

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

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Low gene expression of *folypolyglutamate 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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## 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 *methylenetetrahydrofolate 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 *folypolyglutamate 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). *FPGS* encodes an enzyme that converts reduced folyl monoglutamates to polyglutamates intracellularly. By doing this, the folates are retained better inside the cells. Because we found that *FPGS* gene expression correlated with folate concentration in mucosa, we use it as a surrogate marker for folate. The major transporter

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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 methyl-ene-tetrahydrofolate, 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 pre-malignant 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  $-70^{\circ}\text{C}$  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  $\mu\text{g}$  of purified genomic DNA were denatured at  $97^{\circ}\text{C}$  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  $48^{\circ}\text{C}$ . Next, 500  $\mu\text{L}$  bisulfite solution (1.9 g sodium bisulfite [Sigma, St. Louis, MO, USA] dissolved in 2.5 mL  $\text{H}_2\text{O}$  at  $48^{\circ}\text{C}$ ; 500  $\mu\text{L}$  freshly made 1 M

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

Parameter	All	<i>p16</i> hypermethylation status		<i>P</i>
		negative	positive	
Gender, <i>n</i> (%)				
Female	80 (44.2)	52 (65.0)	28 (35.0)	
Male	101 (55.8)	64 (63.4)	37 (36.6)	0.88 <sup>a</sup>
Mean age (year $\pm$ SD)	69 $\pm$ 14	68 $\pm$ 14	72 $\pm$ 12	0.029 <sup>b</sup>
Tumor location, <sup>d</sup> <i>n</i> (%)				
Right-sided colon	62 (34.4)	37 (58.7)	26 (41.3)	
Left-sided colon	47 (26.1)	34 (72.3)	13 (27.7)	
Rectum	71 (39.5)	45 (63.4)	26 (36.6)	0.33 <sup>c</sup>
Differentiation grade, <i>n</i> (%)				
High/Moderate	119 (65.7)	79 (66.4)	40 (33.6)	
Low	62 (34.3)	37 (59.7)	25 (40.3)	0.42 <sup>a</sup>
Dukes' tumor stage, <i>n</i> (%)				
A	10 (5.5)	7 (70.0)	3 (30.0)	
B	71 (39.2)	42 (59.2)	29 (40.8)	
C	53 (29.3)	38 (71.7)	15 (28.3)	
D	47 (26.0)	29 (61.7)	18 (38.3)	0.50 <sup>c</sup>
Total, <i>n</i> (%)	181 (100)	116 (64.1)	65 (35.9)	

<sup>a</sup>*P* by Fisher's exact test.

<sup>b</sup>*P* by ANOVA *t*-test.

<sup>c</sup>*P* by Pearson's Chi-square test.

<sup>d</sup>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.

hydroquinone solution; 700  $\mu\text{L}$  2 M NaOH) were added, and the solution was incubated in darkness at  $48^{\circ}\text{C}$  for 16 h. Bisulfite-treated DNA was purified using Wizard DNA purification system (Promega Corporation, Madison WI, USA) and eluted in 45  $\mu\text{L}$   $\text{H}_2\text{O}$ . Five  $\mu\text{L}$  3 M NaOH were added to the eluate and the solution was incubated at  $37^{\circ}\text{C}$  for 10 min. Seventy-five  $\mu\text{L}$  5 M ammonium acetate were added and the solution was incubated at room temperature for 5 min. Before DNA precipitation, 7  $\mu\text{L}$  glycogen was added as a carrier. DNA was then precipitated with 330  $\mu\text{L}$  99.5% ethanol at  $-70^{\circ}\text{C}$  for 1 h. After centrifugation at  $4^{\circ}\text{C}$  for 20 min, the DNA pellet was dried at room temperature and then dissolved in 20  $\mu\text{L}$  5 mM Tris, pH 8.0. Modified DNA was stored at  $-20^{\circ}\text{C}$  until used.

### Methylation Analysis

Bisulfite-treated DNA was analyzed on a Sequence 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'-TGGAGTTTTTCGGTTG ATTGGTT-3' (forward primer); 5'-AACAA CGCCCGCACCTCCT-3' (reverse primer); 6FAM5'-ACCCGACCCCGAACC GCG-3'TAMRA (probe); b) *MYOD1*, 5'-CCAAC TCCAAATCCCCTCTCTAT-3' (forward primer); 5'-TGATTAATTTAGATTGGGTT TAGAGAAGGA-3' (reverse primer); 6FAM5'-TCCCTTCCTATTTCCTAAATC CAACCTAAATACCTCC-3'TAMRA

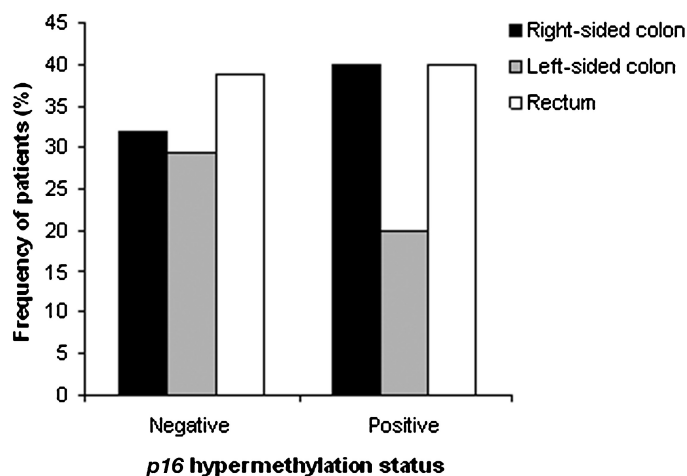
(probe). Real-time PCR was performed as follows: Forty  $\mu\text{L}$  of 2x TaqMan Universal PCR Master mix (without AmpErase UNG enzyme) were mixed with forward and reverse primers (final concentrations 0.6  $\mu\text{M}$ ) and fluorescent probe (final concentration 0.2  $\mu\text{M}$ ) and the mixture was left at room temperature for 10–15 min. Forty  $\mu\text{L}$  of the mixture were then mixed with 10  $\mu\text{L}$  of template DNA. The 50  $\mu\text{L}$  PCR-mixture was split in half and the two 25  $\mu\text{L}$  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



**Figure 1.** Frequency distribution of patients with CRC subgrouped according to *p16* hypermethylation status in mucosa and tumor location ( $n = 181$ ). As shown, the distribution of right- and left-sided colon tumors is almost equal among patients having no *p16* hypermethylation in mucosa. In contrast, twice as many patients have right-sided compared with left-sided tumors among patients having *p16* hypermethylation in mucosa ( $P = 0.16$ ).

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  $P \leq 0.05$  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 ( $72 \pm 12$  years) than patients without hypermethylation ( $68 \pm 14$  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, compared with the left part. However, the difference did not reach significance ( $P = 0.16$ ).

**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  $73 \pm 12$  y) whereas 78 (58%) were still alive (44 women and 34 men; mean age  $67 \pm 13$  y; mean follow-up  $50 \pm 12$  months). The results showed that patients without p16 hypermethylation in mucosa ( $n = 87$ , mean age  $68 \pm 13$  years) tended to live longer than patients with hypermethylation ( $n = 47$ , mean age  $72 \pm 12$  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  $67 \pm 13$  and  $75 \pm 11$  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  $68 \pm 15$  and  $68 \pm 14$  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%,  $P = 0.14$ )

**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.

	Dukes' A-C		Dukes' B		Dukes' C	
	Hazard ratio <sup>a</sup> (95% CI) <sup>b</sup>	P <sup>c</sup>	Hazard ratio <sup>a</sup> (95% CI) <sup>b</sup>	P <sup>c</sup>	Hazard ratio <sup>a</sup> (95% CI) <sup>b</sup>	P <sup>c</sup>
Cancer-specific survival						
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

<sup>a</sup>Multivariate analysis was performed using the co-variables Dukes' tumor stage, tumor differentiation grade, age, gender, tumor location, adjuvant chemotherapy, pre-surgical radiotherapy, and p16 hypermethylation status.

<sup>b</sup>Confidence interval.

<sup>c</sup>P by likelihood ratio tests, multivariate analysis.

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**

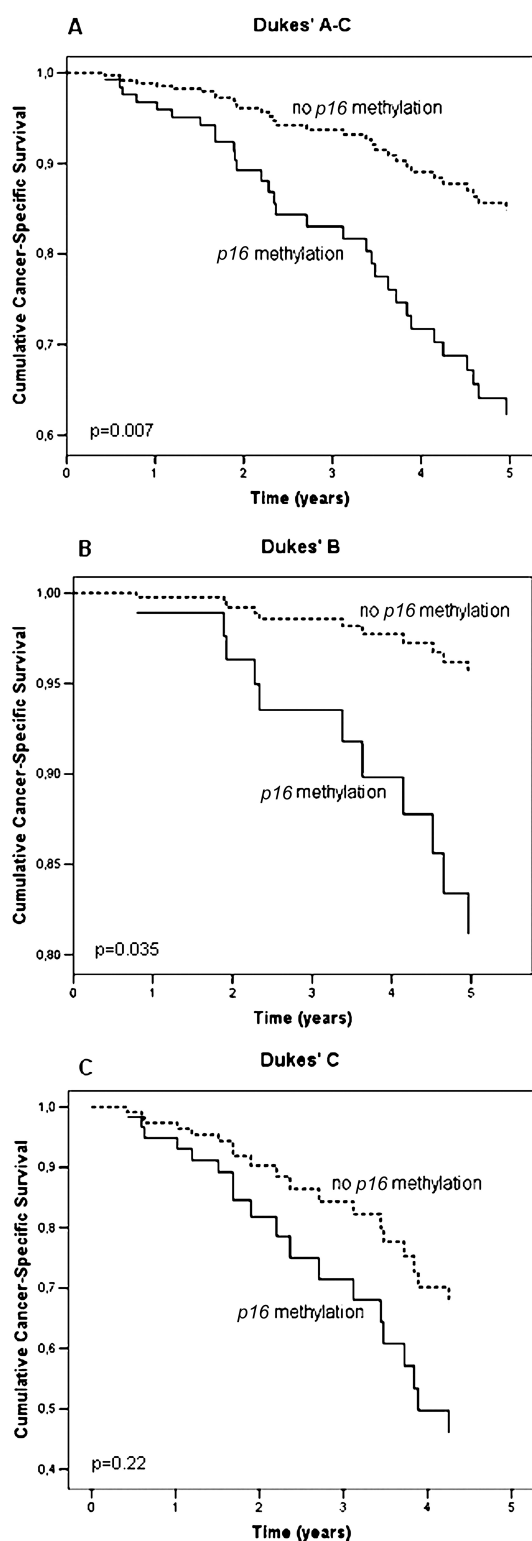
For a multivariate analysis, a Cox regression model was applied to examine whether p16 hypermethylation in mucosa was a risk factor for cancer-specific death independent of known risk factors such as tumor stage, tumor differentiation grade, age, gender, and tumor localization (right-sided colon, left-sided colon, or rectum). The model also was adjusted for whether or not the patients had been treated with radiotherapy prior to surgery and/or adjuvant chemotherapy post surgery (Table 2, Figure 2). As seen, the p16 hypermethylation status in mucosa was an independent prognostic factor for Dukes' A-C patients, the hazard ratio being 2.9 ( $P = 0.007$ ). When Dukes' B patients were analyzed separately, p16 hypermethylation (hazard

ratio = 4.7,  $P = 0.035$ ) and age (hazard ratio = 1.1,  $P = 0.037$ ) were the only co-variables found to be associated with a significantly increased risk of dying. Hypermethylation in mucosa of Dukes' C patients was not found to be a risk factor for cancer-specific death.

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.

**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



**Figure 2.** Multivariate Cox model for *p16* hypermethylation in mucosa and cancer-specific survival 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 and Dukes' B patients, but not for Dukes' C patients.

*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.

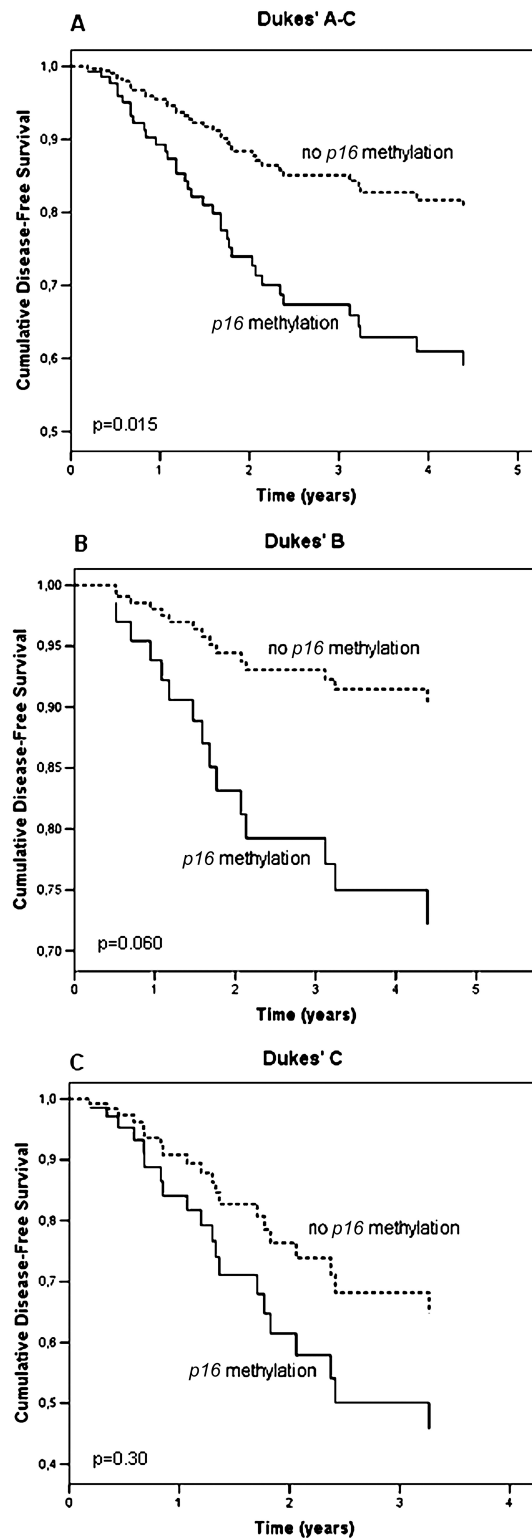
## DISCUSSION

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 (28,44), 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.

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

Gene	<i>n</i> <sup>a</sup>	All	<i>p16</i> hypermethylation			<i>P</i> <sup>b</sup>	
			<i>n</i>	Negative	Positive		
<i>RFC-1</i>	60	0.14 ± 0.17	36	0.15 ± 0.20	24	0.13 ± 0.12	0.89
<i>FPGS</i>	63	0.84 ± 0.61	38	0.92 ± 0.60	25	0.72 ± 0.61	0.079
<i>TS</i>	62	2.6 ± 2.6	37	2.2 ± 2.1	25	3.2 ± 3.2	0.29

<sup>a</sup>Enough material for gene expression analysis could only be obtained for the number of patients specified in the table.

<sup>b</sup>*P* by Kruskal-Wallis' test.

*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 in-

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

	<i>RFC-1</i> and <i>FPGS</i>			<i>FPGS</i> and <i>TS</i>		
	<i>n</i> <sup>a</sup>	<i>r</i> <sup>b</sup>	<i>P</i> <sup>c</sup>	<i>n</i> <sup>a</sup>	<i>r</i> <sup>b</sup>	<i>P</i> <sup>c</sup>
All	60	0.41	0.0013	62	0.57	< 0.0001
<i>p16</i> hypermethylation						
Negative	36	0.57	0.0003	37	0.57	0.0003
Positive	24	-0.0001	1.0	25	0.68	0.0002

<sup>a</sup>Enough material for gene expression analysis could only be obtained for the number of patients that is specified in the table.

<sup>b</sup>*r* = correlation coefficient.

<sup>c</sup>*P* by Pearson.



sion level. The authors speculated that the effect of partial methylation on gene expression in the physiological condition of advancing age may be different from that of promoter hypermethylation in cancer. The mechanistic basis of the apparent paradox of simultaneous hypo- and hypermethylation induced by folate deficiency has been described by James *et al.* (59).

Previous reports have shown that hypermethylation of the *p16* promoter in human tumor tissues leads to inactivation of *p16* expression (20,33). However, a low expression level may result if the *p16* promoter is partially methylated or if only one of the two alleles is being hypermethylated. This scenario might well be the true for some of the patients. Because the method used in the present study detects only a fully methylated amplicon, the importance of *p16* methylation as a prognostic marker in mucosa might be underestimated. At present, we are analyzing *p16* protein expression in the mucosa samples using Western blot technique, correlating the outcoming results 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 questioned. 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 induced by tumor cells *in vivo* (60). Treatment with demethylating agents alone or in combination with histone acetylase

inhibitors and DNA-targeting chemotherapeutic drugs might be useful when attempting to reverse and overcome the inactivation of *p16* and other tumor suppressor genes. The antihypertensive drug hydralazine seems to be well tolerated and effective to demethylate and reactivate the expression of tumor suppressor genes without affecting global DNA methylation (61). The 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 hypermethylation status of *p16* in mucosa seems to be especially useful as a prognostic factor for Dukes' B patients. The results further suggest that there is a connection between folate-associated gene expression and the *p16* methylation status. The potentially reversible nature of CpG island methylation may provide novel therapeutic opportunities to individuals with gene-specific promoter hypermethylation.

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