Research Article

Predicting the progress of colon cancer by DNA methylation markers of the *p16* gene in feces - Evidence from an animal model

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Abstract

A new noninvasive screening tool for colorectal neoplasia detects epigenetic alterations exhibited by gastrointestinal tumor cells shed into stool. There is insufficient existing data to determine temporal associations between colorectal cancer (CRC) progression and aberrant DNA methylation. To evaluate the feasibility of using fecal DNA methylation status to determine CRC progression, we collected stool samples from 14 male SD rats aged six weeks, and administered subcutaneous injections of either 1,2-dimethylhydrazine or saline weekly. *p16* DNA methylation statuses in tumorous and normal colon tissue, and from stool samples were determined using methylation-specific PCR. Additionally, *p16* methylation was detected in stool DNA from 85.7% of the CRC rats. The earliest change in *p16* methylation status in the DMH-treated group stool samples occurred during week nine; repeatabilities were 57.1% in week 19 (p = 0.070) and 85.7% in week 34 (p = 0.005). A temporal correlation was evidenced between progression of CRC and *p16* methylation status, as evidenced by DMH-induced rat feces. Using fecal DNA methylation status to determine colorectal tissue methylation status can reveal CRC progression. Our data suggests that *p16* promoter methylation is a feasible epigenetic marker for the detection and may be useful for CRC screening.

Introduction

Colorectal cancer (CRC) accounts for more than 600,000 deaths each year worldwide (Jemal *et al.*, 2011). In Taiwan in recent years, CRC has become the most common form of cancer occurrence (Promotion, 2010). Effective treatment is possible during the early stages of CRC, but the disease is generally asymptomatic. Unfortunately, the therapy is rarely successful in stage IV after the tumor cells have spread to lymph nodes and other organs (Wong *et al.*, 2004). Thus, an effective screening test and early detection would provide substantial clinical benefits. The present technology CRC screening tools for an average-risk population fall into two categories: (1) physical examination, including colonoscopy, virtual colonoscopy, sigmoidoscopy, double contrast barium enema (DCBE), and digital rectal exam (DRE) (Bretthauer, 2011), and (2) stool tests, including detection of occult blood and exfoliated mutant or epigenetic changes of DNA (Harrison and Benziger, 2011). Colonoscopy and sigmoidoscopy are highly specific and sensitive screening tools; however, these are less acceptable to the public because of invasive medical procedures. Fecal occult blood testing (FOBT), the most efficient noninvasive screening test for colorectal cancer, has a limited impact on survival rate because of its low sensitivity to early stage CRC (Baek *et al.*, 2009).

To provide early detection, it is important to develop a noninvasive and screening tool that is sensitive to CRC characteristics (Krishnan and Wolf, 2011; Labianca and Merelli, 2010). At the molecular level, CRC progression is accompanied by specific genetic and epigenetic changes (Schmid, 2010). Aberrant DNA hypermethylation of CpG islands within promoters is associated with the development and progression of colorectal cancer, which in turn

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leads to silencing of tumor suppressor genes such as *p16INK4a*, *MGMT* and *MLH1*, *SFRP2*, and *vimentin* genes (Chen *et al.*, 2005; Wang and Tang, 2008; Psofaki *et al.*, 2010; Shima *et al.*, 2010).

A novel and non-invasive screening tool for detection of colorectal neoplasia is to assay the methylation alterations present in gastrointestinal tumor cells shed into stool. (Lenhard *et al.*, 2005; Azuara *et al.*, 2010). However, existing data was insufficient to determine temporal correlation between the progression of CRC and aberrant DNA methylation. To address this knowledge gap, we assayed *p16* gene methylation changes in the feces of a rat model with chemically induced colorectal cancer. The assay could provide evidence that feces contain a detectable biomarker to diagnose early stage colorectal cancer. The aim of this study was to evaluate the feasibility of using fecal DNA methylation status to predict CRC progression.

Materials and Methods

Animals

All 14 male Sprague-Dawley rats used in the experiment were obtained from BioLASCO Taiwan Co. Ltd. (Taipei, Republic of China), six weeks of age and weighing 250-300 g. They were individually housed in ventilated stainless-steel cages with soft shavings air-conditioned room (temperature 22 ± 1 °C, humidity 50-60% with 12-hour light-dark cycles, lights on at 0700), and were provided free access to food and water throughout the experiment. All procedures were approved by the Institutional Animal Care and Use Committee of National Defense Medical Center (certificate number IACUC-09-047), and were performed in accordance with National Institute of Health guidelines for the treatment of animals.

Generation of DMH induced tumors

Beginning at seven weeks of age, the rats received subcutaneous (s.c.) injections of either 1,2-dimethyl-hydrazine (DMH) (Sigma Chemical Co) at a dosage of 20 mg/kg body weight (n = 7), or the same volume of saline (n = 7) weekly for 30 weeks. Rats were sacrificed at age 34 weeks through inhaled overdose of CO_2.

Collection of samples

Fecal pellets were collected weekly and placed in 2 mL microcentrifuge tubes for DNA extraction. Tissue samples were harvested after the rats were sacrificed. Samples were resected with scissors and inspected for tumors. Tumor, normal tissue close to a tumor, and normal tissue samples were dissected with a razor blade and placed in 2 mL microcentrifuge tubers for DNA extraction. Each tissue sample was sectioned for standard histological preparation and hematoxylin-eosin staining. Tissue and fecal samples were stored at -80 °C.

DNA extraction and sodium bisulfite treatment

Genomic DNA was extracted either from frozen tissue samples using a DNeasy Blood & Tissue kit (QIAGENE, Germany), or from fecal samples using a QIAamp DNA stool Mini kit (QIAGENE, Germany). The resulting DNA was modified with sodium bisulfite using an EZ DNA Methylation kit (Zymo Research, Orange, County, CA), and a methylated DNA positive control for MSP assays was generated using SssI methylase (Zymo Research, Orange County, CA).

Tissue and fecal DNA methylation studies

The methylation status of tissue samples was determined by methylation-specific PCR (MSP) of bisulfite treated DNA. MSP was carried out in a volume of 15 μL with 7.5 μL of HotStart Taq Premlx (RBC Bioscience), 0.6 μL of each primer, and 0.6 μL of bisulphite treated DNA. PCR conditions included denaturation at 95 °C for 10 min followed by 35 cycles at 95 °C for 30 s, annealing temperature at 55 °C for 35 s, 72 °C for 30 s, and a extension at 72 °C for 4 min.

As only limited quantities of DNA are obtained from fecal samples (Zou *et al.*, 2009) we adopted a nested strategy (Glockner *et al.*, 2009). During the first stage, we carried out amplification by bisulfite-sequencing PCR (BSP) for 35 cycles using primers located in the flanking region of the CDKN2A promoter. In the following stage, MSP amplification was carried out for 30 cycles using 1:100 dilutions of the first-stage products. Table 1 lists the MSP and BSP primer sets, and annealing temperatures.

Table 1 - MSP and BSP primer sets and annealing temperature.

Forward primer (5' → 3')		Annealing temperature (°C)	Product size (bp)
F*	GGGGAGGGTTTTTTAGATATTTT	55	225
R	TTAAACCACCCATACTCACCTAAAC		
U-F**	GTGAATTTGAGGAGAGTGATTTG	57	129
U-R	CAAAACATTTAATAAAACCCCAA		
M-F	AATTCGAGGAGAGCGATTCG	62	123
M-R	AACGTTTAATAAAACCCCGA		

*BSP primer: F, forward; R, reverse. **MSP primer: U, unmethylated; M, methylated.

Statistical analyses

All statistical analyses were performed using the SPSS 21.0 software package for Windows (SPSS Taiwan Corp.). We computed the sensitivity and specificity of the fecal DNA methylation assay by using the McNemar test to compare methylation statuses between fecal and tissue samples for rats treated with DMH (n = 7) and those injected with saline (n = 7); the Fisher's exact test was used to evaluate the differences of *p16* hypermethylation between the two groups in stool samples. A p value ≤ 0.05 was considered statistically significant.

Results

There were no significant differences in body weight between experimental and control rats at euthanasia, and both groups exhibited a healthy appearance. All of the DMH-treated, but none of the control group rates developed colorectal adenomatous cells (Figure 1).

We analyzed the methylation status of the *p16* gene in DNA from tissue samples. A methylated *p16* gene was detected in all intestinal adenoma tissue samples from the DMH-treated group. By contrast, there was no hypermethylation of the *p16* gene in the control group tissue samples (Figure 2). We then assessed the *p16* gene methylation status of tissue and stool samples from corresponding donor rats to ascertain the consistency of *p16* hypermethylation between the two sample types. After euthanasia in week 34, *p16* hypermethylation was found in the stool of 85.7% (6/7) of the DMH-treated rats but in none (0/7) of the control group. The McNemar test *p*-value was 1.000 (Table 2).

When retrospectively assaying stool samples to assess the temporal association between CRC progression and aberrant DNA methylation we detected the earliest instance of *p16* hypermethylation in week nine. Samples from the DMH-treated group showed a *p16* hypermethylation ratio of 71.4% (5/7) against 0% (0/7) from the control group (McNemar test p = 0.500, Table 2); the repeatability of using fecal DNA methylation status for identifying CRC in our rat samples was 57.1% (4/7) during week 19 and 85.7% (6/7) in week 34 (McNemar test p = 0.250 and 1.000, respectively) (Table 2).

From the two groups of this study (DMH-treat and saline control), the methylation status in stool samples of pe-
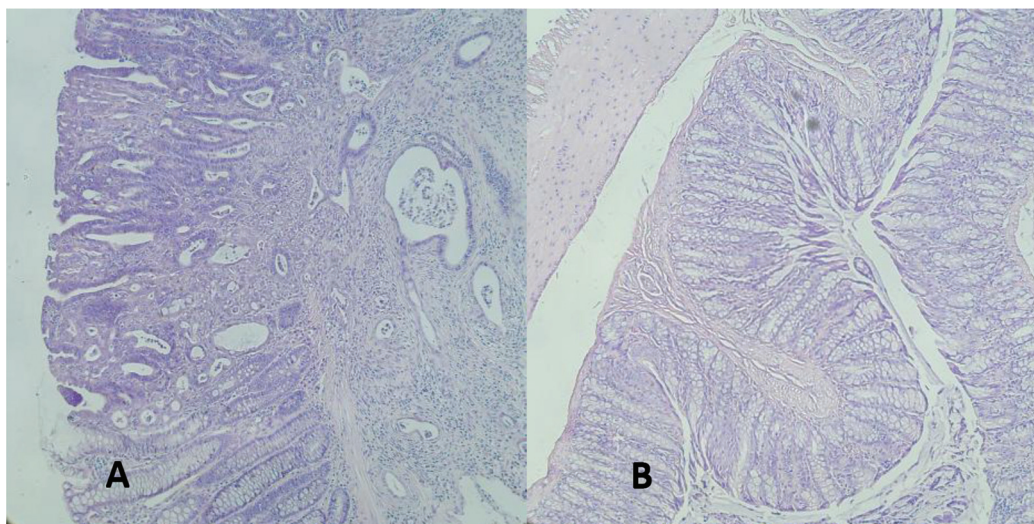

Figure 1 - Histological analysis of (A) DMH-induced rat colonic adenoma and (B) normal tissue. Magnification: x 20; Hematoxylin-eosin staining.

Table 2 - Consistency of *p16* hypermethylation between tissue and stool samples.

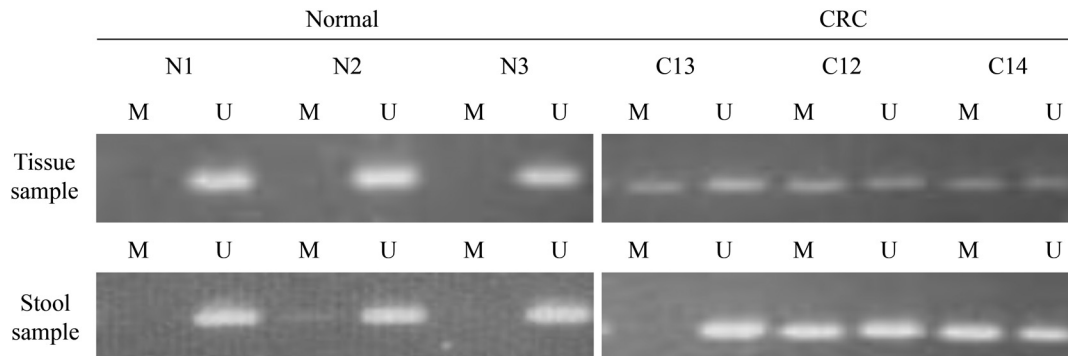|			Tissue samples		
			Methylated	Unmethylated	p-value[*]
Stool samples	Week 9	Methylated	5	0	0.500
		Unmethylated	2	7	
	Week 19	Methylated	4	0	0.250
		Unmethylated	3	7	
	Week 34	Methylated	6	0	1.000
		Unmethylated	1	7	

*McNemar test.

Figure 2 - Methylation status of the *p16* gene in fecal specimens and in CRC tissue taken from the same donor rat. N: normal tissue; C: CRC tissue; M: Methylated; U: Unmethylated.

riods of time was significantly different in week nine and 34 (Fisher's exact test *p*-value were 0.021 and 0.005, respectively) and it was borderline significant in week 19 (p = 0.070) (Table 3). The repeatability of using fecal DNA methylation status was used as an indicator for CRC progression (Figure 3). Samples were taken at the indicated rat age.

Discussion

The development and progression of colorectal cancer follows the "adenoma-carcinoma" sequence, in which colorectal tumor cells develop via a worsening dysplasia of normal colonic mucosa (Fearon and Vogelstein, 1990). An uncomplicated surgical procedure can provide an effective

Table 3 - Differences in *p16* hypermethylation between DMH-treated and saline-control groups in stool samples.

Week		DMH-treated	Saline-control	p-value*
9	Methylated	5(71.4)	0(0)	0.021
	Unmethylated	2(28.6)	7(100)	
19	Methylated	4(57.1)	0(0)	0.070
	Unmethylated	3(42.9)	7(100)	
34	Methylated	6(85.7)	0(0)	0.005
	Unmethylated	1(14.3)	7(100)	

*Fisher's exact test.
The sensitivities in weeks 9, 19 and 34 were 71.4% (5/7), 57.1% (4/7) and 85.7% (6/7), respectively, in the DMH-treated group stool samples; the specificity of each week was 100% (7/7) in the normal saline group.
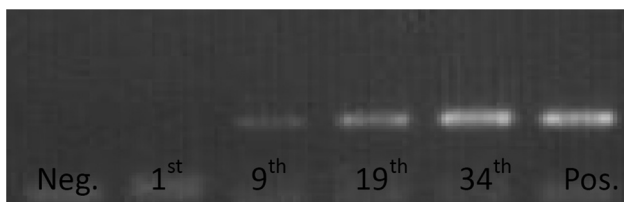

Figure 3 - The repeatability of using fecal DNA methylation status to identify CRC progression. Samples were taken at the indicated rat age.

treatment if diagnosis is made during the early stages before metastasis occurs (Kim *et al.*, 2010). Thus, it is desirable to find biomarkers that have high sensitivity and specificity towards CRC.

There is abundant evidence supporting the efficacy of FOBT in decreasing colorectal cancer mortality. However, the impact of FOBT on CRC incidence is lessened by the method's low sensitivity (Heitman *et al.*, 2010; Levi *et al.*, 2007). Mandel *et al.* (2000) reported that FOBT decreases CRC incidence by 17-20%. Conversely, Heresbach *et al.* (2006) provided evidence that FOBT has an insignificant effect on CRC incidence. The incidence of CRC and its mortality rates are expected to increase due to our aging population, particularly if there are no new screening tools developed to replace FOBT.(Mariotto *et al.*, 2006)

In a normal intestinal tract, the epithelium is constantly and rapidly renewed by the turnover of 5×10^{10} epithelial cells per day (Mehl, 1991). The shedding rate of carcinoma colonocytes is faster than that of normal cells, and we can use this characteristic to identify tumor cells and assay the status of genetic or epigenetic abnormalities. Promoter hypermethylation analysis of stool DNA is a promising noninvasive test for early diagnosis of CRC, and this area has received much research interest. An increasing number of genes is found to undergo promoter region hypermethylation in the tissue and stool of CRC patients, and Glockner *et al.* (2009) found a higher sensitivity to *TFPI2* methylation in patients with CRC (73-89%), in patients with adenomas (21-43%), and a high specificity toward TFPI2 methylation by patients with either CRC or adenomas (93-100%). Other reports observed high sensitivity (> 68%) and specificity (> 84%) towards *SFRP2* and *GATA4* methylation (Oberwalder *et al.*, 2008; Wang and Tang, 2008; Hellebrekers *et al.*, 2009)

There is substantial evidence supporting the value of using methylation analysis of stool DNA as a screening tool, although we cannot exclude the possibility of bias due to the nature of these cross-sectional studies (Thomas *et al.*, 2005); the literature contains no reports of a sequential association between CRC and changes in gene methylation

status in stool. Our findings answer questions about the consistency and temporal association between the progression of CRC and aberrant DNA methylation.

There are currently two animal models commonly employed to investigate the rules of DNA methylation in progression of CRC: (1) using genetic manipulation of animals to overexpress or down-regulate specific genes that are direct regulators of DNA methylation and methylation-related gene expression; and (2) carcinogen-treated animal models with assessment of somatic epigenetic alterations that arise in the resulting tumors (Conerly and Grady, 2010). Our study design adopted the latter category; we used DMH to change the genome methylation status and induce CRC. DMH-induced colon apoptosis in the rat model exhibits a characteristic aberrant crypt foci-adenoma-carcinoma sequence and associated increase in cellular proliferation of colonic epithelial cells similar to that observed in humans (Robertis *et al.*, 2011).

Borinstein *et al.* (2010) used an AOM-induced mice model to investigate the relationship between methylation of candidate genes, CRC, and normal tissues, and these authors reported 25% *p16* methylation in CRC tissue, whereas we found 100% methylation to occur. We attribute the difference between Borinstein's and our own findings to the use of different carcinogens and rat species. In human studies, the extent of *p16* hypermethylation could be as high as 70% (Psofaki *et al.*, 2010), which is in accordance with our results, indicating that *p16* methylation is a high frequency CRC marker.

Although this study reveals a temporal correlation between progression of colorectal cancer and *p16* methylation status in rat feces, the observed high frequency of *p16* methylation in tumor samples may be due to the DMH carcinogenic mechanism (Robertis *et al.*, 2011). Thus, our findings may not represent the true temporal causal relation between the natural history of colorectal cancer and *p16* methylation status in human feces.

Our study demonstrates that *p16* hypermethylation in stool occurs before CRC develops and that changes in the methylation status of the *p16* gene in stool are statistically significant or borderline significant. The consistency of hypermethylated *p16* between stool sample and tumor tissue demonstrates that detecting the hypermethylated *p16* gene in stool to identify colorectal neoplasia provides a sensitive early detection, consistent and noninvasive screening tool.

Acknowledgments

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