently, clinicopathological data were tested for correlated with the methylation score. No significant correlations were observed between the presentation of aberrant methylation in the gastric carcinomas and patient age, maximal tumor size, extent of tumor, histology, or TNM stage (Table I). A significant difference was observed in patient gender ($p$=0.0429) (Table I), suggesting that *HACE1* was frequently methylated in gastric carcinoma derived from male patients.

## Discussion

Gastric cancer is one of the most common malignancies worldwide (15). In order to remove this fatal cancer from patients, we perform surgical operations 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.

We previously examined *HLTF (helicase-like transcription factor)* methylation in colorectal, gastric, and esophageal carcinomas (16). Twenty-five out of 76 colorectal (33%), 11 out of 65 gastric (17%), and 1 out of 40 esophageal (3%) carcinomas demonstrated abnormal methylation of the *HLTF* promoter. This result suggested that *HLTF* might play a variety of roles depending on the tissue type. Subsequently, we examined *CDH13* methylation in esophageal and gastric carcinomas (17). Five out of 37 esophageal (14%) and 23 out of 66 gastric (35%) carcinomas demonstrated abnormal methylation of the *CDH13* promoter. Abnormal methylation was frequently found in gastric cancer of patients at all clinical

stages just as in E-cadherin, another of the cadherin family, suggesting that these types of cancer could be methylated at an early stage. These results suggested that *CDH13* might play a variety of roles depending on the tissue type.

In a recent study, we examined the methylation status of the *HACE1* gene in primary carcinomas and the corresponding normal tissues derived from 32 patients with colorectal cancer (13). Aberrant methylation of the *HACE1* gene was detected in 9 out of the 32 (28%) primary colon carcinomas. A significant increase was observed in the maximal tumor size of the tumors with methylated HACE1 ($p$=0.0304). Moreover, a trend was shown toward preferentially developing lymph node metastasis in the methylated *HACE1* carcinomas ($p$=0.0612), suggesting that *HACE1* might act as a tumor suppressor in colorectal carcinomas and *HACE1* methylation might present a malignant potential in colorectal cancer. In the present study, although a significant difference was observed in patient gender ($p$=0.0429), no significant correlations were observed between the presentation of aberrant methylation in the gastric carcinomas and patient age, maximal tumor size, extent of tumor, histology, or TNM stage. Taken together, these results suggested that the rate of *HACE1* methylation might depend on the type of primary carcinomas.

In conclusion, our results suggest that *HACE1* methylation occurs in gastric carcinomas. Because of frequent methylation of the *HACE1* gene and the high sensitivity of qMSP, it can potentially be used for the detection and monitoring of gastric carcinoma by the detection of *HACE1* methylation in clinical samples such as serum (18, 19).

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