PGP9.5 Methylation as a Marker for Metastatic Colorectal Cancer

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Abstract. *Background: Recently, it has been proven that protein gene product 9.5 (PGP9.5) hypomethylation might play an important role in re-expression of the PGP9.5 gene in gallbladder cancer. We previously examined the expression of PGP9.5 in primary colorectal cancer using immunohistochemistry and found that PGP9.5 expression is related to tumor progression and may be useful as a marker for invasive colorectal cancer. These results prompted us to examine the methylation status of the PGP9.5 gene in colorectal cancer. Materials and Methods: The methylation status of the PGP9.5 gene in primary tumors derived from 49 patients with colorectal cancer using a quantitative methylation-specific polymerase chain reaction (qMSP) and the association between the methylation status and the clinicopathological findings was evaluated. Results: An aberrant methylation of the PGP9.5 gene was detected in 36 out of 49 (73%) primary colon cancer samples. Subsequently, clinicopathological data were tested for their association with the methylation results. Lymph node metastasis was significantly associated with a lower frequency of methylation (p=0.029). Conclusion: These findings indicated that PGP9.5 was less frequently methylated in metastatic colorectal cancer, suggesting that PGP9.5 hypomethylation might play an important role in re-expression of the PGP9.5 gene 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* and *p53* genes, have been identified in colorectal cancer (1-3). In addition, we 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).

Recently, it was proven that protein gene product 9.5 (*PGP9.5*) hypomethylation could be a reliable marker for gallbladder cancer and that DNA hypomethylation might play an important role in re-expression of the *PGP9.5* gene in gallbladder cancer (7). We previously examined the expression of PGP9.5 in primary colorectal cancer using immunohistochemistry and correlated the result with the clinicopathological features (8). Of 74 colorectal cancer specimens examined, 33 cases (46%) showed positive staining with PGP9.5 in most tumor cells, while no PGP9.5 expression was detected in adjacent normal epithelium. Subsequently, we correlated PGP9.5 expression in tumors with the clinicopathological features of affected patients and found two significant positive correlations with maximal tumor size and the extent of tumor (p=0.035 and 0.019, respectively). These results suggests that PGP9.5 expression is related to tumor progression and may be useful as a marker for invasive colorectal cancer. These results prompted us to examine the methylation status of the *PGP9.5* gene in surgically removed colorectal cancer specimens.

In the present study, we examined the methylation status of the *PGP9.5* gene in primary tumors derived from 49 patients with colorectal cancer and evaluated the association between the methylation status and the clinicopathological findings.

Materials and Methods

Sample collection and DNA preparation. Forty-nine primary tumor specimens were collected consecutively at Showa University Fujigaoka Hospital from colorectal cancer patients during colorectal surgery. All specimens were confirmed histologically. Written informed consent, as required by the Institutional Review Board, was obtained from all patients. Collected samples were stored immediately at –80˚C until analysis. DNA was prepared as

Abbreviations: PGP9.5, protein gene product 9.5; qMSP, quantitative methylation-specific PCR.

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Table I. *Clinicopathological features and PGP9.5 promoter methylation in colorectal cancer.*

Clinicopathological feature	Variable	No. of cases	PGP9.5 methylation		*p*-Value
			+	–	
Gender	Male	25	20	5	0.290[a]
	Female	24	16	8	
Age (year[c])		49	63.2±3.3	66.9±2.0	0.356[a]
Maximal tumor size (mm)		49	51.0±4.9	47.8±5.1	0.724[b]
Extent of tumor	<mt	7	6	1	0.428[a]
	≥mt	42	30	12	
Lymph node metastasis[d]	≤N1	40	32	8	**0.029[a]**
	≥N2	9	4	5	
TNM stage[b]	I, II	22	16	6	0.915[d]
	III, IV	27	20	7	
Total		49	36	13	

[a]Chi-square test; [b]Student's *t*-test; [c]mean ± S.D; mt, muscular tunic; [d]by TNM classification.

described elsewhere (9). 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 colorectal cancer specimens was subjected to bisulfite treatment using an Epitect Bisulfite Kit (Qiagen, Hilden, Germany) as described elsewhere (10).

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 1.0 μl of the DNA sample, 100 nM each of the *PGP9.5* 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 *PGP9.5* have been described elsewhere (11) and were: *PGP9.5* MS (sense), 5'-CGGCGAGTGAGATTGTAAGGTT-3', and *PGP9.5* MAS (antisense), 5'-GAACGATCGCGACCAAATAAATAC-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.

PGP9.5 methylation scores. The relative amounts of *PGP9.5* methylated DNA in the colorectal cancer samples normalized to the internal control β-actin were calculated. The *PGP9.5* methylation score in each tissue was defined as follows:

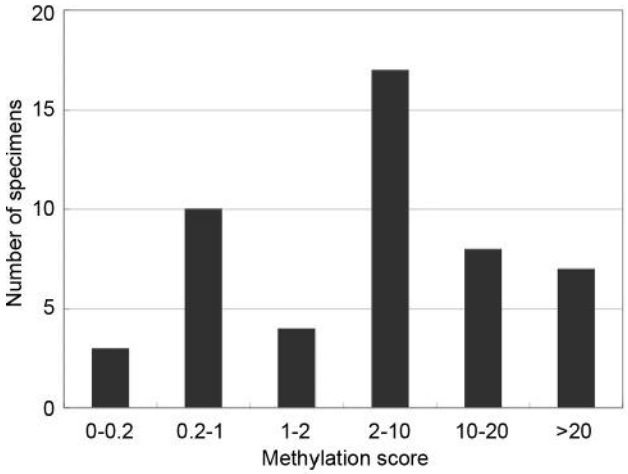

Figure 1. *The distribution of PGP9.5 methylation scores in colorectal cancer (average was 9.26±0.50).*

Methylation score = relative amount of PGP9.5 methylated DNA / relative amount of fully methylated L132 DNA.

PGP9.5 methylation was considered positive when the methylation score was more than 1.0.

Statistical analysis. The associations between *PGP9.5* 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 the *PGP9.5* in primary colorectal cancer samples was examined. An aberrant methylation of the *PGP9.5* gene was detected in 36 out of 49 (73%) primary colon cancer specimens. Figure 1 shows the distribution of *PGP9.5* methylation scores. Our results show that the aberrant methylation of the *PGP9.5* gene was frequent in colorectal cancer samples.

No significant associations were observed between the presentation of abnormal methylation in the colorectal cancer and patient gender or age, maximal tumor size, extent of tumor or TNM stage (Table I). A significant difference was observed in lymph node metastasis (p=0.029) (Table I), indicating that *PGP9.5* was less frequently methylated in metastatic colorectal cancer.

Discussion

Colorectal cancer is one of the most aggressive types of cancer and occurs at a high incidence in most countries (12). In order to eradicate 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.

PGP9.5, a ubiquitin hydrolase, is widely expressed in neuronal tissues and has been suggested as a neuroendocrine marker (13, 14). The ubiquitin-proteasome pathway degrades cytosolic and nuclear proteins *via* an ATP- and ubiquitin-dependent mechanism, which involves the regulation of cell cycle genes (15, 16).

We previously examined the expression of PGP9.5 in primary colorectal cancer using immunohistochemistry and found that PGP9.5 expression was significantly related to tumor progression (8). In this study, *PGP9.5* was less frequently methylated in metastatic colorectal cancer, suggesting that *PGP9.5* hypomethylation might play an important role in re-expression of the *PGP9.5* gene in colorectal cancer. These results were concordant with each other.

Previously, we also evaluated the expression of PGP9.5 using immunohistochemistry in 69 resected ductal carcinomas of the pancreas as well as in normal pancreatic tissue (17). A significant negative correlation was found between overexpression of PGP9.5 and postoperative survival. Multivariate analysis also suggested PGP9.5, along with tumor stage and extrapancreatic plexus invasion, as strong predictors of the outcome of the disease. The study suggests that PGP9.5 expression may be used as a marker for predicting the outcome of resectable pancreatic cancer patients. Using the serial analysis of gene expression (SAGE) method, Hibi *et al*. found that the PGP9.5 transcript was highly expressed in lung cancer (18). They further examined PGP9.5 status in lung cancer and found that PGP9.5 expression was closely associated with the advanced stages of lung cancer (19). Taken together, PGP9.5 expression is closely related to the malignant progression of colorectal, pancreatic and lung cancer. On the other hand, Mandelker *et al*. reported that *PGP9.5* methylation was an independent prognostic factor for esophageal squamous cell carcinoma (11). These results suggested that PGP9.5 might play different roles depending on the tissue type.

In the present study, we observed the frequent methylation of *PGP9.5* in colorectal cancer. We also compared the methylation status of *PGP9.5* in colorectal cancer patients with their clinicopathological features and demonstrated that *PGP9.5* in patients with earlier staged colorectal cancer was more frequently methylated than in metastatic cases. Therefore, *PGP9.5* methylation could be used as a tumor marker in clinical samples such as serum and stool for the early detection of colorectal cancer (20, 21).

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