Aberrant Methylation of the *UNC5C* Gene is Frequently Detected in Advanced Colorectal Cancer

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Abstract. *Background: UNC5C, one of the Netrin-1 receptors, belongs to the functional dependence receptor family, members of which share the ability to induce apoptosis in the absence of their ligands. Recently, two reports indicated that UNC5C methylation was closely associated with loss of gene expression in colorectal cancer. Materials and Methods: The methylation status of the UNC5C gene was examined in primary carcinomas and the corresponding normal tissues derived from 49 patients with colorectal cancer using quantitative methylation-specific polymerase chain reaction (qMSP) and the correlation between the methylation status and the clinicopathological findings was evaluated. Results. 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. Conclusion: UNC5C might act as a tumor suppressor and UNC5C 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).

UNC5C, one of the Netrin-1 receptors, belongs to the functional dependence receptor family, members of which share the ability to induce apoptosis in the absence of their ligands (7-9). Such a trait has been hypothesized to confer a tumor-suppressor activity. Indeed, the loss of *UNC5C* expression is particularly prominent in colorectal cancer (10). However, the molecular mechanisms responsible for the loss of *UNC5C* expression are poorly understood. Recently, two reports indicated that *UNC5C* methylation was closely associated with loss of gene expression in colorectal cancer (11, 12). These results prompted us to examine the methylation status of the *UNC5C* gene in the colorectal carcinomas we surgically removed.

In the present study, the methylation status of the *UNC5C* gene was examined in primary carcinomas and corresponding normal tissues derived from 49 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. Forty-nine 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 (13). 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).

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Table I. *Clinicopathological features and UNC5C methylation in colorectal cancer.*

Clinicopathological feature	Variable	No. of cases	*UNC5C* methylation		*p*-Value
			+	–	
Gender	Male	24	18	6	0.403[1]
	Female	25	16	9	
Age (years)	23-87	49	68.5±10.4[3]	63.2±13.7	0.190[2]
Maximal tumor size (mm)	15-120	49	50.4±27.4[3]	40.5±19.7	0.216[2]
Extent of tumor	confined to mt	11	8	3	0.785[1]
	beyond mt	38	26	12	
Tumor site	C, A, T	21	16	5	0.371[1]
	D, S, R	28	18	10	
Histology	well[4]	35	25	10	0.642[1]
	mod[5]	13	8	5	
	por[6]	1	1	0	
Dukes stage	A, B	25	14	11	0.0380[1]
	C	24	20	4	
Total		49	34	15	

[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. [4]well-differentiated adenocarcinoma, [5]moderately-differentiated adenocarcinoma, [6]poorly-differentiated adenocarcinoma according to Japanese criteria.

qMSP. The bisulfite-treated DNA was amplified with quantitative methylation-specific polymerase chain reaction (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 1.0 μL of the DNA sample, 100 nM each of the *UNC5C* 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 *UNC5C* has been described elsewhere (12) and were: *UNC5C* MS (sense), 5'-CGACTAAAACTTTACCGACGCGACG-3' and *UNC5C* MAS (antisense), 5'-CGGGGGCGGGAGTACGTTCGTTC-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.

UNC5C methylation scores. The relative amounts of *UNC5C* methylated DNA in the colorectal carcinomas and the corresponding normal tissues normalized to the internal control β-actin were calculated. The *UNC5C* methylation score in each tissue was defined as follows: relative amount of *UNC5C* in tumor/average relative amount of *UNC5C* in all corresponding normal tissues.

UNC5C methylation was considered as being positive when the methylation score was more than 1.5.

Statistical analysis. The associations between *UNC5C* 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 *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 carcinomas.

The clinicopathological data were tested for correlation 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 or histology (Table I). A significantly difference was observed in regard to the Dukes' stage (p=0.0380) (Table I), thus indicating that *UNC5C* 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). Only 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 *UNC5C* was observed in colorectal cancer. Moreover, a significant difference was observed in regard to the Dukes' stage (p=0.0380), thus indicating that *UNC5C* was more frequently methylated in advanced colorectal carcinomas. We

previously examined the methylation status of the *p16* and *p14* genes in 86 primary colorectal carcinomas using methylation-specific PCR (MSP) and found that patients with both *p16* and *p14* methylation presented a significantly greater maximal tumor size (p=0.022) when compared to other patients (15). We also investigated the methylation status of the *CDH13* gene and found that poorly-differentiated colorectal carcinomas significantly showed the *CDH13* methylation (p=0.0053) when compared to 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. Poorly-differentiated colorectal carcinomas significantly showed the more number of methylated genes (p=0.0041) when compared to differentiated ones (17). Taken together, the results, in paticular large tumor size and poor differentiation, indicated that the methylated status of *UNC5C* in colorectal carcinomas was significantly correlated with malignant potential.

This study provides solid evidence in further studies of the molecular mechanism of *UNC5C* in colorectal cancer and also suggests that *UNC5C* 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 thus be controlled by inducing the expression of silenced *UNC5C via* demethylating agents.

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