Highly-methylated Colorectal Cancers Show Poorly-differentiated Phenotype

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Abstract. The combined methylation status of p16, p14, HLTF, SOCS-1, CDH13, RUNX3 and CHFR was examined in 58 resected primary colorectal cancers using methylation-specific PCR, and the methylation status was correlated with the clinicopathological features of the affected patients. A significant difference in histology (p=0.0041) was observed when the number of methylated genes of poorly-differentiated colorectal cancers was compared to that of other differentiated ones. Poorly-differentiated colorectal cancers preferentially exhibited gene methylation. There is now strong 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 (1-3). In addition, several other genes were found to be related to the pathogenesis of colorectal cancer (4, 5) and investigation of genetic changes is important in clarifying its tumorigenic pathway (6).

Several tumor suppressor genes contain CpG islands in their promoters, prompting many investigations into the role of methylation in silencing these genes. Many tumor suppressor genes show evidence of methylation silencing, providing a new potential pathway for the deactivation of tumor suppressor genes. At first, a tumor suppressor gene, p16, was found to harbor promoter hypermethylation associated with the loss of protein expression in cancer cells (7). Though homozygous deletions of the p16 locus were not present (8), p16 promoter methylation was detected in colorectal cancer (9). Subsequently, it has been found that human p14 was also silenced by promoter hypermethylation in colorectal cancer (10). p14 interacts with the MDM2 protein and neutralizes the MDM2-mediated degradation of p53. Thus, p14 acts as a tumor suppressor gene via inhibition of p53 degradation. These studies indicated that p16 and p14 inactivation due to promoter methylation was important for colorectal tumorigenesis. We previously examined the methylation status of these genes in 86 primary colorectal cancers using methylation-specific PCR (MSP) (11). Aberrant promoter methylation of p16 and p14 genes was detected in 43 out of 86 (50 %) and 25 out of 86 (29%) colorectal cancer specimens, respectively.

The loss of expression of a helicase-like transcription factor (HLTF), a SWI/SNF family member gene, accompanied by HLTF promoter methylation was reported in colon cancers (12). In this report, all colon cancer cell lines that lacked the HLTF gene expression demonstrated methylation of CpG sites within the putative HLTF promoter, while methylation was not detected in the HLTF-expressing cell lines. Moreover, HLTF methylation was detected in 25 out of 76 primary colon cancers, suggesting that HLTF is a common target for methylation and epigenetic gene silencing in colon cancer and qualifies as a potential colon cancer suppressor gene (13).

Yoshikawa et al. demonstrated that suppressor of cytokine signaling-1 (SOCS-1) was silenced frequently by methylation of the CpG island in human HCC (14). SOCS-1 is an intracellular protein that negatively regulates the Janus kinase (JAK) and the signal transducer and activation of transcription (STAT) signaling pathway, a principal cytokine signaling transduction pathway. The JAK/STAT pathway is also known to play an important role in the regeneration of hepatocytes (15, 16) and additional studies indicated that both JAKs and STATs were involved in the oncogenesis of several tumors (17, 18). These findings suggested a potential role of the SOCS protein, a growth suppressor in hepatocarcinogenesis through negative regulation of the JAK/STAT pathway. The aberrant methylation of the SOCS-1 CpG island in 6 out of 74 (8%) colorectal cancer specimens was also detected in our laboratory (19). The correlation between the clinicopathological features
and SOCS-1 aberrant methylation was subsequently analyzed and younger age was found to be significantly related to SOCS-1 methylation (p=0.048).

Subsequently, it also recently became clear that CDH13 (H-cadherin, T-cadherin) expression is frequently silenced by aberrant methylation in colorectal cancers and adenomas (20, 21). CDH13 encodes a protein belonging to the cadherin family of cell surface glycoproteins responsible for selective cell recognition and adhesion (22). Ubiquitous methylation of CDH13 in colorectal cancers and adenomas indicated that such methylation occurs at an early stage in the multistage process of oncogenesis.

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 (23). In this report, between 45% and 60% of human gastric cancer cells did not significantly express RUNX3 due to hemizygous deletion and hypermethylation of the RUNX3 gene. The tumorigenicity 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. Aberrant promoter methylation of the RUNX3 gene was detected in 31 out of 92 (34%) colorectal cancers (24).

In recent years, the CHFR (checkpoint with the FHA and RING finger) gene was reported as a new mitotic checkpoint gene. CHFR protein delayed chromosome condensation and entry into metaphase in response to the mitotic stress induced by a microtubule inhibitor (25). However, cancer cell lines lacking CHFR entered into metaphase without any delay after treatment with a microtubular inhibitor, thus, suggesting the function of CHFR as a mitotic checkpoint. Recently, the aberrant methylation of the CHFR gene associated with gene silencing was reported in several primary tumors (26-30). The aberrant methylation of the CHFR gene was also detected in 25 out of 98 (26%) colorectal cancers. This finding suggested that an aberrant methylation of the CHFR gene occurs frequently in colorectal cancers.

In this study, the combined methylation status of p16, p14, HLTF, SOCS-1, CDH13, RUNX3 and CHFR genes was examined in colorectal cancer and corresponding normal tissue specimens using MSP, as described previously (11, 13, 19, 21, 24). The methylation status of p16, p14, HLTF, SOCS-1, CDH13, RUNX3 and CHFR genes were originally examined in 86, 86, 76, 74, 84, 92, and 98 colorectal cancer specimens, respectively. Fifty-eight specimens were tested for the methylation status of all 7 genes. Aberrant methylation of p16, p14, HLTF, SOCS-1, CDH13, RUNX3 and CHFR genes was detected in 34 (58%), 20 (34%), 18 (31%), 6 (8%), 22 (37%), 22 (37%) and 25 (26%) of 58 colorectal cancer specimens, respectively (Figure 1). All tumors also exhibited unmethylation of each gene, which might be the result of the contamination of nontumorous cells in the tumor specimens. Otherwise, it might be possible that these gene expressions had not been completely inhibited in these cancer specimens.

The correlation between the methylation status and clinicopathological data was subsequently analyzed (Table I). Colorectal cancers with more than 4 methylated genes were included in the highly-methylated group (HMG), while colorectal cancers with less than 3 methylated genes made the low-methylated group (LMG). A significant difference in histology (p=0.0041) was found when the number of methylated genes of poorly-differentiated colorectal cancers was compared to that of other differentiated ones (Table I), suggesting that poorly-differentiated colorectal cancers preferentially exhibited gene methylation. No other factors such as gender, age, tumor size, extent of tumor, tumor site, or lymph node metastasis were correlated with the number of methylated genes.

**Materials and Methods**

Sample collection and DNA preparation. Fifty-eight primary tumors and corresponding normal tissues were collected consecutively at Nagoya University Hospital from colorectal cancer patients. All tissues were confirmed histologically. The collected samples were stored immediately at –80°C until analysis. DNA was prepared as described previously (31).

Sodium bisulfite modification. One µg of genomic DNA extracted from tumors and corresponding normal colorectal tissues was subjected to bisulfite treatment, as described previously (32). Briefly, alkali-denatured DNA was modified by 2.1 M sodium bisulfite / 0.5 mM hydroquinone at pH 5.0. The bisulfite-reacted DNA was then treated by NaOH, purified using the Wizard DNA Clean-Up System® (Promega, Madison, WI, USA), precipitated with ethanol and resuspended in distilled water.

MSP. The bisulfite-treated DNA was amplified with MSP. The primers and PCR conditions were described previously (7, 10, 12, 14, 20, 23). Ten µl of each PCR product were loaded directly onto nondenaturing 8% polyacrylamide gels, stained with ethidium bromide and visualized under UV illumination.

Statistical analysis. The possible association between the number of methylated genes and clinicopathological features was examined using the Chi-squared test or Student’s t-test. The computed p-values were two-tailed. Statistical significance was considered as p<0.05.

**Results**

The methylation status of p16, p14, HLTF, SOCS-1, CDH13, RUNX3 and CHFR genes was examined in colorectal cancer and corresponding normal tissue specimens using MSP, as described previously (11, 13, 19, 21, 24). The methylation status of p16, p14, HLTF, SOCS-1, CDH13, RUNX3 and CHFR genes were originally examined in 86, 86, 76, 74, 84, 92, and 98 colorectal cancer specimens, respectively. Fifty-eight specimens were tested for the methylation status of all 7 genes. Aberrant methylation of p16, p14, HLTF, SOCS-1, CDH13, RUNX3 and CHFR genes was detected in 34 (58%), 20 (34%), 18 (31%), 6 (8%), 22 (37%), 22 (37%) and 25 (26%) of 58 colorectal cancer specimens, respectively (Figure 1). All tumors also exhibited unmethylation of each gene, which might be the result of the contamination of nontumorous cells in the tumor specimens. Otherwise, it might be possible that these gene expressions had not been completely inhibited in these cancer specimens.

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Discussion

Our results indicated that poorly-differentiated colorectal cancer specimens preferentially exhibited a higher number of methylated genes including \(p16, p14, \) \(HLTF, SOCS-1, CDH13, RUNX3\) and \(CHFR\) genes in 58 colorectal cancers. The methylated gene is shown as a closed box. Colorectal cancers with more than 4 methylated genes were included in the highly-methylated group (HMG) while colorectal cancers with less than 3 methylated genes in the low-methylated group (LMG).

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 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 (34).

Colorectal cancer, one of the most aggressive cancers, occurs at a high incidence rate in most countries (35). To eliminate this fatal cancer, surgery and subsequent chemotheraphy and radiotherapy are generally performed. For this purpose, it is important to seek new genetic and epigenetic alteration parameters to estimate cancer malignancy.

References


Note: Table I. Clinicopathologic features and methylation status in colorectal cancer patients.

<table>
<thead>
<tr>
<th>Clinicopathological features</th>
<th>Variables</th>
<th>No. of cases</th>
<th>Methylation status</th>
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| Gender                      | Male      | 32           | 6                  | 26      | 0.451^a
|                             | Female    | 26           | 3                  | 23      |         |
| Age                         | 22-83     | 58           | 64±10^b            | 61±10   | 0.230^b
| Maximum tumor size          | 15-100 mm | 58           | 38±11              | 47±18   | 0.906^b
| Extent of tumor             | ≤pm^d     | 17           | 3                  | 14      | 0.773^a
|                             | pm<       | 41           | 6                  | 35      |         |
| Histology                   | Well^e    | 53           | 6                  | 47      | 0.0041^a
|                             | Poor      | 5            | 3                  | 2       |         |
| Tumor site                  | C, A, T^f | 18           | 3                  | 15      | 0.871^a
|                             | D, S, R^g | 40           | 6                  | 34      |         |
| Lymph node                  | –         | 34           | 5                  | 29      | 0.839^a
| metastasis                  | +         | 24           | 4                  | 20      |         |
| Total                       |           | 58           | 9                  | 49      |         |

^a chi-squared test; ^b Student’s t-test; ^c mean±S.D.; ^d proper muscle; ^e well- or moderately differentiated adenocarcinoma; ^f poorly-differentiated or mucinous adenocarcinoma; ^g C, cecum; A, ascending colon; T, transverse colon; D, descending colon; S, sigmoid colon; R, rectum.

Figure 1. Methylation status of \(p16, p14, \) \(HLTF, SOCS-1, CDH13, RUNX3\) and \(CHFR\) genes in 58 colorectal cancers. The methylated gene is shown as a closed box. Colorectal cancers with more than 4 methylated genes were included in the highly-methylated group (HMG) while colorectal cancers with less than 3 methylated genes in the low-methylated group (LMG).


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