Methylation of the *p16* Gene Is Frequently Detected in Lymphatic-invasive Gastric Cancer

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Abstract. *Background: A tumor suppressor gene, p16, was found to harbor promoter methylation associated with the loss of protein expression in cancer cells, suggesting that p16 inactivation due to promoter methylation may be important for gastric tumorigenesis. Patients and Methods: The methylation status of the p16 gene was examined in primary carcinomas and the corresponding normal tissues derived from 49 patients with gastric 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 p16 gene was detected in 17 out of the 49 (34%) primary gastric carcinomas, suggesting that the aberrant methylation of p16 is frequently observed in gastric carcinomas. The clinicopathological data were then correlated with these results. Significant differences were observed with lymphatic invasion (p=0.046) and tumor site (p=0.010). Conclusion: p16 might act as a tumor suppressor in gastric carcinomas and appears to be more frequently methylated in lymphatic-invasive gastric carcinomas.*

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 human mutL homolog 1 (*hMLH1*), *O*-6-methylguanine-DNA methyltransferase (*MGMT*), tissue inhibitor of metalloproteinase 3 (*TIMP-3*), and other genes by promoter hypermethylation has been demonstrated (3-8). There has been substantial interest in attempting to adapt such cancer-associated aberrant gene methylation for clinical use.

It has recently become clear that alterations in DNA methylation are very common and are capable of directly modifying carcinogenesis (9). A tumor suppressor gene, *p16*, was found to harbor promoter methylation, associated with the loss of protein expression in cancer cells (10). Although homozygous deletions of the *p16* locus are not usually found (11), *p16* promoter methylation has been detected in colorectal cancer (12, 13). These studies indicated that *p16* inactivation due to promoter methylation was important for tumorigenesis. These results prompted us to examine the methylation status of the *p16* gene in the gastric carcinomas we surgically removed.

In the present study, the methylation status of the *p16* gene was examined in primary carcinomas and the corresponding normal tissues derived from 49 patients with gastric cancer, and the correlation between the methylation status and the clinicopathological findings was evaluated.

Patients and Methods

Sample collection and DNA preparation. Forty-nine primary tumor and corresponding normal tissue specimens were collected consecutively at Showa University Fujigaoka Hospital from gastric cancer patients during 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 qMSP that was conducted in a Thermal Cycler Dice® Real-Time System TP800 (Takara Bio Inc., Otsu,

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Key Words: p16, quantitative methylation-specific PCR, gastric cancer.

Table I. *Clinicopathological features and p16 methylation in gastric cancer.*

Clinicopathological feature	Variable	No. of cases	*p16* Methylation		*p*-Value
			+	–	
Gender	Male	39	15	24	0.257[a]
	Female	10	2	8	
Age (years)	51-82		70.5±9.9[c]	69.4±8.4[c]	0.680[b]
Max. tumor size (mm)	5.7-150		60.1±28.3[c]	55.0±27.7[c]	0.547[b]
Extent of tumor	<mt	3	0	3	0.102[a]
	≥mt	46	17	29	
Tumor site	U	10	1	9	0.010[a]
	M	11	8	3	
	L	24	6	18	
	R	4	2	2	
Histology	Well	25	9	16	0.844[a]
	Poor	24	8	16	
Lymphatic invasion	+	35	15	20	0.046[a]
	–	14	2	12	
Venous invasion	+	29	11	18	0.564[a]
	–	20	6	14	
Lymph node metastasis	+	27	9	18	0.824[a]
	–	22	8	14	
TNM stage	1	19	6	13	0.714[a]
	2-4	30	11	19	
Total		49	17	32	

[a]Chi-square test; [b]Student's *t*-test; [c]mean±S.D; mt, muscular tunic; U, upper part of stomach; M, middle part of stomach; L, lower part of stomach; R, remnant gastric cancer; Well, well- or moderately differentiated adenocarcinoma according to Japanese criteria; Poor, poorly differentiated, mucinous, or signet ring cell adenocarcinoma according to Japanese criteria.

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 *p16* 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 *p16* have been described elsewhere (15) and were: *p16* MS (sense), 5'-TTATTAGAG GGTGGGGCGGATCGC-3'; and p16 MAS (antisense), 5'-GACCCCGAACCGCGACCGTAA-3'. The PCR amplification consisted of 40 cycles (95˚C for 5 s and 68˚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.

p16 Methylation scores. The relative amounts of *p16* methylated DNA in the gastric carcinomas and the corresponding normal tissues that were normalized to the internal control β-actin were calculated. The *p16* methylation score in each tissue was defined as follows: relative amount of *p16* in tumor/relative amount of *p16* in corresponding normal tissue.

p16 methylation was considered as positive when the methylation score was more than 1.0.

Statistical analysis. The associations between *p16* methylation and clinicopathological parameters were analyzed using Chi-square or Student's *t*-tests. A *p*-value <0.05 indicated statistical significance.

Results

Aberrant methylation of the *p16* gene was detected in 17 out of the 49 (34%) primary gastric carcinomas, suggesting that the methylation of *p16* is frequently observed in gastric carcinomas.

The clinicopathological data were correlated with the methylation results. No significant correlations were observed between the presentation of abnormal methylation in the gastric carcinomas and patient gender or age, maximal tumor size, tumor extent, venous invasion, or histology (Table I). *p16* methylation was significantly observed in lymphatic-invasive (p=0.046) or middle-part gastric carcinoma (p=0.010) (Table I).

Discussion

Gastric cancer is one of the most common malignancies worldwide (16, 17). The identification of the genetic alterations as a new parameter to estimate the process of the neoplastic process is important to improve the success of treatment.

In the present study, frequent methylation of *p16* gene was observed in gastric cancer. Lymphatic invasion was significantly observed (p=0.046). Additionally, methylation

was significantly more frequently observed in the middle part of stomach (p=0.010). We have previously examined the methylation status of the *p16* gene in primary carcinoma and the corresponding normal tissues derived from 50 patients with colorectal cancer using qMSP and evaluated the correlation between the methylation status and the clinicopathological findings was evaluated (13). Methylation of the *p16* gene was detected in 20 out of 50 (40%) primary colorectal carcinomas and significantly associated with Dukes' stage (p=0.0495) and lymphatic invasion (p=0.0277). These results indicated that *p16* methylation was commonly observed in lymphatic-invasive digestive tract tumors.

Recent studies have shown that it is possible to reverse epigenetic changes and restore gene function to a cell (18). Treatment with DNA methylation inhibitors can restore the activities of *p16* gene and decrease the growth rate of cancer cells (19). The administration of drugs such as cytosine analogs might soon be able to restore the function of these tumor suppressor genes and slow the rate of gastric cancer progression.

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Received March 29, 2010
Revised June 11, 2010
Accepted June 16, 2010