p16INK4a Gene Promoter Hypermethylation in Mucosa as a Prognostic Factor for Patients with Colorectal Cancer

Yvonne Wettergren, Elisabeth Odin, Staffan Nilsson, Göran Carlsson, and Bengt Gustavsson

INTRODUCTION

Colorectal cancer (CRC) is the third leading cause of cancer deaths in Western countries. In Sweden, approximately 6,000 new cases of CRC will be diagnosed each year, and about 3,000 individuals will die of the disease (1). A major cause of death in CRC is the development of distant metastases through the spreading of tumor cells from the primary tumor site. Thus, it is important to find biological markers that can identify CRC patients who would benefit from adjuvant treatment due to an increased risk of recurrence. It also would be of great value to identify premalignant alterations in macroscopically normal-appearing colorectal mucosa that could be used in screening tests for patients at risk of developing CRC.

Several studies have shown that the colorectal mucosa in patients with CRC is characterized by alterations at the DNA, RNA, and protein levels that can be associated with premalignant behavior (2–5), metastatic potential (6), and prognosis (7–9). Dietary factors such as folate, alcohol, and methionine in combination with polymorphisms in genes like *methylentetrahydrofolate reductase* may be associated with CRC because of their influence on DNA methylation processes (10,11). In a previous study, we have shown that low expression of the folate-associated gene *folylpolyglutamate synthase* (*FPGS*, EC 6.3.2.17) in colorectal mucosa adjacent to surgically removed tumors correlated with poor survival of CRC patients and could be used as a prognostic marker (12).

Low gene expression of *folylpolyglutamate synthase* (*FPGS*) in colorectal mucosa correlates with low folate levels and poor survival of colorectal cancer (CRC) patients. Because gene-specific hypermethylation is affected by the folate level, the hypermethylation status in mucosa may also be linked to clinical outcome of CRC patients. The tumor suppressor gene *p16INK4a* (*p16*) regulates the cell cycle and angiogenic switch. In human neoplastic tissues, the main mechanism of *p16* inactivation is promoter methylation. The aim of the study was to determine whether hypermethylation of the *p16* promoter could be detected in mucosa of CRC patients (*n* = 181) and to analyze if hypermethylation was related to survival. The relation between *p16* hypermethylation and expression of *FPGS* and two other folate-associated genes, *reduced folate carrier 1* (*RFC-1*), and *thymidylate synthase* (*TS*), was analyzed (*n* = 63). The results showed that *p16* was hypermethylated in 65 (36%) of the mucosa samples and that hypermethylation was age-related (*P* = 0.029). After adjustment for known risk factors, Cox regression analysis showed that Dukes’ A–C patients with *p16* hypermethylation in mucosa had an increased risk of cancer-related death (hazard ratio = 2.9, *P* = 0.007) and shorter disease-free survival (hazard ratio = 2.5, *P* = 0.015) compared with patients with no *p16* hypermethylation. *RFC-1* and *FPGS* gene expression levels were significantly correlated in patients lacking *p16* hypermethylation in mucosa (*P* = 0.0003), but not at all correlated in patients having hypermethylation in mucosa (*P* = 1.0). In conclusion, *p16* hypermethylation in mucosa of CRC patients was identified as an independent prognostic parameter for cancer-specific survival as well as an independent predictor of DFS. The results suggest that there might be a connection between folate-associated gene expression and *p16* methylation status.

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of reduced folates into the cells is the reduced folate carrier, RFC-1. In colorectal mucosa, there usually is a correlation between the RFC-1 and FPGS gene expression levels, possibly indicating a balance between uptake and polyglutamation of folates. Within the cells, folate polyglutamates are further converted to methyltetrahydrofolate, which is required as a methyl donor in the synthesis of dTMP from dUMP. The reaction requires the catalytic activity of the enzyme thymidylate synthase (TS, EC 2.1.1.45). Because all of these folate-associated genes can be expected to affect DNA methylation indirectly by interacting with the methyl-group metabolism (12), we wanted to analyze the relationship between their expression and gene-specific hypermethylation in mucosa of CRC patients.

The tumor suppressor gene p16 is a cyclin-dependent kinase inhibitor that acts as a negative regulator of cell growth and proliferation in the G1 phase of the cell cycle (13). In addition, previous research has implicated p16 as an important regulator of the angiogenic switch (14). The recent finding that p16 controls epigenetic changes such as DNA hypermethylation in homeobox genes (15) makes the gene very interesting as a biomarker of pre-malignant alterations. While p16 methylation has not been detected in normal colorectal mucosa obtained at autopsy from individuals without colorectal cancer (16), p16 gene promoter methylation has been found in colorectal dysplasia, adenomas, malignant tumors, and normal mucosa adjacent to tumors (17–19). In fact, the major mechanism of p16 gene inactivation seems to be promoter region methylation (20).

Although hypermethylation of some genes seems to start early and to increase with age, the rate of hypermethylation may be enhanced during certain pathological conditions. For instance, the results of Hsieh et al. (21) suggested that hypermethylation of the p16 gene promoter region is a frequent and early event during neoplastic progression in ulcerative colitis. Furthermore, Jang et al. (22) showed that non-tumorous tissues adjacent to gastric cancers displayed a close association among the grade of chronic inflammation and p16 gene promoter hypermethylation. Their results suggested that inactivation of the p16 gene by promoter hypermethylation was an early event in gastric carcinogenesis that might serve as a prognostic marker for the risk of gastric cancers.

Methylation of p16 has been reported to occur at a frequency of 7% to 53% in malignant colorectal tumors (19,23–25). Some results implicate p16 methylation as being an early event during colorectal carcinogenesis, whereas others suggest that it is a late event (26,27). Although the absence of p16 methylation in mucosa adjacent to tumors has been reported (27), most studies have shown that the frequency of p16 methylation is higher in mucosa than in corresponding colorectal tumors (19,28,29). Methodological differences may be one explanation for obtaining conflicting results. However, discrepant results also may be attributed to ethnic variations caused by genetic and/or dietary factors, or could be linked to clinicopathological differences among different patient cohorts, such as age and tumor location. Possibly, a high p16 hypermethylation frequency in normal mucosa reflects a premalignant stage that precedes tumor development. A lower hypermethylation frequency in tumors compared with adjacent mucosa could then result from reversal of CpG island methylation or deletion of the hypermethylated allele in the growing tumor.

The gene expression level in macroscopically normal-appearing mucosa adjacent to tumors is frequently used as a baseline for comparison. Already 20 y ago, however, an abnormal pattern of cell proliferation was found in the entire colonic mucosa of patients with adenoma or cancer (30). The finding indicated that although neoplastic lesions develop in a limited area of the colon, the entire large bowel may be at risk for tumor growth. Several recent studies have shown that the adjacent mucosa is not normal metabolically when compared with mucosa of healthy controls (31–34). Alterations have been found at different distances from the site of the growing tumor, and may occur in a patch-like manner (31) or as a ‘‘field-change’’ surrounding the tumor (34). Polley et al. (35) analyzed protein expression in morphologically normal colonic mucosa from healthy subjects and patients with adenomatous polyps or colon cancer (obtained more than 10 cm from the tumor margin). Sixty-one proteins were found to differ significantly between mucosa from healthy subjects and all other tissue types (polyp mucosa, cancer mucosa, or tumor tissue), while 206 differed significantly between healthy mucosa and polyp mucosa. These findings indicate that protein expression in the apparently normal colonic mucosal field is modified in individuals with neoplastic lesions at sites distant from the lesion.

It is now becoming increasingly clear that some of the changes detected in grossly normal-appearing mucosa using molecular methods can be used to predict the prognosis of CRC patients. Barrier et al. (9) recently showed that microarray gene expression profiles of non-neoplastic mucosa could be used to predict the postoperative prognosis of stage II colon cancer patients. Previous studies at our laboratory have shown that the expression of folate-associated genes in mucosa is associated with the survival of CRC patients (8,12). Possibly, alterations in the normal-appearing mucosa represent pre-malignant changes associated with an enhanced risk of tumor cell spreading. To distinguish these changes from normal inter-individual variations at the tissue level, the characterization of a healthy mucosa is needed. The aim of the present study was to analyze the p16 gene promoter hypermethylation status in mucosa adjacent to colorectal tumors and relate it to the expression of the folate-associated genes RFC-1, FPGS, and TS, and to clinicopathological parameters.
MATERIALS AND METHODS

Patients and Study Design

Surgically resected specimens from macroscopically normal-appearing mucosa were obtained from 181 patients with non-hereditary colorectal adenocarcinoma diagnosed at the Sahlgrenska University Hospital/Östra during the period between 1994 and 2004. The ethic committee of Göteborg University approved the study and informed consent was obtained from each of the patients. The tissue samples were obtained from areas approximately 10 cm from the primary tumors by surgical resection. All samples were snap frozen in liquid nitrogen immediately after surgical excision and stored at –70° C until use. Surgical and pathological records were reviewed for patients’ gender, age at surgery, tumor location, tumor differentiation grade, and tumor stage. Tumors were graded histopathologically by experienced pathologists as recommended by the World Health Organization (36), and classified as right-sided when proximal and left-sided when distal to the splenic flexure. Malignant tumors were classified according to MAC staging system as Dukes-MAC A (TNM I), Dukes-MAC B (TNM II), Dukes-MAC C (TNM III), or Dukes’ D (TNM IV) (37). Twenty-nine of the patients were subjected to pre-surgical radiotherapy, 24 Dukes’ C and 2 Dukes’ B patients received in the adjuvant setting 5-fluorouracil-based chemotherapy and 28 patients were subjected to palliative treatment. Patients’ characteristics are presented in Table 1.

Bisulfite Modification of DNA

Genomic DNA was extracted from mucosa samples using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Two μg of purified genomic DNA were denatured at 97° C for 10 min and then cooled on ice for 5 min. The DNA was then treated with 0.3 M NaOH for 20 min at 48° C. Next, 500 μL bisulfite solution (1.9 g sodium bisulfite [Sigma, St. Louis, MO, USA] dissolved in 2.5 mL H2O at 48° C; 500 μL freshly made 1 M hydroquinone solution; 700 μL 2 M NaOH) were added, and the solution was incubated in darkness at 48° C for 16 h. Bisulfite-treated DNA was purified using Wizard DNA purification system (Promega Corporation, Madison WI, USA) and eluted in 45 μL H2O. Five μL 3 M NaOH were added to the eluate and the solution was incubated at 37° C for 10 min. Seventy-five μL 5 M ammonium acetate were added and the solution was incubated at room temperature for 5 min. Before DNA precipitation, 7 μL glycogen was added as a carrier. DNA was then precipitated with 330 μL 99.5% ethanol at –70° C for 1 h. After centrifugation at 4° C for 20 min, the DNA pellet was dried at room temperature and then dissolved in 20 μL 5 mM Tris, pH 8.0. Modified DNA was stored at –20° C until used.

Methylation Analysis

Bisulfite-treated DNA was analyzed on a Sequencing Detector ABI Prism 7700 (Applied Biosystems, Foster City, CA, USA) using a fluorescence-based, real-time methylation specific PCR method (38). Primers and probes specifically amplifying bisulfite-converted DNA representing the p16 gene promoter (GenBank accession number NM_000077, amplicon location 66-133 bp) and the internal reference gene MYOD1 (GenBank accession number AF027148, amplicon location 9889-9962 bp) were used. The specificity of the reactions for methylated DNA was confirmed using CpGenome Universal Methylated DNA (Intergen Company, New York City, NY, USA). The primer and probe sequences used were the following: a) p16, 5′-TGGAGTGGTTCGATTTGATTTGTTG-3′ (forward primer); 5′-AACAA GGCCCCGACCTCTCCT-3′ (reverse primer); 6FAM-′ACCCGACCCCGAACC CGG-3′TAMRA (probe); b) MYOD1, 5′-CAAC GCTCAATCCCCCTCTCTCAGT-3′ (forward primer); 5′-TGATTAATTTAGGTTGGTAGAGAAGGA-3′ (reverse primer); 6FAM-′TCTCACTTCTATTCTAAATC CAACTAATACCTCC-3′TAMRA

### Table 1. Clinicopathological features and hypermethylation status of the 181 CRC patients included in the study.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>All</th>
<th>p16 hypermethylation status</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>negative</td>
<td>positive</td>
</tr>
<tr>
<td></td>
<td>n (%)</td>
<td>p (%)</td>
</tr>
<tr>
<td>Gender, n (%)</td>
<td>181 (100)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>80 (44.2)</td>
<td>52 (65.0) 28 (35.0)</td>
</tr>
<tr>
<td>Male</td>
<td>101 (55.8)</td>
<td>64 (63.4) 37 (36.6)</td>
</tr>
<tr>
<td>Mean age (year ± SD)</td>
<td>69 ± 14</td>
<td>68 ± 14 72 ± 12 0.029b</td>
</tr>
<tr>
<td>Tumor location, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right-sided colon</td>
<td>62 (34.4)</td>
<td>37 (58.7) 26 (41.3)</td>
</tr>
<tr>
<td>Left-sided colon</td>
<td>47 (26.1)</td>
<td>34 (72.3) 13 (27.7)</td>
</tr>
<tr>
<td>Rectum</td>
<td>71 (39.5)</td>
<td>45 (63.4) 26 (36.6)</td>
</tr>
<tr>
<td>Differentiation grade, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High/Moderate</td>
<td>119 (65.7)</td>
<td>79 (66.4) 40 (33.6)</td>
</tr>
<tr>
<td>Low</td>
<td>62 (34.3)</td>
<td>37 (59.7) 25 (40.3)</td>
</tr>
<tr>
<td>Dukes’ tumor stage, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>10 (5.5)</td>
<td>7 (70.0) 3 (30.0)</td>
</tr>
<tr>
<td>B</td>
<td>71 (39.2)</td>
<td>42 (59.2) 29 (40.8)</td>
</tr>
<tr>
<td>C</td>
<td>53 (29.3)</td>
<td>38 (71.7) 15 (28.3)</td>
</tr>
<tr>
<td>D</td>
<td>47 (26.0)</td>
<td>29 (61.7) 18 (38.3)</td>
</tr>
<tr>
<td>Total, n (%)</td>
<td>181 (100)</td>
<td>116 (64.1) 65 (35.9)</td>
</tr>
</tbody>
</table>

αP by Fisher’s exact test.
βP by ANOVA t-test.
γP by Pearson’s Chi-square test.

One tumor was located at the borderline between the right and left side of the colon and could not be categorized with regard to tumor location.
Real-time PCR was performed as follows: Forty μL of 2x TaqMan Universal PCR Master mix (without AmpErase UNG enzyme) were mixed with forward and reverse primers (final concentrations 0.6 μM) and fluorescent probe (final concentration 0.2 μM) and the mixture was left at room temperature for 10–15 min. Forty μL of the mixture were then mixed with 10 μL of template DNA. The 50 μL PCR-mixture was split in half and the two 25 μL samples were run in parallel. Thermal cycling started with two incubation steps, the first at 50°C for 2 min and the second at 95°C for 10 min, and proceeded with 50 cycles of 95°C for 15 s, and 58°C for 1 min. Negative (water) and positive (CpGenome Universal Methylated DNA, Intergen Company, New York City, NY, USA) controls were used in each set of PCR reactions. All samples scored positive for DNA input as measured by the control gene MYOD1. Samples with no detectable peak at PCR cycle 50 were judged as being negative. These samples were analyzed at two different occasions.

**cDNA Preparation and Real-Time Quantitative PCR**

At the start of the RNA extraction, tissue samples were placed under liquid nitrogen and pulverized with a steel mortar and pestle that had been thoroughly pre-cooled. Total RNA was isolated according to Chomczynski and Sachi (39) and then reverse-transcribed to cDNA according to Horikoshi et al. (40). The gene expression levels of RFC-1, FPGS, and TS were determined using quantitative PCR as described previously (12).

**Statistics**

Cancer-specific survival and disease-free survival (DFS) were estimated by the Kaplan-Meier method (41). Cancer-specific survival time was calculated from the date of surgery to the last time of follow-up or to the date of death caused by cancer. Four patients who died within 1 month post-surgery were censored, as were 28 patients whose deaths were considered not to be associated with CRC, and three patients for whom survival data could not be obtained. DFS time was calculated from the date of surgery to the last time of follow-up or to the date of recurrence. Survival outcomes were calculated using the Cox proportional hazard model. Data were analyzed by statistical modeling using the commercial software programs JMP (version 7.0, SAS) or SPSS (version 16.0). Statistical differences between groups were tested using Analysis of Variance (ANOVA) t-test, Kruskal-Wallis’ test, Fisher’s exact test, or Pearson’s Chi-square test as indicated in the tables. To compare sets of continuous parameters measured in the same tissue, the Pearson correlation coefficient (r) was used. Statistical values of P ≤ 0.05 were judged as significant. No corrections for multiple testing were done.

**RESULTS**

**Hypermethylation of p16 in Mucosa**

p16 hypermethylation was detected in 65 (36%) of the samples whereas the remaining 116 (64%) were judged as negative for hypermethylation. The association of p16 hypermethylation in mucosa and clinicopathological data is presented in Table 1. As shown, no significant differences were detected when patients were sub-grouped by gender, tumor location, tumor differentiation grade, or Dukes’ stages and stratified by p16 hypermethylation status. However, patients with p16 hypermethylation in mucosa were significantly older (72 ± 12 years) than patients without hypermethylation (68 ± 14 years, P = 0.029).

**p16 Hypermethylation According to Tumor Location in Colon**

Because previous studies (24,42) have shown that methylation of tumor suppressor genes in general is more frequent in right- compared with left-sided colon, we analyzed the association of p16 hypermethylation in mucosa with tumor location. The results showed that the frequency of p16 hypermethylation was highest in right-sided colon (41%) followed by rectum (37%), and left-sided colon (28%). The frequency distribution of patients sub-grouped according to p16 hypermethylation status in mucosa and tumor location is presented in Figure 1. As shown, p16 hypermethylation was more common in mucosa obtained from patients having tumors in the right part of the colon.
p16 Hypermethylation in Mucosa and Cancer-Specific Survival

The relationship between p16 hypermethylation in mucosa and cancer-specific survival was analyzed for Dukes’ A-C patients (n = 134). Fifty-six (42%) of these patients had died during follow-up (24 women and 32 men; mean age 73 ± 12 y) whereas 78 (58%) were still alive (44 women and 34 men; mean age 67 ± 13 y; mean follow-up 50 ± 12 months). The results showed that patients without p16 hypermethylation in mucosa (n = 87, mean age 68 ± 13 years) tended to live longer than patients with hypermethylation (n = 47, mean age 72 ± 12 y). The estimated 5-year survival rate was 72% and 61%, respectively, in the two groups (P = 0.063). When Dukes’ B patients were analyzed separately, p16 hypermethylation was found to be associated with a significantly shorter survival time. The estimated 5-year survival rate was 90% for patients negative for p16 hypermethylation (n = 42) and 66% for patients positive for hypermethylation (n = 29, P = 0.0028). The mean age of the Dukes’ B patients was 67 ± 13 and 75 ± 11 years in the two groups, respectively (P = 0.017). The estimated 5-year survival time for Dukes’ C patients was 44% and 57% for patients without (n = 38) and with (n = 15) hypermethylated p16 in mucosa, respectively. This difference in survival time was not significant however (P = 0.26). The mean age of the Dukes’ C patients was 68 ± 15 and 68 ± 14 y in the two groups, respectively (P = 1.0).

p16 Hypermethylation in Mucosa and Disease-Free Survival (DFS)

During follow-up, one of the Dukes’ A patients relapsed. This patient was one of three who had p16 hypermethylation in mucosa. Out of the 42 Dukes’ B patients with no signs of p16 hypermethylation in mucosa, only 6 (14%) relapsed. In contrast, 9 out of 29 (31%, P = 0.14) Dukes’ B patients who showed p16 hypermethylation relapsed. The estimated 5-year DFS rates for Dukes’ B patients sub-grouped by negative or positive p16 hypermethylation in mucosa were 83% and 67%, respectively (P = 0.079). Twenty-one out of 53 patients (39.6%) with Dukes’ C stage tumors relapsed during follow-up. The estimated 5-year DFS rates for Dukes’ C patients sub-grouped by negative or positive p16 hypermethylation in mucosa were 55% and 59%, respectively (P = 0.46).

Cox Regression Multivariate Analysis

For a multivariate analysis, a Cox regression model was applied to examine whether p16 hypermethylation in mucosa was a risk factor for cancer-specific death independent of known risk factors such as tumor stage, tumor differentiation grade, age, gender, tumor location, adjuvant chemotherapy, pre-surgical radiotherapy, and p16 hypermethylation status. Cox regression analysis of the relationship between the clinicopathological parameters and p16 hypermethylation on DFS revealed that the relative risk of Dukes’ A-C patients relapsing was 2.5 times higher (P = 0.015) if the p16 gene was hypermethylated in mucosa (Table 2, Figure 3). A tendency toward an increased risk of relapsing associated with p16 hypermethylation also was seen when Dukes’ B patients were analyzed separately (hazard ratio = 3.2, P = 0.060). No association between age and DFS was found among Dukes’ B patients, however, p16 hypermethylation in mucosa was not found to be a risk factor for cancer recurrence in patients with Dukes’ C stage tumors.

| Table 2. Cox multivariate analyses demonstrating the influence of p16 hypermethylation status in mucosa on cancer-specific survival and DFS of Dukes’ A-C, Dukes’ B, and Dukes’ C patients, respectively. |
|-----------------|-----------------|-----------------|
| Cancer-specific survival | Hazard ratio | (95% CI) | P | Hazard ratio | (95% CI) | P | Hazard ratio | (95% CI) | P |
| No p16 hypermethylation | 1 | | | 1 | | | 1 | | |
| p16 hypermethylation | 2.9 (1.3–6.2) | 0.007 | 4.7 (1.1–19.5) | 0.035 | 2.0 (0.67–5.9) | 0.22 | |
| DFS | No p16 hypermethylation | 1 | | | 1 | | | 1 | | |
| p16 hypermethylation | 2.5 (1.2–5.1) | 0.015 | 3.2 (0.95–11) | 0.060 | 1.8 (0.60–5.4) | 0.30 | |

a Multivariate analysis was performed using the co-variates Dukes’ tumor stage, tumor differentiation grade, age, gender, tumor location, adjuvant chemotherapy, pre-surgical radiotherapy, and p16 hypermethylation status.

bConfidence interval.

p by likelihood ratio tests, multivariate analysis.

Gene Expression of RFC-1, FPGS, and TS According to p16 Hypermethylation Status

No significant differences were detected when the gene expression levels of RFC-1, FPGS, and TS were compared in p16 hypermethylation positive and negative mucosa (Table 3). The mean...
FPGS gene expression level was non-significantly lower, however, in samples with p16 hypermethylation compared with those without hypermethylation. When the correlation between RFC-1, FPGS, and TS gene expression was analyzed, RFC-1 and FPGS gene expression was found to be significantly correlated in patients that lacked p16 hypermethylation in mucosa (Table 4), but not at all correlated if the p16 promoter was hypermethylated. In contrast, FPGS and TS expression was correlated significantly regardless of the p16 hypermethylation status. No correlation between RFC-1 and TS expression was found.

DISCUSSION

Aging may predispose human tissues to neoplasia through alterations in expression of genes involved in cell growth, adhesion, differentiation, migration, and apoptosis. Age-associated inactivation of tumor suppressor genes may occur through mutation of one allele and LOH or promoter hypermethylation of the other allele (43). It has been reported that age-related methylation begins in normal tissues and eventually progresses to hypermethylation in cancer (26). Waki et al. (16) studied age-related promoter methylation of several genes in non-neoplastic cells derived from different human tissues obtained at autopsy. The incidence of age-related methylation paralleled the reported methylation incidence in malignant counterparts in the majority of organs. In normal colorectal mucosa samples (28 colonic and 26 rectal) obtained from 35 individuals without CRC (aged 0.7 to 87 y), p16 methylation was not detected.

In the present study, the hypermethylation status of the p16 gene promoter in normal-appearing mucosa obtained 10 cm from malignant colorectal tumors was analyzed. The results showed that p16 was hypermethylated in 36% of the samples. In line with previous results (26,44), patients with p16 hypermethylation in mucosa were found to be significantly older than patients without hypermethylation. Also in agreement with...
previous studies (24,42), p16 hypermethylation tended to be more common in mucosa of patients with right-sided, compared with left-sided, colon tumors. We did not detect any differences according to gender, however. Cox regression analysis showed that p16 hypermethylation in colorectal mucosa of Dukes’ A-C patients was an independent prognostic parameter for cancer-related death as well as an independent predictor of DFS. When Dukes’ B and C patients were analyzed separately, however, an association between p16 hypermethylation status in mucosa and survival was seen only for Dukes’ B patients. The increased risk was independent of all clinicopathological parameters, including age, in spite of the fact that patients with p16 hypermethylation in mucosa were significantly older than those without hypermethylation.

The reason for the association of p16 hypermethylation in adjacent mucosa with the increased risk of dying in CRC may be linked to a number of events initiated in the mucosa when the p16 gene is inactivated. The normal function of p16 is to decelerate the cell cycle by inactivating cyclin-dependent kinases phosphorylating the Rb protein (13). Hence, p16 is involved directly in controlling the rate of cell proliferation. Downregulation of p16 would lead to a hyperproliferative state which is characteristic of the inflamed mucosa. Recent research also has implicated that the methylation status of p16 plays an important role in the regulation of angiogenesis (45). Inactivation of p16 leads to upregulation of the very potent pro-angiogenic factor vascular endothelial growth factor (VEGF) which has multiple roles in angiogenesis: it increases the permeability (46) and promotes survival (47) of existing vessels, as well as stimulates new vessel growth (48). Thus, inactivation of p16 would have a major impact on vascularization in mucosa. Malignant tumor cells surrounded by a mucosa where vascularization is abnormal might migrate more easily and reach microvessels even far from the primary tumor site. Normally,

Figure 3. Multivariate Cox model for p16 hypermethylation in mucosa and DFS of patients with A) Dukes’ A-C, B) Dukes’ B, and C) Dukes’ C tumor stages. Survival curves for patients with and without p16 hypermethylation in mucosa are significantly different for Dukes’ A-C patients and a similar trend is seen for Dukes’ B patients.
p16 inhibits the expression of matrix metalloproteinase-2 (MMP-2) in human cancer cells to suppress tumor invasion and metastasis (49). Inactivation of p16 would lead to an increased MMP-2 expression, further enhancing the capacity of the growing tumor to invade blood vessels. Thus, the risk of tumor cell spreading and invasion to the surroundings would be elevated substantially through p16 inactivation.

Interestingly, Chen et al. found that several inflammation markers were altered in mucosa of patients with CRC as compared with controls (31). These alterations could be found in mucosa several cm from the tumor site. It is known that a long-standing chronic inflammation in the colorectal epithelium will lead to hyperproliferation and oxidative stress (50), a state that increases the need for folate utilized in DNA synthesis and repair. Ultimately, folate deficiency may occur. Folate deficiency has been associated with decreased expression of adhesion molecules and increased expression of urokinase in the colon mucosa of rats, suggesting that cell detachment and migration may be modulated by folate status (51). Thus, folate deficiency in combination with p16 hypermethylation in mucosa may lead to major environmental alterations increasing the ability of tumor cells to migrate, infiltrate microvessels, and, eventually, to metastasize.

The relationship between promoter methylation of genes involved in colorectal carcinogenesis, including p16, and folate was investigated recently by van Engeland et al. (10). Their results suggested that folate and alcohol intake were associated with changes in gene promoter hypermethylation in CRC. In the present study, we have analyzed the correlation between the hypermethylation status of the p16 gene promoter region and the gene expression level of the folate-associated enzymes RFC-1, FPGS, and TS in mucosa. Because we have shown previously that the FPGS gene expression level correlates with the folate concentration in mucosa (12), we use it as a surrogate marker of the folate level. Assuming that a correlation between RFC-1 and FPGS gene expression indicates a balance between uptake and polyglutamation of folates, the results showed that such a balance only existed in mucosa negative for p16 hypermethylation. A strong correlation between FPGS and TS gene expression was found in mucosa, however, regardless of p16 hypermethylation status. Because TS expression correlates with the proliferation rate in tissues (52–54), our results indicate a correlation between the folate level and the proliferation rate in the mucosa. The superior survival of patients lacking p16 hypermethylation in mucosa might then be related to the correlation between the RFC-1 and FPGS gene expression levels in the mucosa. In a healthy mucosa, the uptake and retention of folates is expected to meet the need of the cellular proliferation rate, as seems to be the case in mucosa negative for p16 hypermethylation.

Folate deficiency in combination with oxidative stress, which is common among the elderly (55), may result in global hypomethylation concomitant with gene-specific hypermethylation as has been described previously (56). When Iacopetta et al. (57) used LINE-1 repeat methylation as a surrogate marker of the genomic methylation level in CRC, no associations between methylation levels in LINE-1 repeats and CpG island loci were seen in cancer tissues. In normal colonic mucosa, however, the methylation level of the repeats was inversely correlated with CpG island methylation of the MLH1, p16, TIMP3, APC, ER, and MYOD genes. With advancing age, genomic LINE-1 methylation decreased while gene-specific CpG island methylation increased. Furthermore, Keyes et al. (58) found that aging decreased genomic DNA methylation, but increased p16 promoter methylation in mouse colons, and that the effect depended on the level of dietary folate. In old mice, however, methylation of the p16 promoter was associated with an increased gene expres-

### Table 3. Mean gene expression levels in colorectal mucosa of Dukes’ A-D patients stratified by p16 hypermethylation status.

<table>
<thead>
<tr>
<th>Gene</th>
<th>n⁰</th>
<th>All</th>
<th>n</th>
<th>Negative</th>
<th>n</th>
<th>Positive</th>
<th>P⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFC-1</td>
<td>60</td>
<td>0.14 ± 0.17</td>
<td>36</td>
<td>0.15 ± 0.20</td>
<td>24</td>
<td>0.13 ± 0.12</td>
<td>0.89</td>
</tr>
<tr>
<td>FPGS</td>
<td>63</td>
<td>0.84 ± 0.61</td>
<td>38</td>
<td>0.92 ± 0.60</td>
<td>25</td>
<td>0.72 ± 0.61</td>
<td>0.079</td>
</tr>
<tr>
<td>TS</td>
<td>62</td>
<td>2.6 ± 2.6</td>
<td>37</td>
<td>2.2 ± 2.1</td>
<td>25</td>
<td>3.2 ± 3.2</td>
<td>0.29</td>
</tr>
</tbody>
</table>

⁰Enough material for gene expression analysis could only be obtained for the number of patients specified in the table.
⁺P by Kruskal-Wallis’ test.

### Table 4. Correlation between RFC-1, FPGS, and TS gene expression in colorectal mucosa of Dukes’ A-D patients stratified by p16 hypermethylation status.

<table>
<thead>
<tr>
<th>RFC-1 and FPGS</th>
<th>FPGS and TS</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>60</td>
</tr>
<tr>
<td>p16 hypermethylation</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>36</td>
</tr>
<tr>
<td>Positive</td>
<td>24</td>
</tr>
</tbody>
</table>

⁰Enough material for gene expression analysis could only be obtained for the number of patients specified in the table.
⁺⁺ by Spearman’s rank correlation test.
⁺⁺⁺P by Pearson.
amplicon, the importance of study detects only a fully methylated cause the method used in the present be the true for some of the patients. Be-permethylated. This scenario might well

permethylation of the p16 a low expression level may result if the human tumor tissues leads to inactiva-

tion with demethylating agents alone might be underestimated. At present, we are analyzing p16 protein expression in the mucosa samples using Western blot technique, correlating the outgoing re-
sults to the level of methylation in the p16 promoter region. The results of the present study, as well as those of previous recent studies (8,9,12,31–35), indicate that the use of matching normal-appearing mucosa as control material in CRC may be ques-
tioned. Clearly, the colorectal mucosa of patients with CRC differs from that of healthy individuals on several levels. What is particularly interesting is that the alterations in the mucosa can be used as prognostic markers. Giving attention to changes in the normal-appearing mucosa instead of focusing on alterations found in surgically excised tumors, which usually are very heterogenous, might improve the understanding of CRC pathogenesis. Restoration of wild-type p16 into human p16-deleted glioma cells has been shown to reduce the expression of VEGF and to inhibit neovascularization in-
duced by tumor cells in vivo (60). Treat-

ment with demethylating agents alone or in combination with histone acetylase inhibitors and DNA-targeting chemotherapeutic drugs might be useful when attempting to reverse and over-
come the inactivation of p16 and other tumor suppressor genes. The antihyper-
tensive drug hydralazine seems to be well tolerated and effective to demethy-
late and reactivate the expression of tumor suppressor genes without affect-

ing global DNA methylation (61). The ef-

effect of epigenetic cancer therapy on breast, lung, cervical, ovarian, head and neck, prostate, and testicular cancer is being evaluated presently in phase II and III studies (62). If the results of these studies turn out to be promising, similar studies on gastrointestinal cancer would be most valuable.

In conclusion, the present study showed that p16 hypermethylation in mucosa was associated with inferior survival of CRC patients with Dukes’ A-C stage tumors. The hypermethyla-
tion status of p16 in mucosa seems to be especially useful as a prognostic fac-
tor for Dukes’ B patients. The results further suggest that there is a connection between folate-associated gene expression and the p16 methylation sta-
tus. The potentially reversible nature of CpG island methylation may provide novel therapeutic opportunities to indi-

viduals with gene-specific promoter hypermethylation.

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