Modulation of Genetic and Epigenetic Biomarkers of Colorectal Cancer in Humans by Black Raspberries: A Phase I Pilot Study

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Abstract

Purpose: This study evaluated the effects of black raspberries (BRBs) on biomarkers of tumor development in the human colon and rectum including methylation of relevant tumor suppressor genes, cell proliferation, apoptosis, angiogenesis, and expression of Wnt pathway genes.

Experimental Design: Biopsies of adjacent normal tissues and colorectal adenocarcinomas were taken from 20 patients before and after oral consumption of BRB powder (60 g/d) for 1–9 weeks. Methylation status of promoter regions of five tumor suppressor genes was quantified. Protein expression of DNA methyltransferase 1 (DNMT1) and genes associated with cell proliferation, apoptosis, angiogenesis, and Wnt signaling were measured.

Results: The methylation of three Wnt inhibitors, SFRP2, SFRP5, and WIFI, upstream genes in Wnt pathway, and PAX6a, a developmental regulator, was modulated in a protective direction by BRBs in normal tissues and in colorectal tumors only in patients who received BRB treatment for an average of 4 weeks, but not in all 20 patients with 1–9 weeks of BRB treatment. This was associated with decreased expression of DNMT1. BRBs modulated expression of genes associated with Wnt pathway, proliferation, apoptosis, and angiogenesis in a protective direction.

Conclusions: These data provide evidence of the ability of BRBs to demethylate tumor suppressor genes and to modulate other biomarkers of tumor development in the human colon and rectum. While demethylation of genes did not occur in colorectal tissues from all treated patients, the positive results with the secondary endpoints suggest that additional studies of BRBs for the prevention of colorectal cancer in humans now appear warranted. Clin Cancer Res; 17(3); 598–610. ©2010 AACR.

Introduction

Colorectal cancer is the third most common cancer in both men and women in the United States (1). An estimated 49,920 deaths from colorectal cancer were expected to occur in 2009, accounting for 9% of all cancer deaths. Mortality rates from colorectal cancer have declined in both men and women over the past two decades, a reflection of declining rates in incidence and an increase in early screening for the disease. The 5-year survival from this disease is 64% and continues to decline to 57% at 10 years after diagnosis. For persons with distant metastases at diagnosis, the 5-year survival is only 10%. Therefore, the prevention of colorectal cancer remains an important goal, and chemoprevention is one approach to achieve this goal.

Our laboratory has shown that the administration of BRB powder at 2.5%, 5%, and 10% of a synthetic diet to azoxymethane (AOM)-treated Fischer 344 (F-344) rats resulted in an average 42%, 45%, and 71% (P < 0.05 for all groups) decline, respectively, in tumor number relative to the AOM-only group in a 36-week assay (2). We also found significant reductions in urinary levels of the oxidative DNA adduct, 8-hydroxy-2'-deoxyguanosine (8-OHdG) in these same rats. It is also well known that heat-processed black raspberries contain substances that reduce inflammation, reduce proliferation, and angiogenesis, and reduce oxidative stress. The mechanism(s) by which BRBs reduced AOM-induced tumors in rat colon were not investigated. However, in studies using a rat model of nitrosamine-induced esophageal squamous cell carcinoma (SCC), BRBs were found to reduce proliferation, inflammation, and angiogenesis, and
Translational Relevance

We are developing strategies for the chemoprevention of colorectal cancer using freeze-dried berries. The present trial was conducted to determine, in a small cohort of colorectal cancer patients, if short-term treatment with freeze-dried black raspberries (BRBs) would result in modulating biomarkers of tumor development. Twenty colorectal cancer patients were administered 60 g/d of BRBs orally for 1–9 weeks. Biopsies of adjacent normal and colorectal tumor tissues were taken before and after the berry treatment. BRBs were found to protectively modulate biomarkers of cell proliferation, apoptosis, angiogenesis, and Wnt pathway in tumor and adjacent normal specimens. BRBs caused demethylation of the promoter regions of relevant tumor suppressor genes in adjacent normal tissues and colorectal tumors, and this effect was dependent upon total dose. These data, along with our preclinical data demonstrating the ability of BRBs to prevent tumor development in the rat colon and mouse small intestine, provide more rationale for the further evaluation of BRBs for the prevention of colorectal cancer in humans.

stimulate apoptosis and differentiation of premalignant cells and tissues, resulting in reduced tumor development. Genes associated with these cellular functions were also protectively modulated by BRB diets (3).

DNA methylation in mammalian cells is regulated by a family of highly related DNA methyltransferase enzymes (DNMT1, DNMT3a, and DNMT3b) that mediate the transfer of methyl groups from S-adenosylmethionine to the 5 position of cytosine bases in the dinucleotide sequence CpG. DNMT1 functions as the maintenance DNA methyltransferase in mammalian cells and is responsible for accurately replicating genomic DNA methylation patterns during the S phase of the cell cycle (4). In contrast, de novo methylation of DNA is believed to be performed by DNMT3a and DNMT3b enzymes that possess both maintenance and de novo DNA methylation activities (5). Both groups of enzymes, however, have been shown to exhibit some level of both maintenance and de novo methylation in vitro, suggesting that this classification of the DNMTs may be oversimplified (6). Confirming the importance of DNA methylation in tumorigenesis, studies have shown all 3 DNMTs to be overly expressed in several tumor types, including tumors of the colon and rectum, bladder, and kidney (7). When DNMT1 and DNMT3b are knocked out in colon cancer cell lines, methylation of tumor suppressor genes such as p16 is almost entirely eliminated and the gene is re-expressed (8). Inhibition of DNMTs, therefore, may lead to demethylation and reactivation of the silenced genes. Aberrant methylation of tumor suppressor genes by DNMTs may be a promising target for chemoprevention. DNMT inhibitors are currently being developed as potential therapeutic agents for cancer (9, 10).

Aberrant Wnt/β-catenin (Wnt) pathway signaling occurs in about 85% of sporadic colorectal cancers and is due principally to mutations in the Apc gene (11). Recent findings have demonstrated an important role of the Wnt pathway in cell proliferation, differentiation, and stem cell maintenance. Disruption of Wnt signaling due to dysregulated gene methylation is also a common event in human colorectal cancer, for example, frequent methylation of the negative Wnt regulators, secreted frizzled-related proteins 2 and 5 (SFRP2 and SFRP5), and Wnt inhibitory factor 1 (WIF1) (11). Methylation of SFRP-gene promoter regions has been detected in aberrant crypt foci (ACF) and persists in primary colon cancer cells (12). Re-expression of SFRPs in colon cancer cells blocks Wnt signaling and results in apoptosis (12). Importantly, these events occur in colon cancer cells that exhibit β-catenin mutations, a gene in the downstream Wnt pathway (12). Silencing of p16, also known as CDKN2A, a cell cycle regulator, and PAX6a, a development regulator, in colorectal cancer is reported to be associated with promoter hypermethylation (13, 14). Unlike mutational changes in DNA bases, the potential reversibility of DNA methylation patterns of tumor suppressor genes suggests that these are a viable target for the prevention and/or treatment of colorectal cancer.

The goal of this study was to investigate the effects of short-term treatment with dietary BRBs on promoter methylation of the Wnt inhibitors, SFRP2, SFRP5, and WIF1, as well as p16 and PAX6a, and on modulating DNMT family enzymes in human colorectal cancer tissues. The effects of the berries on protein expression of downstream genes in the Wnt pathway; that is, β-catenin, E-cadherin, and c-Myc, and on biomarkers of cell proliferation (Ki-67), apoptosis (TUNEL), and angiogenesis (CD-105) were also evaluated. Our results indicate that BRBs, administered orally on a daily basis for an average of approximately 4 weeks, led to the protective modulation of multiple epigenetic and cellular biomarkers in human colorectal tissues. These effects likely occurred through local absorption of berry bioactives into colorectal tissues.

Materials and Methods

Clinical trial

The present trial to evaluate the effects of BRBs on biomarkers of colorectal cancer development was approved by the Institutional Review Boards of the Ohio State University Comprehensive Cancer Center and the University of Texas, San Antonio. Written informed consent was obtained from all patients before participation in the trial. Most of the patients accrued to this trial were individuals who were diagnosed with colorectal cancer elsewhere and sent to Ohio State University James Cancer Hospital for surgery. Therefore, in order to be eligible for this trial, they had to agree to undergo a second colonoscopy or sigmoidoscopy (depending upon tumor location) in order to obtain baseline colorectal tumor and adjacent normal tissues for the study.

A. Inclusion criteria. Patients accrued to the trial had one of the following: (i) Patients with a pathological diagnosis of early-stage colorectal cancer...
(primary or recurrent), who had a primary lesion in the colon or rectum, and were considered as candidates for elective colorectal surgery. (ii) Patients who had a diagnosis of stage IV colorectal cancer with metastatic lesions in the liver or abdomen (carcinomatosis) were also eligible, providing that the primary lesion was present in the colon or rectum, and a surgery to resect the colorectal lesion was planned. (iii) Patients with a colon polyp, or pathological diagnosis of adenoma, not removable by initial colonoscopy or sigmoidoscopy, due to size or other reasons (such as receiving anti-carcinoma treatment), who required a repeat colonoscopy or sigmoidoscopy or surgery for removal. (iv) Patients who were considered to have a high likelihood for colorectal malignancy by the discretion of the surgeon, with histories such as anemia, rectal bleeding, weight loss, bowel movement habit changes, X-ray evidence of colorectal mass, etc. were considered eligible. (v) Patients with rectal cancers were eligible prior to proceeding with chemoradiation or surgery if they did not have obstructive lesions or obstructive symptoms.

B. Exclusion criteria. Patients excluded from the trial had one of the following: (i) Clinical symptoms of obstruction or bleeding and considered for immediate surgery. (ii) Patients with rectal cancer or obstructive lesions who were considered candidates for immediate neoadjuvant chemoradiation treatment or surgery. (iii) Patients currently receiving chemotherapy or radiation therapy (must be > 4 weeks since last chemotherapy treatment and > 3 weeks after radiation treatment). (iv) Patients who took nonsteroidal anti-inflammatory drugs (NSAIDs) and could not be taken off the medication due to their clinical condition. (v) Pregnant or lactating patients. (vi) Patients with uncontrolled, uncompensated cardiac, pulmonary or hepatic diseases, or uncontrolled infectious diseases or diabetes mellitus.

Black raspberries

Fresh frozen BRBs (Jewel variety, 2004 harvest) were purchased from an Ohio farm and shipped frozen to Van Drunen Farms in Momence, Illinois for freeze-drying as described (15). Nutrient analysis and packing of BRBs are detailed in Supplementary Methods.

Administration of black raspberries

One dose, 20 g, of freeze-dried berry powder was mixed with approximately 100 mL of water and consumed orally 3 times a day (60 g/d total) for 1–9 weeks. Berry powder was consumed regularly at 3 times throughout the day, 6 hours apart. Sixty grams of berry powder approximates 1.3 lbs of fresh BRBs per day and is equivalent to a rodent diet of ~7% berry powder (16). BRB powder was found to be chemopreventive in the rat colon when provided in the diet at concentrations of 2.5% to 10% (3).

Evaluations before and during berry treatment

Initially, all patients were given a physical examination during which their medical/surgical history was obtained, and laboratory tests for lactic dehydrogenase (LDH), carcinoembryonic antigen (CEA), complete blood count (CBC), and a complete metabolic panel were completed. They then participated in a 24-hour verbal food recall to establish consumption patterns of phenol-rich foods, including berry products, before treatment with berry powder. During berry treatment, patients were evaluated once per week for compliance and any adverse event(s). Patients were asked to avoid consumption of berry types other than black raspberry powder during the trial.

Tissue and urine collection

All patients accrued to the trial had a diagnosis of colorectal cancer before entry into the trial. Because tissue taken for initial diagnosis was not available for study, it was necessary to obtain additional tissue specimens before treatment of the patients with berries. Initially, patients signed a consent form, after which they discontinued use of NSAIDs. After approximately 5 days, 3 biopsies each of adjacent normal (<2 cm from tumors) and tumor tissues were taken from patients with either rectal or colon cancer. One-half of the each biopsy was placed in 10% buffered formalin and the other half was frozen in liquid nitrogen. Patients began taking BRB powder approximately 24 hours after the tissue biopsies were taken and daily until ~12–36 hours before surgery to remove the tumor. At surgery, an additional 3 biopsies each of adjacent normal and tumor tissues were taken from each patient and placed in buffered formalin or frozen as described for the pretreatment biopsies. All tissue specimens were classified histopathologically as either normal or tumor by Dr. W. Frankel, a medical pathologist. All tumors were adenocarcinomas.

Pre- and posttreatment urine specimens (~50 mL each) were collected at baseline and after berry treatment. To ensure the stability of berry anthocyanins, the specimens were stored at −80°C after adding 5% trifluoroacetic acid to reduce the pH to acidity.

Measurement of berry anthocyanins in colorectal tissues and urine

Sample preparation, and HPLC–MS/MS analysis and quantification of anthocyanins (17) are detailed in Supplementary Methods.

Analysis of DNA methylation

A. DNA extraction and bisulfite conversion. Adjacent normal and tumor tissues were used for extraction of genomic DNA as described in Supplementary Methods.

B. MassARRAY. High-throughput MassARRAY was used to quantify methylation levels of the CpG islands of p16, PAX6a, SFRP2, SFRP5, and WIF1 as described (18) and detailed in Supplementary Methods.
C. **Pyrosequencing.** The LINE-1 repetitive element bisulfite/pyrosequencing assay was used to estimate global methylation (19) as detailed in Supplementary Methods.

**Immunohistochemical staining and computer-assisted image analysis**

Immunohistochemical staining procedures and quantification of staining of β-catenin, E-cadherin, c-Myc, Ki-67, TUNEL, p16, CD105, or DNMT1 are detailed in Supplementary Methods.

**Statistics**

Methylation and immunohistochemical staining data were quantified by determining pre and post berry treatment, and the percent change of each variable from baseline. Differences in mean of pre and post, percent change, and anthocyanin levels were compared by Student’s t-test. All analyses were two-sided, and a P value of less than 0.05 was considered to be significant. Linear regression was used to determine the correlation between the percent change of methylation and the percent change of DNMT1 expression, berry dose, and age.

**Results**

**Patient characteristics**

Twenty patients were included into the trial and their characteristics are summarized in Table 1. Seventeen patients were accrued at the Ohio State University and 3 were accrued at the University of Texas, San Antonio. Seventeen of the 20 patients were male. The average age of the study population was 59 years. Six patients had colon cancer (30%) and the other 14 (70%) had rectal cancer. Two patients had metastatic disease.

**Berry treatment and compliance**

Patients were treated with BRB powder orally for 1–9 weeks (Table 1). Patient compliance to berry treatment was excellent with each patient consuming >90% of the stipulated daily doses, based upon self-reporting and return of empty bags. The berry powder was generally well tolerated with 7 patients reporting mild disturbances of the gastrointestinal tract; that is, diarrhea and constipation, that resolved within 2–3 days. Patient LDH, CBC, CEA, and metabolic profiles were not altered by BRB treatment during the study.

**Analysis of anthocyanins in urine and colorectal tissue**

Anthocyanins were not present in the urine of any patient before berry treatment. All 4 anthocyanins were detected in the urine of all 20 patients after berry treatment (Supplementary Table S1). The amounts of the 4 anthocyanins in the urine of all patients ranged from 1.7 to 2011.5 fmol/mg tissue (Supplementary Table S1). The levels of total anthocyanins in adjacent normal tissues versus tumor tissues were 299.9 ± 754.9 and 55.4 ± 60.8 fmol/mg tissue (mean ± S.D.), respectively. Anthocyanin levels in adjacent normal tissues were not significantly different from those in tumor tissues (P = 0.21).

**Effect of BRBs on promoter demethylation of tumor suppressor genes and DNMT1 protein expression**

Preliminary analysis of combined methylation data from adjacent normal and colorectal tumor tissues from all 20 patients indicated that BRB treatment for different

<table>
<thead>
<tr>
<th>Table 1. Characteristics of colorectal cancer patients in this study</th>
<th>No. of patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>3 (15)</td>
</tr>
<tr>
<td>Male</td>
<td>17 (85)</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
</tr>
<tr>
<td>&lt;45</td>
<td>2 (10)</td>
</tr>
<tr>
<td>45–60</td>
<td>8 (40)</td>
</tr>
<tr>
<td>&gt;60</td>
<td>10 (50)</td>
</tr>
<tr>
<td><strong>Range 32–82</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Tumor location</strong></td>
<td></td>
</tr>
<tr>
<td>Transverse, descending, and sigmoid colon</td>
<td>3 (15)</td>
</tr>
<tr>
<td>Cecum and ascending colon</td>
<td>3 (15)</td>
</tr>
<tr>
<td>Rectum</td>
<td>14 (70)</td>
</tr>
<tr>
<td><strong>Metastatic disease</strong></td>
<td></td>
</tr>
<tr>
<td>Lymph node involved</td>
<td>2 (10)</td>
</tr>
<tr>
<td>No evidence</td>
<td>18 (90)</td>
</tr>
<tr>
<td><strong>Berry treatment (wks)</strong></td>
<td></td>
</tr>
<tr>
<td>&lt;2</td>
<td>7 (35)</td>
</tr>
<tr>
<td>2–4</td>
<td>4 (20)</td>
</tr>
<tr>
<td>&gt;4</td>
<td>9 (45)</td>
</tr>
<tr>
<td><strong>Range 1–9</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Berry doses</strong> (20 g/dose, 3 doses/d)</td>
<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td>8 (40)</td>
</tr>
<tr>
<td>50–85</td>
<td>8 (40)</td>
</tr>
<tr>
<td>&gt;85</td>
<td>4 (20)</td>
</tr>
<tr>
<td><strong>Range 10–189</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Compliance (%)</strong></td>
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<tr>
<td>80–90</td>
<td>1 (5)</td>
</tr>
<tr>
<td>90–100</td>
<td>8 (40)</td>
</tr>
<tr>
<td>100</td>
<td>11 (55)</td>
</tr>
<tr>
<td><strong>Toxicities reported</strong></td>
<td></td>
</tr>
<tr>
<td>Diarrhea</td>
<td>3 (15)</td>
</tr>
<tr>
<td>Constipation</td>
<td>4 (20)</td>
</tr>
<tr>
<td>None</td>
<td>13 (65)</td>
</tr>
<tr>
<td><strong>Improved bowel movements</strong></td>
<td>15 (75)</td>
</tr>
</tbody>
</table>
periods of time (1–9 weeks; average = 3 weeks) failed to produce significant effects on promoter methylation of SFRP2, PAX6a, p16, SFRP5, and WIF1, and on LINE-1 repetitive element in colorectal tumor (adenocarcinoma) tissues (Fig. 1). In fact, the only significant effects observed were decreased SFRP2 and SFRP5 promoter methylation in adjacent normal tissues. It is apparent from Figure 1, that the effects of berry treatment on promoter methylation of genes in both adjacent normal and tumor tissues were quite variable from one patient to another.

Significant differences in promoter methylation of these genes were observed, however, when the patients were divided into two groups based upon: (a) the total number of berry doses taken and, (b) the extent of changes in DNMT1 protein expression. The data from analyses of adjacent normal tissues are shown in Figure 2. N/H refers to adjacent normal tissues taken from patients who had received an average of 83 berry doses (~4 weeks of treatment). N/L refers to adjacent normal tissues taken from patients who had received an average of 52 berry doses (~2 weeks of treatment). Figure 2 shows significant decreases in percent methylation change from baseline of the SFRP2 and PAX6a tumor suppressor genes, as well as DNMT1 protein expression, in N/H tissues compared to N/L tissues. The box labeled "All" indicates that the reduction in methylation of all 5 tumor suppressor genes (SFRP2, PAX6a, p16, SFRP5, and WIF1) combined was significant in the N/H group versus the N/L group.

Figure 3 illustrates data from analyses of colorectal adenocarcinomas from patients who had received different doses of berries. T/H refers to adenocarcinomas from patients who had received an average of 85 berry doses (~4 weeks of treatment). T/L refers to adenocarcinomas taken from patients who had received an average of 53 berry doses (~2 weeks of treatment). Significant decreases in percent methylation change from baseline of the SFRP2, PAX6a, and WIF1 genes, as well as DNMT1...
protein expression, were observed in T/H group tumors versus T/L group tumors. When promoter methylation data from all 5 suppressor genes were combined, the T/H tumor data were significantly different from the T/L tumor data. These results suggest that the degree of change in promoter methylation of tumor suppressor genes and in DNMT1 protein expression was influenced by the total dose of BRB powder consumed.

In the heat-maps in Figures 2B and 3B, green and red colors depict decreased and increased methylation, respectively. When comparing these figures, adjacent normal colorectal tissues generally responded more favorably to berry-induced promoter demethylation than colorectal adenocarcinomas as evidenced by the relative amount of green color. Further, there was a positive correlation between changes in DNMT1 protein expression and...
A

Berry doses

\[ P = 0.0476 \]

\[ \text{T/H} \]
\[ 85 \]
\[ \text{T/L} \]
\[ 53 \]

% change from baseline

\[ \text{T/H} \]
\[ -21 \]
\[ \text{T/L} \]
\[ +36 \]

\[ \text{SFRP2} \]
\[ P = 0.001 \]

\[ \text{T/H} \]
\[ +22 \]
\[ \text{T/L} \]
\[ -8 \]

DNMT1

\[ P = 0.025 \]

\[ \text{T/H} \]
\[ -2 \]
\[ \text{T/L} \]
\[ +39 \]

\[ P = 0.028 \]

\[ \text{T/H} \]
\[ -19 \]
\[ \text{T/L} \]
\[ +26 \]

\[ P = 0.064 \]

\[ \text{T/H} \]
\[ -10 \]
\[ \text{T/L} \]
\[ +7 \]

\[ P < 0.0001 \]

Pax6a

\[ P = 0.189 \]

\[ \text{T/H} \]
\[ +6 \]
\[ \text{T/L} \]
\[ +28 \]

\[ P = 0.032 \]

\[ \text{T/H} \]
\[ -14 \]
\[ \text{T/L} \]
\[ +29 \]

\[ All \]
\[ P < 0.0001 \]

\[ \text{T/H} \]
\[ -19 \]
\[ \text{T/L} \]
\[ +26 \]

B

Promoter methylation

<table>
<thead>
<tr>
<th>SFRP2</th>
<th>PAX6a</th>
<th>p16</th>
<th>SFRP5</th>
<th>WIF1</th>
<th>DNMT1</th>
</tr>
</thead>
<tbody>
<tr>
<td>T/H</td>
<td>pts</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T/L</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

Protein

% change from baseline

Adenocarcinoma

\[ -10 \]
\[ +7 \]

\[ +36 \]
\[ +28 \]

\[ 0.0 \]
\[ -50.0 \]
promoter methylation (Fig. 4A, $P = 0.006, R^2 = 0.181$). In general, treatment with BRBs for ~4 weeks (high dose) but not ~2 weeks (low dose) led to decreases in promoter methylation in both adjacent normal and adenocarcinoma tissues as indicated in Figure 4B ($P = 0.035, R^2 = 0.118$). Berry treatment did not cause significant changes from baseline in global methylation in either adjacent normal tissues or colorectal tumors from high and low berry dose groups as measured by the LINE-1 repetitive element bisulfite/pyrosequencing assay (Figure 4C). There were no significant differences in the age of patients treated with either high- or low-dose berries (Supplementary Fig. S1A) and berry-induced promoter methylation changes were not age dependent (Supplementary Fig. S1B).

**Differential responses of colon and rectal tissues to BRB treatment**

Because the exposure of colon tissues to BRB compounds administered orally might differ from that of rectal tissues, we determined if adjacent normal colon and colon tumors might respond differently to the demethylation effects of berry treatment than adjacent normal rectum and rectal tumors. Figure 4D depicts the percent change in methylation from baseline of all 5 tumor suppressor genes (SFRP2, PAX6a, p16, SFRP5, and WIF1) combined in adjacent normal colon and rectal tissues from the high-dose (N/H) and low-dose (N/L) treatment groups. When adjacent normal tissues from the two dose groups were compared, only the methylation changes in rectal tissues in the N/H versus N/L treatment groups were significantly different (Figure 4D, $P = 0.011$). Similar results were obtained with tumor specimens in that methylation changes in rectal tumors in the T/H group were significantly different from the T/L group (Figure 4E, $P < 0.0001$). Interestingly, T/L group tumors located in the colon responded more favorably to BRBs than those in the rectum (Fig. 4E).

**Effect of BRBs on Wnt signaling, cell proliferation, apoptosis, angiogenesis, and cell cycle regulation**

Quantitative immunohistochemistry was used to measure the expression of three proteins associated with the Wnt pathway (β-catenin, E-cadherin, c-Myc) as well as proteins associated with cell proliferation (Ki-67), apoptosis (TUNEL), angiogenesis (CD105), and cell cycle regulation (p16) in adjacent normal tissues and colorectal tumors. Representative staining of these proteins is shown in Figure 5A. Combined data from all 20 patients before and after 1–9 weeks of BRB treatment indicated that the berries protectively modulated β-catenin, Ki-67, TUNEL, CD105, and DNMT1 in colorectal tumor tissues, and CD105 and DNMT1 in adjacent normal tissues (Fig. 5B).

When BRBs were evaluated for their effects on these tissue biomarkers as a function of total dose, they were also found to be effective. In adjacent normal tissues from the high-dose (N/H; ~4 weeks treatment) berry group, berry treatment led to a decrease in β-catenin expression and an increase in E-cadherin expression when compared to the low-dose (N/L; ~2 weeks treatment) group (Fig. 6A, $P < 0.05$). This was accompanied by a decrease in Ki-67 protein expression and an increase in TUNEL staining in the N/H as compared to the N/L group ($P < 0.05$). CD105 was decreased and p16 was increased in both N/H and N/L groups but the differences between groups were not significant ($P > 0.05$).

In colorectal adenocarcinomas, BRB treatment led to significant decreases in percent protein expression change from baseline of β-catenin, Ki-67, and CD105, and increases in the expression of TUNEL and p16 in T/H tissues versus T/L tissues (Fig. 6B). Again, these data suggest that the responses to BRBs are related to the cumulative berry dose.

**Discussion**

The results from this study indicate that treatment of colorectal cancer patients with BRBs led to positive modulation of both genetic and epigenetic biomarkers in colorectal adenocarcinomas and also adjacent normal tissues. Immunohistochemical data from analysis of biomarkers such as Ki-67, TUNEL, β-catenin, CD105, and DNMT1 indicated that these markers were modulated protectively in tissues from all 20 patients after BRB treatment. In contrast, positive modulation of the DNA methylation markers occurred only in tissues from patients who were treated with BRBs for an average of ~4 weeks (high dose), suggesting that berry treatment had to occur for longer periods of time to be effective. The reason for these differences might be related to the fact that whole tissue specimens, containing multiple cell types, were used for the methylation studies whereas the immunohistochemical data were obtained largely from analysis of the epithelium. Therefore, if the effects of berries are more pronounced in the epithelium than in other tissues, such as stroma, then one might expect to detect berry effects more readily using the immunohistochemical markers. It is also possible that the inhibition of DNA methyltransferases such as DNMT1 might occur only after prolonged treatment with berries. These observations suggest that BRBs may well be effective in...
modulating promoter methylation of tumor suppressor genes in long-term phase II and III clinical trials where the berries would be administered for months to years. We should also point out that, although we made equal attempts to recruit men and women to our trial, we found that men were more likely to participate. Nevertheless, more effort will be made to reduce the gender imbalance in future trials.

Results from the present study suggest that BRBs may have potential for inhibition of DNA methylation in addition to their many other mechanisms of action (20). BRBs elicit minimal or no toxicity when administered.
orally to humans at high daily doses making them attractive for chemoprevention. Further, we recently showed that the oral administration of BRB powder (60 g/d—equivalent to about 1.3 lbs of fresh berries) in patients with familial adenomatous polyposis for 9 months produced only minor gastrointestinal disturbances in some patients that were resolved in 2–3 days (21).

Although global DNA hypomethylation is closely linked to chromatin restructuring and nuclear disorganization in cancer cells leading to chromosomal instability (22), the effects of global DNA hypomethylation in animals have been controversial. For example, in contrast to the overall inhibition of intestinal tumorigenesis in these animals, hypomethylation caused the development of multifocal liver tumors. These results clearly demonstrate the opposing effects of DNA hypomethylation on intestinal and liver carcinogenesis (23). In humans, a study examining global methylation in cancer cell lines, including breast, central nervous system, colon, leukemia, liver, lung, ovary, and prostate showed that 85% of tested cell lines (51/60) were globally hypomethylated (24). Interestingly, the same study demonstrated that global methylation in colorectal cancers is highly variable with increased, no change, or decreased global methylation when comparing colorectal carcinomas with their adjacent tissues; global hypomethylation is partially reversed in cancers with microsatellite instability that may reflect alternative progression pathways in cancer. Therefore, the concept of

Figure 5. Effects of BRB consumption on expression of proteins (β-catenin, E-cadherin, and c-Myc) downstream of the Wnt pathway as well as biomarkers of cell proliferation (Ki-67), apoptosis (TUNEL), and angiogenesis (CD105) in adjacent normal tissues and colorectal adenocarcinomas from 20 patients before and after treatment with BRBs for 1–9 weeks. A, representative staining of β-catenin, E-cadherin, c-Myc, Ki-67, TUNEL, p16, and CD105 and B, dot-line plots of quantitative staining of these proteins. *P < 0.05.
global DNA hypomethylation in cancers might be too simplified; alterations of global DNA methylation patterns in carcinogenesis are organ and tumor type specific. DNA methylation inhibitors cause hypomethylation in promoters and global repeat sequences, and yet they exert therapeutic activities.

Further, based on Issa (9), although demethylation of tumor suppressor genes may have a beneficial effect, decreased methylation of oncogenes could lead to their reactivation and a subsequent adverse effect. It has been shown however, that hypomethylation agents elicit therapeutic effects that may be due to the fact that tumors are more dependent upon gene silencing to maintain their phenotype than normal adult cells. Thus, the overall effect of decreasing methylation appears to be positive. In addition, methylation is age dependent, and age-related methylation appears to be gene and tissue specific. However, the methylation of some genes, for example, p16 and SFRP2, and genes associated with DNA repair, is not affected by aging; rather their methylation levels are increased with cancer progression. This is especially true of colorectal cancer (25).

In the present study, BRBs showed differential effects on promoter methylation of SFRP2, SFRP5, WIF1, p16, and PAX6a genes. For example, while BRBs reduced methylation of the SFRP2 and PAX6a genes in the N/H versus N/L groups (Fig. 2), they had no effect on methylation of the other 3 genes in these same groups. Thus, it would appear that BRBs exhibit some selectivity in their effects on methylation of different genes; however, the data are preliminary and the mechanism(s) for this effect are unknown.

The effects of BRBs on gene demethylation are likely through the localized absorption of berry active compounds into colorectal tissues. This is evidenced by the detection, albeit at low levels, of BRB anthocyanins in colorectal tissues following berry treatment. The metabolites of cyanidin-based anthocyanins include protocatechuic acid, the predominant degradation product in cultured cells, and both 2,4-dihydroxybenzoic acid and 2,4,6-trihydroxybenzoic acid (26). This same study reported that 50 μM of protocatechuic acid exerted antioxidant activity but not the other 2 metabolites. We are determining if these metabolites can be detected in blood from animals and humans. The large patient variation in

![Figure 6](image_url)
the amounts of anthocyanins (1.7–2011.5 fmol/mg) detected in colorectal tissues is likely due to the fact that the patients took their last berry dose at different times (12–36 hours) before surgery. Therefore, the biomarker data could not be correlated with anthocyanin levels. Pharmacokinetic studies indicate that BRB anthocyanins reach peak levels in the blood and urine at 1 and 4 hours, respectively, after oral administration of berry powder (27). Therefore, one might expect large differences in colorectal tissue levels in patients who consumed berries as long as 12–36 hours before surgery.

The "field effect" model of carcinogenesis suggests that cells adjacent to tumors harbor at least some of the genetic alterations present in the tumors themselves (28). Indeed, epigenetic alterations in adjacent normal tissues have been associated with cancer progression in the human colon and breast (29, 30). As indicated in Figures 1, 2, 5, and 6, however, BRBs caused demethylation of tumor suppressor genes and protectively modulated biomarkers of cell proliferation and apoptosis in adjacent "normal" colorectal tissues that could well have restored these tissues to a higher degree of "normalcy."

In conclusion, our results suggest that BRBs protectively modulate both genetic and epigenetic biomarkers in tissues from colorectal cancer patients. The berries appeared to demethylate tumor suppressor genes (SFRP2 and WIF1) upstream, and protectively modulate the expression of genes (β-catenin, E-cadherin) downstream, of the Wnt pathway. Gene demethylation was likely a result of inhibition of DNMT1, although other methyltransferase enzymes might also have been affected. Colon and rectal tissues showed differential responses to berry treatment; in particular, rectal adenocarcinomas from patients who were treated with BRBs for a short period of time (~2 weeks) had reduced responses. This suggests that longer term treatment of colorectal cancer patients with berries may be beneficial. It also suggests that BRBs might be more effective in patients with rectal tumors if a method were developed for localized delivery of the berries to rectal tissues.

While the data are supportive of the ability of BRBs to demethylate tumor suppressor genes, the fact that the primary endpoint; that is, gene demethylation in tissues from all 20 treated patients was, by large, not positive means that the data need to be interpreted with caution. Furthermore, given that methylation is not a validated endpoint in colorectal cancer chemoprevention, one has to be circumspect in extrapolating these results. Although we do not recommend berries for cancer treatment, certainly not in lieu of standard methods of therapy, they might be considered for use in combination with other therapeutic modalities, such as chemotherapy, immunotherapy, radiotherapy, and/or surgery. Alternatively, it would be interesting to determine if the dietary consumption of BRBs on a regular basis following therapy for colorectal cancer reduces the risk for recurrent disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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