Aberrant Methylation of the *p16* Gene Is Frequently Detected in Advanced Colorectal Cancer

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Abstract. *Background: A tumor suppressor gene, p16, was found to harbor promoter hypermethylation associated with the loss of protein expression in cancer cells, suggesting that p16 inactivation due to promoter methylation was important for colorectal tumorigenesis. Materials and Methods: The methylation status of the p16 gene was examined in primary carcinomas and the corresponding normal tissues derived from 50 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 p16 gene was detected in 20 out of the 50 (40%) primary colon carcinomas, suggesting that the aberrant methylation of p16 was frequently observed in colorectal carcinomas. The clinicopathological data were then correlated with these results. Significant differences were observed with Dukes' stage (p=0.0495) and lymphatic invasion (p=0.0277). Conclusion: p16 might act as a tumor suppressor in colorectal carcinomas and was more frequently methylated in advanced colorectal carcinomas.*

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).

It has recently become clear that alterations in DNA methylation are very common and are capable of directly modifying carcinogenesis (7). A tumor suppressor gene, *p16*, was found to harbor promoter hypermethylation associated with the loss of protein expression in cancer cells (8). Though homozygous deletions of the *p16* locus are not found (9), *p16* promoter methylation was detected in colorectal cancer (10). These studies indicated that *p16* inactivation due to promoter methylation was important for colorectal tumorigenesis. These results prompted us to examine the methylation status of the *p16* gene in the colorectal 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 50 patients with colorectal cancer and the correlation between the methylation status and the clinicopathological findings was evaluated.

Materials and Methods

Sample collection and DNA preparation. Fifty 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 (11). 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).

qMSP. The bisulfite-treated DNA was amplified with quantitative methylation-specific PCR (qMSP) 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

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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 (12) and were: *p16* MS (sense), 5'-TTATTAGAGGGTGGGGCGGATCGC-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 colorectal 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 tests or Student's *t*-tests. A *p*-value <0.05 indicated statistical significance.

Results

Aberrant methylation of the *p16* gene was detected in 20 out of the 50 (40%) primary colon carcinomas, suggesting that the aberrant methylation of *p16* 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, maximal tumor size, tumor extent, tumor site, venous invasion, or histology (Table I). Significant differences were observed in the Dukes' stage (*p*=0.0495) and lymphatic invasion (*p*=0.0277) (Table I), thus indicating that *p16* 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 (13). One treatment of 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.

In the present study, frequent methylation of *p16* was observed in colorectal cancer. Methylation was significantly observed in Dukes' C colorectal cancer (*p*=0.0495). Moreover, lymphatic invasion was also significantly observed (*p*=0.0277). We have previously examined the methylation status of the *CDH13* gene and found a significant difference in histology (*p*=0.0053) comparing the *CDH13* methylation of poorly differentiated colorectal carcinomas to that of differentiated ones (12). We also investigated the methylation status of *HACE1* gene in colorectal cancer. A significant increase was observed in the maximal tumor size in the methylated *HACE1* tumors (*p*=0.0304) (14). Recently, We examined the methylation status of the *UNC5C* gene in primary carcinomas and the corresponding normal tissues derived from 49 patients with colorectal cancer. Aberrant methylation of the *UNC5C* gene was detected in 34 out of the 49 (69%) primary colon carcinomas, suggesting that the aberrant methylation of *UNC5C* was frequent in colorectal cancer. The clinicopathological data were then tested for correlation with this result. A significantly greater proportion of cases with methylated *UNC5C* was found in Dukes' stage C (*p*=0.0380) than in earlier stages (15). The methylation status of the *Vimentin* gene was also examined in primary carcinomas and the corresponding normal tissues derived from 48 patients with colorectal cancer. Aberrant methylation of the *Vimentin* gene was detected in 31 out of 48 (65%) primary colorectal

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

Clinicopathological feature	Variable	No. of cases	*p16* Methylation +	*p16* Methylation –	*p*-value
Gender	Male	27	11	16	0.908[1]
	Female	23	9	14	
Age (years)	23-87		65.9±2.63	65.6±2.2	0.934[2]
Max. tumor size (mm)	15-120		50.7±25.73	46.9±22.7	0.590[2]
Extent of tumor	<mt4	10	5	5	0.471[1]
	mt≤	40	15	25	
Tumor site	C, A, T	20	10	10	0.239[1]
	D, S, R	30	10	20	
Histology	Well	35	14	21	0.700[1]
	Mod	14	6	8	
	Poor	1	0	1	
Lymphatic invasion	+	23	13	10	0.0277[1]
	–	27	7	20	
Venous invasion	+	28	12	16	0.132[1]
	–	22	8	14	
Lymph node metastasis	+	18	10	8	0.0922[1]
	–	32	10	22	
Dukes' stage	A, B	26	7	19	0.0495[1]
	C	24	13	11	
Total	50	20	30		

[1]Chi-square test; [2]Student's *t*-test; [3]mean±S.D; mt, muscular tunic; C, cecum; A, ascending colon; T, transverse colon; D, descending colon; S, sigmoid colon; R, rectum; Well, well-differentiated adenocarcinoma according to Japanese criteria; Mod, moderately differentiated adenocarcinoma according to Japanese criteria; Poor, poorly differentiated or signet ring cell adenocarcinoma according to Japanese criteria.

carcinomas. A significant difference was observed in age and Dukes' stage (p=0.001 and p=0.034, respectively). Moreover, a trend was shown toward preferentially developing liver metastasis and peritoneal dissemination in colorectal carcinomas with *Vimentin* methylation (p=0.052 and p=0.080, respectively) (16). 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.

We previously examined the methylation status of *p16* in colorectal cancer using conventional MSP (17). Although 44 out of 94 (47%) cancer cases exhibited abnormal methylation of *p16* gene, the clinical stage, histology, and tumor size were not correlated with representations of methylation in colorectal cancer. In this study, we used qMSP, which is more accurate than conventional MSP. This might be the reason we were able to detect significant differences between the presentation of abnormal *p16* methylation in the colorectal carcinomas with the Dukes' stage and lymphatic invasion.

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 the *p16* gene and decrease the growth rate of cancer cells. 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 colorectal cancer progression.

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