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RUNX3 Promoter Region is Specifically Methylated in Poorly-differentiated Colorectal Cancer

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Abstract. *Background, Materials and Methods: It has recently become clear that RUNX3 expression is frequently silenced by aberrant methylation in gastric cancers. In this study, we investigated the methylation status of the RUNX3 gene in 92 resected primary colorectal cancers using methylation-specific PCR (MSP) and correlated the results with the clinicopathological features of affected patients. Results: Aberrant promoter methylation of the RUNX3 gene was detected in 31 out of 92 (34%) colorectal cancers. A significant difference in histology (p=0.028) was also found on comparing the RUNX3 methylation of poorly-differentiated colorectal cancers to that of other differentiated ones. Conclusion: RUNX3 aberrant methylation might play an important role in colorectal cancers, especially in poorly-differentiated colorectal cancers.*

Advances in molecular genetics have established that several genetic changes, such as activation of the K-*ras* oncogene and inactivation of the *p53* tumor suppressor gene, are involved in the pathogenesis of colorectal and other cancers (1-3). Recently, a growing number of cancer genes have been recognized that harbor methylation in normally unmethylated promoter CpG islands (4, 5). This epigenetic change results in no expression of the tumor suppressor gene and plays a key role in an epigenetically-mediated loss-of-gene function that is as critical for tumorigenesis as mutations in coding regions. In fact, it has been confirmed that hypermethylation of a normally unmethylated CpG island in the promoter region of *p16* correlates with its loss of transcription in various cancers (6-8). On the other hand, 70-80% of colorectal cancers with microsatellite instability show aberrant promoter hypermethylation and lack expression of hMLH1 (9).

Recently, the loss of expression of *RUNX3*, a transcription factor containing a highly conserved domain, designated as a 'runt domain', accompanied by *RUNX3* gene methylation was reported in gastric cancers (10). In this report, between 45% and 60% of human gastric cancer cells do not significantly express *RUNX3* due to hemizygous deletion and hypermethylation of the *RUNX3* gene. Tumorigenecity of human gastric cancer cell lines in nude mice was inversely related to their level of *RUNX3* expression, suggesting that *RUNX3* is a common target for methylation and epigenetic gene silencing in gastric cancer and qualifies as a potential gastric cancer suppressor gene.

These results prompted us to examine the methylation status of *RUNX3* in colorectal cancers. It might be possible that *RUNX3* was also inactivated by methylation in other digestive tract cancers and is related to the tumorigenic pathway. In this study, we examined *RUNX3* methylation in colorectal cancers using the methylation-specific PCR (MSP). The results obtained were then compared to the clinicopathological features.

Materials and Methods

Sample collection and DNA preparation. Ninety-two primary tumors were collected at the Nagoya University School of Medicine, Japan, from Japanese colorectal cancer patients who had been diagnosed histologically. The tissue samples were obtained during surgery, quickly frozen in liquid nitrogen and stored at –80˚C until analysis. Tumor samples were digested overnight by proteinase K and DNA was prepared by extraction with phenol. Oral or written informed consent, as indicated by the institutional review board, was obtained from all patients. There was no family history of cancer

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Abbreviations: Methylation-specific PCR, MSP.

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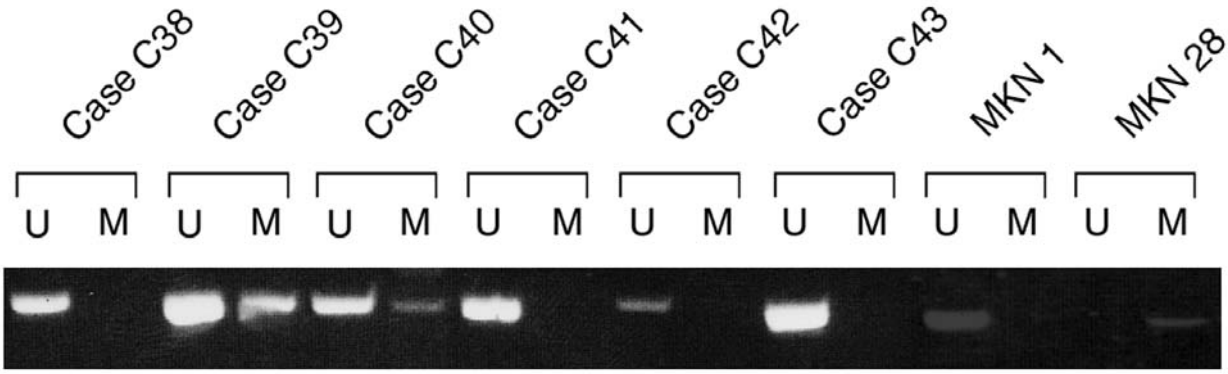

Figure 1. *Representative MSP of RUNX3 promoter in colorectal cancer samples. The presence of a visible PCR product in Lane U indicates the presence of unmethylated genes; the presence of PCR product in Lane M indicates the presence of methylated genes. RUNX3 promoter methylation was present in cases 39 and 40. In each case, modified DNA from MKN1 and MKN28 was used as positive controls of RUNX3 for unmethylated and methylated alleles, respectively.*

in the poorly-differentiated colorectal cancer patients. The tumor sites of 7 poorly-differentiated colorectal cancers were rectum (4 patients), sigmoid colon (1 patient) and cecum (2 patients).

Bisulfite modification and MSP. DNA from the colorectal cancer specimens was subjected to bisulfite treatment, as described previously (11). The modified DNA was used as a template for MSP. The primers for the unmethylated reaction were: RUNX3UMS (sense), 5'-GGTAGGTTGTGGGTGGTTGTT, and RUNX3UMAS (anti-sense), 5'-TCCTCAACCACCACTAC CACA, which amplify a 146 bp product. The primers for the methylated reaction were: RUNX3MS (sense), 5'- GGTAGG TTGCGGGCGGTCGTC, and RUNX3MAS (anti-sense), 5'-CCTCGACCGCCGCTACCGCG, which amplify a 145 bp product. The PCR amplification of the modified DNA samples consisted of 1 cycle at 95˚C for 5 min; 33 cycles at 95˚C for 30 sec, 69˚C for 1 min and 72˚C for 1 min; 1 cycle at 72˚C for 5 min. DNA from MKN1 and MKN 28 (gastric cancer cell lines) was used as positive controls for *RUNX3* amplification of unmethylated and methylated alleles, respectively. The methylation status of the MKN1 and MKN 28 cells has been examined previously (10). Control reactions without DNA were performed for each set of PCR. Ten μl of each PCR product was directly loaded onto non-denaturing 6% polyacrylamide gels, stained with ethidium bromide and visualized under UV illumination. Each MSP was repeated at least 3 times. We considered that the presence of a visible PCR product in *Lane U* or *M* indicated the presence of unmethylated or methylated genes, respectively.

Statistical analysis. The χ^2 test and Student's *t*-test were used to examine the association between *RUNX3* promoter methylation and clinicopathological features.

Results

We first examined the methylation status of the *RUNX3* promoter in tumors using the MSP technique. Aberrant promoter methylation of the *RUNX3* gene was detected in 31 out of 92 (34%) colorectal cancers, indicating that *RUNX3* aberrant methylation might play an important role in these cancers. A representative MSP analysis of *RUNX3* gene promoter methylation from the tumors is shown in Figure 1, illustrating that in no cases was methylation of the colorectal cancers complete. Therefore, it might be possible that the *RUNX3* gene expression was not completely inhibited in these cancers.

To determine the role of *RUNX3* methylation in colorectal cancer, we examined the correlation of methylation status with the clinicopathological features. There was no significant difference in the distribution of patients with positive or negative methylation of *RUNX3* in terms of sex, maximal tumor size, the extent of tumor, lymph node metastasis, or Dukes' stage. However, we found a significant difference in histology ($p=0.028$) when we compared the *RUNX3* methylation of poorly-differentiated colorectal cancers to that of other differentiated ones (Table I). These results suggest that poorly-differentiated colorectal cancers specifically exhibited *RUNX3* methylation.

Discussion

Colorectal cancer, a most aggressive cancer, occurs with a high incidence in most countries (12). Standard therapy involves tumor resection followed by chemotherapy and radiotherapy. It is also important to determine the genetic alterations as a new parameter for estimation of colorectal cancer. Colorectal cancers are classified histopathologically as either differentiated carcinomas forming tubular or papillary structures, or poorly-differentiated carcinomas including mucinous adenocarcinoma, in which such structures are inconspicuous. Poorly-differentiated

Table I. *Clinicopathological features and methylation status of RUNX3 promoter region in colorectal cancer patients.*

Clinicopathological features	Variable	No. of cases	*RUNX3* methylation		*p*-value
			+	–	
sex	male	55	22	33	0.119[a]
	female	37	9	28	
maximal tumor size	15-100 mm	92	50.2±3.6[c]	47.4±2.5	0.519[b]
histology	poorly-differentiated[d]	7	5	2	0.028[a]
	other differentiated[e]	85	26	59	
extent of tumor	≤mt[f]	23	6	17	0.373[a]
	mt<	69	25	44	
lymph node	+	33	11	22	0.956[a]
metastasis	–	59	20	39	
Dukes' stage	A, B	61	20	41	0.796[a]
	C, D	31	11	20	
total		92	31	61	

[a]χ^2 test; [b]Student's *t*-test; [c]mean±S.D; [d]poorly-differentiated or mucinous adenocarcinoma according to Japanese criteria; [e]well- or moderately-differentiated adenocarcinoma according to Japanese criteria; [f]muscular tunic.

colorectal carcinomas are quite rare, comprising only 3-5% of all colorectal carcinomas. It is well known that mucinous carcinoma is frequently observed in colorectal cancer with genetic instability, but the difference in genetic pathways between these histological types is mostly unknown because of the very small number of cases (13). In this study, we investigated the methylation status of *RUNX3* in colorectal cancers and found that most (71%) poorly-differentiated colorectal cancers presented *RUNX3* methylation, while only 31% of other differentiated colorectal cancers did. We have previously examined the methylation status of the *CDH13* gene and found a significant difference in histology ($p=0.0053$) on comparing the *CDH13* methylation between poorly-differentiated and differentiated colorectal cancer (14). These results suggested that inactivation of the *RUNX3* gene or/and the *CDH13* gene play an important role in the poor differentiation of colorectal cancers.

As described, the incomplete methylation of the *RUNX3* gene suggests that this gene has not been inhibited completely in primary colorectal cancers. Zheng *et al.* previously reported that the partial methylation pattern was associated with relatively low levels of $p14^{ARF}$ in colorectal cancer cell lines (15). $p14^{ARF}$ mRNA was expressed at extremely low levels in fully methylated cell lines. $p14^{ARF}$ expression in the partially-methylated LoVo cell line was intermediate. Moreover, partial methylation of $p14^{ARF}$ was the most common pattern observed in primary colorectal cancers. Taken together, it was suggested that *RUNX3* gene expression might also be controlled by methylation in colorectal cancers.

Recent studies have shown that it is possible to reverse epigenetic changes and restore gene function. Treatment with DNA methylation inhibitors can restore the activities of the *RUNX3* gene and decrease the growth rate of cancer cells. The administration of drugs, such as cytosine analogs, might soon enable the functional restoration of these tumor suppressor genes and slow the rate of colorectal cancer progression.

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