RUNX3 Promoter Methylation in Colorectal Cancer: Its Relationship with Microsatellite Instability and its Suitability as a Novel Serum Tumor Marker

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Abstract. *Background/Aim: RUNX3 is a novel gastric cancer tumor suppressor. RUNX3 promoter hypermethylation is associated with many types of cancer, including colorectal cancer. Furthermore, the RUNX3 promotor is one of the CpG island methylator phenotype (CIMP)-specific promotors. CIMP is a distinct phenotype associated with microsatellite instability (MSI) in colorectal cancer. In this study, the suitability of the quantitative analysis of RUNX3 promoter hypermethylation as a novel serum tumor marker was investigated. Moreover, we investigated the relationship between RUNX3 promoter methylation and MSI in colorectal cancer. Patients and Methods: A RUNX3 real-time quantitative methylation-specific PCR (RTQ-MSP) technique we developed was used to analyze the CpG sites in the RUNX3 promoter of 119 colorectal tumors and 344 sera from colorectal cancer patients. MSI analysis of 119 colorectal tumors was performed with five microsatellite markers (BAT25, BAT26, D5S346, D2S123, and D17S250). Results: Proximal colon tumors exhibited significantly higher RUNX3 methylation than their paired normal tissues (p=0.0438). Analysis of the clinicopathological parameters revealed that a proximal location (p=0.0054), lymphatic invasion (p<0.0001), and an advanced pathological stage (p=0.0018) were associated with significantly higher RUNX3 methylation. Assessment of the relationship between RUNX3 methylation and tumor MSI revealed 11 out of 13 tumors with high-frequency MSI (85%) were positive for RUNX3 hypermethylation, significantly more than the tumors with low-frequency MSI or which were microsatellite stable (34%, p=0.0070). In preoperative sera from 344 colorectal cancer patients, significantly higher RUNX3 methylation was associated with lymphatic invasion (p=0.0487) and an advanced pathological stage (p=0.0466). Post-operative follow-up data revealed that recurrence cases exhibited significantly higher preoperative serum RUNX3 methylation than non-recurrence cases (p=0.0003). Concomitant analysis of carcinoembryonic antigen (CEA) levels in the preoperative sera showed that 17.7% (61/344) were CEA-negative but RUNX3 methylation-positive, which means assessing both serum RUNX3 methylation and CEA should improve diagnosis of colorectal carcinoma. Conclusion: RTQ-MSP-based quantification of serum RUNX3 methylation is useful for the detection and monitoring of colorectal cancer.*

To detect and monitor cancer and determine the likely prognosis, it is necessary to identify molecular markers of the disease that can be used in the clinic. In this regard, nucleic acid markers in serum have recently become a subject of great interest. These markers include oncogene mutations and

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amplifications, microsatellite alterations, and gene rearrangements, and have been associated with many types of cancer, including those of the lung, colon, and breast (1, 2).

Epigenetic alterations, such as aberrant promoter methylation, can also be detected by serological analysis. Thus, circulating nucleic acids are an emerging class of molecular tumor markers (3-5). Their wide applicability and clinical association with malignancy mean that they are likely to become clinically important.

The members of the Runt-related gene family, namely *RUNX1, RUNX2,* and *RUNX3*, are developmental regulators that appear to play important roles in the development of human cancer and tumors in murine experimental systems (6). It has been shown that lack of functional *RUNX3* promotes gastric carcinogenesis by disrupting normal gastric mucosal development and inhibiting the differentiation of gastrtic mucosal cells; thus, *RUNX3* appears to be a novel tumor suppressor of gastric cancer (7). Our recent studies have also shown that the transcriptional silencing of *RUNX3* through promoter methylation is associated with many types of cancer, including lung cancer, hepatocellular carcinoma and esophageal squamous cell carcinoma (8-10). It was also found recently that patients with cancer of the stomach, breast, and bladder often exhibit *RUNX3* promoter hypermethylation as determined in the serum (11).

In relation to this, it has been shown that in colon cancer, various tumor suppressor genes can be silenced by promoter CpG island methylation (12, 13), with a subset of colon tumors exhibiting widespread promoter methylation; the latter are referred to as having the CpG island methylator phenotype (CIMP) (12, 14, 15). The CIMP phenotype is associated with microsatellite instability (MSI) (16, 17), which is characterized by genome-wide alterations in repetitive DNA sequences and is caused by defects in mismatch repair (MMR) genes. The MMR genes that are most commonly affected are human mutL homolog 1 *(hMLH1)* and human mutS homolog 2 *(hMSH2)*. MSI is found in ~90% of hereditary non-polyposis cancer-associated tumors and ~15% of sporadic colorectal carcinomas (18-20). MSI-associated colorectal cancer has a distinct phenotype that is characterised by MMR deficiency, a proximal location, poor differentiation, a mucinous nature, dense lymphocytic infiltration, a greater than average number of tumors, an older disease onset, a preponderance amongst females, and, paradoxically, a favourable prognosis (18, 19). However, some studies have not found that MSI in colorectal carcinoma correlates with a better survival (21, 22). Recently, we developed a *RUNX3* real-time quantitative methylation-specific PCR (RTQ-MSP) method to analyze the CpG sites in *RUNX3* promoter regions whose hypermethylation induces transcriptional silencing. We showed that this method is clinically useful for the diagnosis and monitoring of gastric cancer (23). In the study reported here, we used this method to determine the degree of *RUNX3* promoter hypermethylation in the cancer tissue and paired normal mucosae of colorectal cancer patients. We also used *RUNX3* RTQ-MSP to analyze the sera of another cohort of colorectal cancer patients. In addition, we investigated how *RUNX3* promoter hypermethylation in the tumors of colorectal cancer patients relates to tumor MSI.

Patients and Methods

Patients and controls. In total, 463 colorectal cancer patients who were treated at the Kyoto Prefectural University of Medicine were enrolled in this study. All provided informed consent. This study was also approved by the Ethics Committee of the University. Of these 463 patients, 344 provided preoperative peripheral blood samples, from which the sera were obtained. Surgically removed tumors and the paired surrounding mucosae were collected from the remaining 119 patients. In all cases, the diagnosis of colorectal cancer was confirmed histologically. As controls, serum samples were collected from 56 healthy volunteers: 29 were male, 27 were female, and they ranged in age from 38 to 83 years, with a mean of 63.87 years.

DNA extraction from tissue and serum. Prior to DNA extraction, the tissue samples were frozen and stored at –20˚C and the serum samples were stored at 4˚C. DNA was prepared by proteinase-K digestion and phenol-chloroform extraction according to standard procedures by using a Dneasy Blood & Tissue Kit® (QIAGEN, Tokyo, Japan).

Bisulfite conversion of DNA. A total of 2 μg DNA was diluted in 50 μl dH_2O and 5.5 μl 2 M NaOH were added. After incubation of the mixture at 37˚C for 10 minutes, 30 μl of freshly prepared 10 mM hydroquinone (Sigma, St. Louis, MO, USA) and 520 μl of 3 M sodium bisulfite (pH 5.0) were added. The mixture was incubated at 50˚C for 16 hours and the DNA was purified with Wizard® DNA Clean-Up System (Promega, Pittsburgh, PA. USA), washed with 2 ml of 80% isopropanol, and placed in a clean 1.5 ml tube, to which 50 μl of 70˚C heated water were added. After 5 minutes, the tube was spun for 5 minutes at 15000 rpm and 5.5 μl of 3 M NaOH were added. After another 5 minutes, 1.0 μl of glycogen as a carrier, 1.0 μl of 10 M NH_4Ac, and three volumes of 100% ethanol were added. The DNA was placed in a freezer at –20˚C overnight and then spun for 30 minutes at 15000 rpm. The precipitated DNA was washed with 500 μl of 70% ethanol by centrifugation for 30 minutes at 15000 rpm. The DNA pellet was then dissolved in 20 μl H_2O.

Development of RTQ-MSP. RTQ-MSP is based on continuous optical monitoring during fluorogenic PCR, where one fluorescent dye serves as a reporter (6-carboxylfuorescein, FAM) and its emission spectrum is quenched by a second fluorescent dye (6-carboxytetramethyl-rhodamine, TAMRA). During the extension phase of PCR, the 5' to 3' exonuclease activity of the Taq DNA polymerase cleaves the reporter from the probe, thus releasing it from the quencher, resulting in an increase in fluorescence emission at 518 nm.

Using this system, we have developed a quantitative real-time two-step MSP system. In the first step of the PCR reaction, a cocktail of gene-specific primer pairs is used to co-amplify the DNA of various genes in a manner that is independent of their DNA

methylation status. In the second step, quantitative real-time PCR is performed with the end product DNA template of the first-step PCR reaction (diluted $1:10^4$ and placed in separate wells), the TAMRA quencher, methylation status-specific forward and reverse gene-specific primers, and a FAM-labeled probe.

For this study, two real-time MSP systems were developed: the first was used to quantify bisulfite-converted methylated *RUNX3* sequences, while the second quantified actin sequences, which served as an internal control. In the first system, bisulfite-modified DNA was first amplified by using specifically designed sense and antisense primers that amplify the methylated sequence of *RUNX3*, namely *RUNX3* MF (5'-GGTATAGTTTCGCGCGGGT-3') and *RUNX3* MR (5'-TATTCTCGCCCATCTTACCGC -3'), respectively. In the second PCR, the sense and antisense primers for the methylated sequence were *RUNX3* MF2 (5'-GGTATAGTTTCG CGCGGGT-3') and *RUNX3* MR2 (5'-TATTCTCGCCCATCTTA CCGC-3'), respectively, while the fluorogenic probe was *RUNX3* [5'-(FAM)-CGTCGGGTTAGCGAGGTTTCGTAGCGG-(MGB)-3']. In the actin system, the forward and reverse primer sequences were (5'-TGGTGATGGAGGAGGTTTAGTAAGT -3') and R (5'-AACCAATAAAACCTACTCCTCCCTTAA -3'), respectively, and the fluorogenic probe was [5'-(FAM)-ACCACCACCCAACA CACAATAACAAACACA-(TAMRA)-3']. Both fluorogenic probes were custom-synthesized by Perkin-Elmer Applied Biosystems (Foster City, CA, USA).

Briefly, the RTQ-MSP assay involves setting up the fluorogenic PCRs in reaction volumes of 25 μl with components supplied in the TaqMan PCR Core Reagent Kit (Perkin-Elmer Applied Biosystems). Each reaction contained 100 mM Tris-HCl, 50 mM NaCl, 5 mM $MgCl_2$, 300 nM of each amplification primer, 25 nM of the corresponding fluorogenic probe, 200 μM each of dATP, dCTP and dGTP, 400 μM dUTP, and 1.25 units of AmpliTaq Gold polymerase. In total, 1 μl of bisulfite-converted DNA from the serum or tissue was used in each RTQ-MSP assay. DNA amplification was carried out in a 0.2 ml reaction plate format in an Applied Biosystems 5700 Sequence Detector. Thermal cycling was initiated with a denaturation step consisting of 5 minutes at 94˚C. For RUNX3 RTQ-MSP, this step was followed by 45 cycles consisting of 95°C for 15 seconds, 70˚C for 50 seconds, and 72˚C for 1 minute. The amplification data generated from the tissue and serum samples and multiple negative water blanks were then collected by the 5700 Sequence Detector, stored in a Macintosh computer (Apple Computer, Cupertino, CA, USA), and analyzed by using the Sequence Detection System software (Version 1.6.3) developed by Applied Biosystems. The methylation index in each sample was expressed as the ratio $M/actin \times 10^3$, where M is the concentration of methylated *RUNX3* sequences (as measured by *RUNX3* M RTQ-MSP) and actin is the relative number of actin sequences (as measured by actin RTQ-MSP) after bisulfite conversion. Receiver operating characteristic (ROC) curve analysis using the serum samples of the 344 colorectal cancer patients and the 56 volunteers was performed to determine the limits of the positive and negative cut-off values.

MSI analysis. DNA from the surgically resected colorectal tumors and paired surrounding mucosae of the 119 colorectal cancer patients was obtained by using the Dneasy Blood & Tissue Kit (QIAGEN, Tokyo, Japan). MSI analysis was performed with five microsatellite markers recommended by the National Cancer Institute (BAT25, BAT26, D5S346, D2S123, and D17S250). PCR was performed in a total reaction volume of 25 μl that contained 50 ng genomic DNA, 2.5 μl of 10×PCR Gold buffer (150 mmol/l Tris-HCl, pH 8.0, 500 mmol/l KCl), 10 pmol fluorescent-labeled sense microsatellite primer, 10 pmol antisense microsatellite primer (not labeled with a fluorescent dye), 5 nmol of each dNTP, 37.5 nmol of $MgCl_2$, 2.5 U AmpliTaq Gold polymerase, and 16.5 μl of distilled water. The PCR conditions involved an initial denaturation step at 95˚C for 5 minutes followed by 40 cycles of 95˚C for 30 seconds, 55˚C for 30 seconds, 72˚C for 1 minute, and a final extension step at 72˚C for 30 minutes. The amplified PCR products were diluted 1:10 and run on an automated electrophoresis DNA sequencer (ABI PRISM® 310 Genetic Analyzer). The allelic sizes were estimated with GeneScan software (ABI PRISM® 310 Data Collection version 2.1). The tumors were characterized as having high-frequency MSI (MSI-H) if two or more of the five microsatellite markers showed instability, or as low-frequency MSI (MSI-L) if only one of the five microsatellite markers showed instability. If none of the five microsatellite markers showed instability, the tumors were determined to be microsatellite stable (MSS) (24).

Serum collection and tumor marker assay. Serum samples were collected preoperatively. Serum carcinoembryonic antigen (CEA) levels were determined by using CIS RIA MoAb-Centocor kits (CIS International, Gif-sur-Yvette, Cedex, France), which are based on an immunoradiometric assay. The cut-off values for the normal CEA serum assay were set at 5 ng/ml, as indicated by the instructions supplied with the kits.

Statistical analyses. Statistical analyses were carried out by using JMP 8.0.1 for Windows software (SAS Institute, Inc, Cary, NC, USA). Group differences were analyzed by using the Fisher's exact test and *t*-test. A value of $p<0.05$ was considered to indicate statistical significance. The log-rank test was used to statistically analyze the difference in survival.

Results

LightCycler validation of the RUNX3 RTQ-MSP technique. We have described the quantitative *RUNX3*-MSP technique previously, when this method was used to analyze the association between *RUNX3* methylation and gastric cancer (23). In this technique, real-time fluorescence PCR with fluorescent dye-labeled hybridization probes is monitored by an Applied Biosystems 5700 Sequence Detector, which permits the rapid and sensitive quantification of *RUNX3* methylation in patient samples (tissues and sera). This method can detect 5 fg-500 ng MKN28-derived DNA that contains methylated *RUNX3* sequences per μg of human placental DNA that contains unmethylated *RUNX3*. *RUNX3* methylation is quantified by determining the crossover point (Ct), *i.e*. the cycle when the fluorescence of a given sample rises above the background level to yield the maximal slope with respect to log-linear amplification. We constructed a standard curve by plotting the log number of serially diluted MKN28-derived DNA against their respective Cts. *RUNX3* methylation values for patient samples whose methylation status was unknown were calculated by referring to the calibration curve.

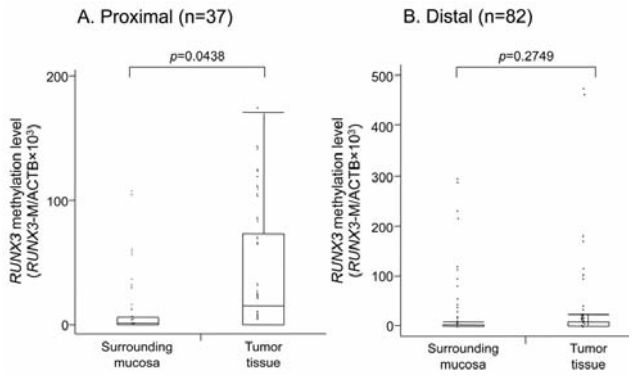

Figure 1. *Comparison of RUNX3 methylation in the tumors of patients with proximal (A) and distal (B) colorectal cancer with that in the paired surrounding mucosal tissues. The proximal colon includes the cecum, ascending colon and transverse colon. The distal colon includes the descending colon, sigmoid colon and rectum. There was significantly higher RUNX3 methylation in the proximal colon tumors than in the paired surrounding mucosae (*p=0.0438).*

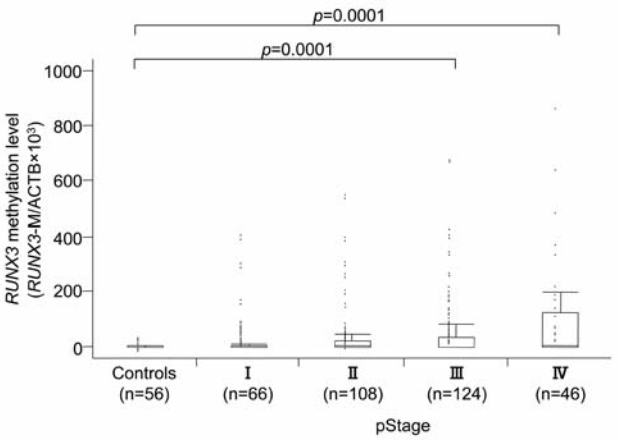

Figure 2. *Comparison of RUNX3 methylation in the serum of healthy volunteers and colorectal cancer patients divided according to disease stage. The stage III, and IV patients had significantly higher RUNX3 methylation than did the controls (p=0.0001).*

Table I. *Associations between RUNX3 methylation in the tumors of colorectal cancer patients and their clinicopathological parameters.*

Variable cases	Number of (Average±SD)	*RUNX3* Methylation level×10³	*p*-Value
Gender (mean age, years±S.D.)			0.0768
Male (56.47±15.01)	72	26.64±66.54	
Female (63.55±13.24)	47	14.11±33.05	
Tumor location			0.0054
Proximal	37	37.91±50.36	
Distal (including rectum)	82	14.38±57.05	
Tumor size (cm)			0.5302
≤4	57	20.22±42.92	
>4	62	23.05±65.99	
Lymphatic invasion			<0.0001
Negative	55	3.24±13.46	
Positive	64	37.55±71.77	
Vessel invasion			0.7635
Negative	36	28.07±85.63	
Positive	83	18.93±36.68	
Tumor differentiation			0.4785
Differentiated	109	16.50±33.87	
Not differentiated	10	78.33±152.80	
Pathological stage			0.0018
I	7	4.47±6.57	
II	47	9.00±24.46	
III	51	32.54±77.34	
IV	14	33.4-1±43.52	

Table II. *Relationship between RUNX3 methylation and microsatellite instability in the tumors of colorectal cancer patients.*

RUNX3-Methylation/MSI status	Tumor location						Total
	Proximal			Distal			
	Cecum	Ascending	Transverse	Descending	Sigmoid	Rectum	
RUNX3-M(+)/MSI-H	4 (36%)	6 (55%)	0	0	0	1 (9%)	11
RUNX3-M(+)/MSI-L	0	1 (50%)	0	0	0	1 (50%)	2
RUNX3-M(+)/MSS	7 (21%)	1 (3%)	1 (3%)	1 (3%)	14 (41%)	10 (29%)	34
RUNX3-M(–)/MSI-H	1 (50%)	1 (50%)	0	0	0	0	2
RUNX3-M(–)/MSI-L	3 (100%)	0	0	0	0	0	3
RUNX3-M(–)/MSS	4 (6%)	2 (3%)	6 (9%)	3 (4%)	18 (27%)	34 (51%)	67
Total	19	11	7	4	32	46	119

RUNX3-M(+) : RUNX3 methylation-positive; RUNX3-M(–): RUNX3 methylation-negative; MSI-H: high frequency of microsatellite instability; MSI-L: low frequency of microsatellite instability; MSS: microsatellite stable.

RUNX3 methylation in primary colorectal tumors and paired surrounding mucosae. By using *RUNX3* RTQ-MSP, aberrant *RUNX3* promoter methylation was detected in 39% (47 out of 119) of the surgically resected colorectal tumors. However, when all primary tumors were considered together, the *RUNX3* methylation was not significantly higher than that in the paired surrounding mucosae (p=0.8212). This was also the case when only the limited primary distal (including descending colon, sigmoid colon, and rectum) tumors were examined (p=0.2749, respectively). However, the limited primary proximal (including cecum, ascending colon, and transverse colon) tumors had significantly higher *RUNX3* methylation than the paired surrounding mucosae (p=0.0438) (Figure 1).

Associations between RUNX3 methylation in the tumors of colorectal cancer patients and clinicopathological factors. The *RUNX3* methylation values were calculated by determining the *RUNX3* methylation/β-actin ratios. Table I summarizes the *RUNX3* methylation ratios of the 119 colorectal tumors and their association with various clinicopathological factors. In terms of tumor location, the average methylation ratio of the proximal colon in tumors were statistically significently higher than that of distal tumors (p=0.0054). Tumors that exhibited lymphatic invasion had higher average ratio than those that did not and this difference was statistically significant (p<0.0001). In terms of pathological stage classification, the average ratio increased with increasing tumor stage. These differences were also statistically significant (p=0.0018). However, there were no significant differences in the ratios when patient gender (p=0.0768), tumor size (p=0.5302), vessel invasion (p=0.7635), and tumor differentiation (p=0.4785) were considered.

Relationship between the colorectal cancer RUNX3 methylation index and MSI status. ROC curve analysis of the *RUNX3* methylation/β-actin ratios revealed that this measurement has a sensitivity of 75.6%, a specificity of 76.3%, and an area under the curve (AUC) of 0.7606 when the optimal cut-off point of 7.546 was used. The patients were divided into *RUNX3* methylation-positive and -negative patients on the basis of this cut-off point (data not shown).

The MSI status of the resected tumors and their surrounding mucosae from 119 colorectal cancer patients was evaluated by PCR employing the five microsatellite markers recommended by the National Cancer Institute. Table II shows how the *RUNX3* methylation ratios of the 119 colorectal cancer tissues relate to their MSI status when the tumors are classified on the basis of location. There were 11 tumors that were both *RUNX3* methylation-positive and MSI-H. The *RUNX3* methylation-positive MSI-H tumors were predominantly located in the proximal colon (10 out of 11). There were two *RUNX3* methylation-positive MSI-L tumors, one each in the ascending colon and rectum, respectively. Thus, this type of tumor did not show a preference for either portion of the colon. In contrast, the majority (74%, 25 out of 34) of the remaining *RUNX3* methylation-positive tumors, which were MSS, were located in the distal colon. The majority (82%, 55 out of 67) of the *RUNX3* methylation-negative MSS tumors were also located in the distal colon.

Table III shows the proportions of *RUNX3* methylation-positive and -negative tumors that were MSI-H, MSI-L, or MSS. *RUNX3* promoter methylation was approximately 2.5 times more common in MSI-H tumors (85% of MSI-H tumors showed *RUNX3* promoter methylation) than in MSI-L or MSS tumors (34% of the MSI-L and MSS tumors showed such methylation; chi-square p=0.0070).

Table III. *Frequencies with which sporadic colorectal tumors exhibited both RUNX3 promoter hypermethylation and microsatellite instability.*

	RUNX3 Methylation level $\times 10^3 > 7.5$	*RUNX3* Methylation level $\times 10^3 \leq 7.5$
MSI-H	11/13 (85%)*	2/13 (15%)
MSI-L	2/5 (40%)	3/5 (60%)
MSS	34/101 (34%)	67/101 (66%)
Total	47/119 (39%)	72/119 (61%)

*Significantly different when compared with MSI-L and MSS tumors (p=0.0070).

Table IV. *Associations between RUNX3 methylation in the sera of colorectal cancer patients and their clinicopathological variables.*

Variable cases	Number of (Average±S.D.)	*RUNX3* Methylation level$\times 10^3$	p-Value
Gender (mean age, years±S.D.)			0.6277
Male (62.71±11.38)	205	23.64±55.09	
Female (65.69±11.29)	139	14.51±37.88	
Tumor location			0.2551
Proximal	114	22.90±62.90	
Distal (including rectum)	230	18.50±40.50	
Tumor size (cm)			0.1618
≤4	228	18.18±44.28	
>4	116	23.45±57.24	
Lymphatic invasion			0.0487
Negative	191	12.76±49.58	
Positive	153	40.25±79.41	
Vessel invasion			0.8655
Negative	104	25.50±66.19	
Positive	240	17.56±39.25	
Tumor differentiation			0.6626
Differentiated	311	20.29±50.84	
Not differentiated	33	16.83±26.43	
Pathological stage			0.0466
I	66	10.53±30.59	
II	108	15.04±37.45	
III	124	19.75±43.69	
IV	46	45.57±86.86	

Table V. *Relationship between CEA and RUNX3 methylation levels in the sera of colorectal cancer patients.*

CEA (ng/ml)	*RUNX3* Methylation level$\times 10^3 > 7.5$	*RUNX3* Methylation level$\times 10^3 \leq 7.5$
>5.0	39/344 (11.3%)	143/344 (41.6%)
≤5.0	61/344 (17.7%)	101/344 (29.4%)

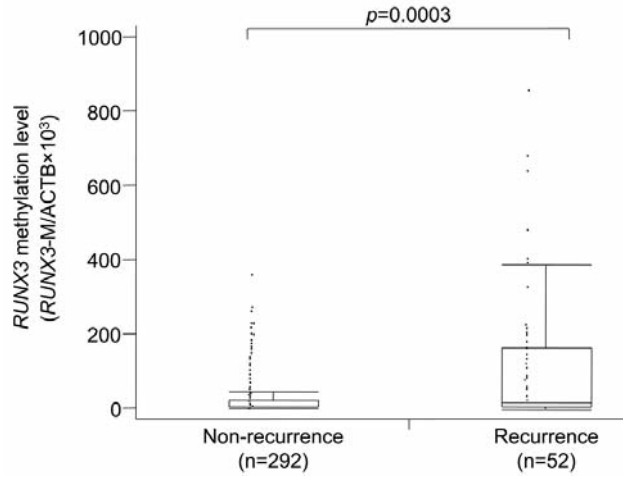

Figure 3. *Comparison of serum RUNX3 methylation in colorectal cancer patients who had (n=52) and did not have (n=292) disease recurrence during the three years after surgical resection. The cases with recurrence had significantly higher RUNX3 methylation than those without (p=0.0003).*

Overall survival curves. The overall survival curves of the colorectal cancer patients whose tumors were (n=47) and were not (n=72) positive for *RUNX3* hypermethylation were generated by the Kaplan-Meier method. The log-rank test was used to statistically analyze the difference in survival of the two groups. The two groups did not differ significantly in terms of their five-year survival rates.

Relationship between the RUNX3 methylation indices of the sera from colorectal cancer patients and the clinicopathological factors of the patients. Sera were obtained from another cohort of 344 colorectal cancer patients and their *RUNX3* methylation/β-actin ratios were determined. Table IV summarizes how the serum *RUNX3* methylation relates to the clinicopathological factors of the 344 patients. Methylated *RUNX3* was detected in the serum of 29% (100 of 344) of the colorectal cancer patients. The patients whose tumors showed lymphatic invasion had significantly higjer (p=0.0487) average serum *RUNX3* methylation/β-actin ratios than those who did not. When the tumors of the 344 patients were divided according to pathological stage classification, the average serum ratio again increased with increasing stage. These differences were statistically significant (p=0.0466). As shown in Figure 2, when the *RUNX3* methylation in the sera of the 344 colorectal cancer patients was compared to that in the sera from 56 healthy volunteers, the ratios of the stage III and stage IV patients were significantly higher than those of the controls (p=0.0001). However, there were no significant differences in serum ratios when the patients were divided according to gender (p=0.6277), tumor location (p=0.2551), tumor size (p=0.1618), vessel invasion (p=0.8655), or tumor differentiation (p=0.6626).

We investigated how the preoperative serum *RUNX3* methylation values related retrospectively to tumor recurrence in the three years following surgical resection (Figure 3). On average, the 52 patients who exhibited recurrence had significantly higher serum *RUNX3* methylation indices than the 292 non-recurrence cases (p=0.0003). In addition, Table V shows how the CEA levels in the preoperative serum relate to the preoperative serum *RUNX3* methylation levels. Of the 344 cases, 61 (17.7%) were negative for CEA but nevertheless exhibited serum *RUNX3* methylation. Thus, if *RUNX3* methylation is assessed together with CEA, another 17.7% of colorectal cancer patients can be diagnosed on the basis of serology alone.

Discussion

Colorectal cancer accounts for 10-15% of all cancer cases and is the second leading cause of cancer deaths in developed countries (25). In Japan, the incidence of colon cancer is increasing rapidly (26). The genetic alterations that are associated with colorectal cancer have been described as Vogelstein cascades (27) and include the loss of genes encoding p53, adenomatous polyposis coli *(APC)*, or deleted in colorectal cancer *(DCC)*, or involve a point mutation of *KRAS* (28, 29). Furthermore, MSI has been reported in some colorectal tumors (30, 31). However, the significance of each of these changes is unclear, as is the identity of the specific genes that are involved.

The aberrant hypermethylation of CpG islands is a hallmark of cancer (5, 32-34), and the clinical applications and prognostic value of this feature have been investigated for many types of cancer (5, 32-36). It has been shown that aberrant promoter methylation of *RUNX3*, which is a novel tumor suppressor of gastric cancer, is a common phenomenon in gastric cancer as well as in other types of cancer (8-10, 37-40). It was also reported recently that *RUNX3* promoter hypermethylation may participate in colorectal tumorigenesis (11, 41, 42). It is possible that such aberrant CpG island hypermethylation may precede and promote the further development of genetic mutations, including MSI (43). MSI due to CpG island hypermethylation occurs in approximately 15% of sporadic colorectal cancer, with a significant positive relationship being found between the prevalence of MSI and proximal tumor location (43-45). In this study, we investigated whether the methylation of *RUNX3* in colorectal tumors is associated with MSI. We also asked whether *RUNX3* methylation is a useful serum marker that can be used to detect colorectal cancer at an earlier stage and to identify patients at risk of recurrence.

We found that tumors of the proximal colon were significantly more likely to exhibit aberrant *RUNX3* promoter methylation than the paired surrounding mucosae. However, this was not observed when the tumors in the whole colon (including the rectum) or the tumors of the distal colon were examined. We also found that 18 out of the 119 colorectal tumors examined (15%) were MSI-H or MSI-L tumors, and that the vast majority of these (89%, 16/18) were located in the proximal colon. Thus, both MSI and *RUNX3* promoter hypermethylation predominate in tumors of the proximal colon. Of the 119 colorectal cancer cases, 11% (13/119) had MSI-H, which is similar to previously reported frequencies (46, 47), and 11 of these 13 (87%) exhibited *RUNX3* hypermethylation. We found that *RUNX3* promoter methylation was ~2.5 times more common in MSI-H tumors (85%) than in MSI-L/MSS tumors (34%; chi-square p=0.0070). While a study by Goel *et al*. found a lower rate of aberrant *RUNX3* methylation in MSI-H sporadic colon tumors (13/40, 33%) (48), they also observed that *RUNX3* promoter methylation was ~3 times more common in MSI-H tumors than in MSI-L/MSS tumors (48), which is consistent with our findings.

The loss of *RUNX3* expression is strongly associated with a poor prognosis for many types of cancer (9, 49-55). However, it seems that this association is not true for colorectal cancer because the patients with hypermethylated *RUNX3*-bearing tumors did not differ significantly in terms of their five-year survival rates from patients whose tumors had normal *RUNX3* promoters. These observations are consistent with those of Soong *et al*. (55). Survival was not significantly associated with the localization or expression of *RUNX3* in immunostaining and *RUNX3* methylation (data not shown).

It has been reported that conventional MSP analysis of serum samples can detect the methylation of *RUNX3* in various types of cancer (11). We showed here that our highly sensitive RTQ-MSP technique can detect and measure the circulating, tumor-derived, methylated *RUNX3* sequences in a significant proportion (29%) of 344 colorectal cancer patients. In addition, the high specificity of the assay was proven by the absence of methylated *RUNX3* sequences in the serum of most of the controls (23). None of the controls exhibited methylated *RUNX3*.

RTQ-MSP analysis of the 119 colorectal tumors revealed for the first time a significant association between higher *RUNX3* methylation and an advanced cancer stage, lymphatic invasion, and a proximal tumor location. Similarly, RTQ-MSP analysis of the preoperative sera of the 344 colorectal cancer patients revealed that significantly higher *RUNX3* methylation was associated with an advanced cancer stage and lymphatic invasion. These associations are similar to those detected when we analyzed gastric cancer (23).

Our serum analysis also revealed that compared with 292 non-recurrent cases, the 52 cases with recurrence within three years of their first operations had significantly higher *RUNX3* methylation. Histologically, most of these patients had mucinous, poorly differentiated carcinomas with lymph node involvement.

We found that combining the CEA and *RUNX3* methylation data can facilitate the serology-based diagnosis of colorectal cancer, as 61 out of the 344 patients (17.7%) were negative for CEA but positive for *RUNX3* promoter hypermethylation. While approximately 40% of patients with colorectal cancer are positive for CEA detection in the serum (56), CEA positivity is low for patients with poorly differentiated adenocarcinomas (57). Therefore, combining CEA and *RUNX3* methylation can improve the sensitivity and accuracy of serology-based cancer diagnostic tests.

In summary, these data show that *RUNX3* is a valuable supplementary tumor marker and that RTQ-MSP for *RUNX3* may be a powerful diagnostic and prognostic tool for the detection of colorectal cancer recurrence. Further studies investigating the clinical utility of *RUNX3* as a diagnostic and prognostic marker are warranted.

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