A Phase Ib Study of the Effects of Black Raspberries on Rectal Polyps in Patients with Familial Adenomatous Polyposis

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Abstract

Familial adenomatous polyposis (FAP) is characterized by the early onset of colonic polyposis and a high risk for colorectal cancer. FAP is treated by colectomy followed by lifelong removal of rectal polyps. This study determined whether black raspberries (BRBs) might regress rectal polyps in patients with FAP. Fourteen patients with FAP were treated with BRBs daily for 9 months. Seven patients received BRB powder orally plus two BRB suppositories inserted into the rectum at bedtime. The other 7 received an oral placebo plus the suppositories. Rectal polyp counts and polyp sizes were obtained at time zero and after 9 months of BRB treatment. Polyps and adjacent normal tissue were collected at both time points. The burden (P = 0.036) but not number (P = 0.069) of rectal polyps was significantly decreased. No benefit was noted with the addition of oral BRBs. Three patients were nonresponders. BRBs significantly decreased cellular proliferation, DNA methylation methyl transferase 1 protein expression, and p16 promoter methylation, but not promoter methylation of the Wnt pathway antagonists, SFRP2 and WIF1, in rectal polyps (adenomas) from responders but not from nonresponders. The MBD-seq assay revealed more demethylated transcription start sites (TSS), including those for miRNAs, in BRB-treated adenomas from the responders. In conclusion, BRB suppositories seem sufficient for regressing rectal polyps in patients with FAP. Cancer Prev Res; 7(7); 666–74. ©2014 AACR.

Introduction

Familial adenomatous polyposis (FAP) is characterized by colonic polyposis and a lifetime risk of subsequent colorectal cancer of nearly 100% due to inherited germline mutations in the adenomatous polyposis coli (APC) gene. Total abdominal colectomy with ileorectal anastomosis or total proctocolectomy with ileal pouch anal anastomosis are the traditional management strategies for colonic polyposis. Lifelong endoscopic surveillance of the rectum is required for the management of recurrent polyposis and does not obviate the development of uncontrolled rectal polyposis or rectal cancer that may require proctectomy (1).

Nonsteroidal anti-inflammatory drugs (NSAIDs) were first reported to cause regression of colonic polyps in subjects with FAP over two decades ago. Case series and many randomized controlled trials have confirmed this observation for nonselective NSAIDs such as sulindac (2–6). However, the gastrointestinal toxicity of nonselective NSAIDs led to the development of selective COX-2 inhibitors. Celecoxib and rofecoxib have been shown in randomized controlled trials to induce regression of colonic adenomas in patients with FAP (7, 8). Celecoxib is the FDA approved for the regression of adenomatous colocolorectal polyps in patients with FAP, as an adjunct to standard endoscopic management. Unfortunately, the increased risk of cardiovascular, thromboembolic, and cerebrovascular events led to the withdrawal of rofecoxib from the market and remains a concern for celecoxib (9). Therefore, an effective chemopreventive agent with no or minimal systemic toxicity would be a substantial advance for patients with FAP.

In one small study, the combined use of the dietary supplements curcumin and quercetin led to a reduction in the number and size of rectal polyps in patients with FAP with minimal side effects or toxicity (10). Black raspberries (BRBs) are a source of multiple nutritive and nonnutritive compounds, including vitamins A, C, E, and folic acid; calcium and selenium; β-sitosterol, ellagic acid, ferulic acid, quercetin, and several anthocyanins (11). Many of these compounds have demonstrated chemopreventive activity,
both in vitro and in animal models; for example, ellagic acid and the anthocyanins (11). Two mouse models of colorectal cancer were used to evaluate the effects of BRBs on colorectal tumor development and to investigate the underlying mechanisms (12, 13). A 12-week feeding of BRBs significantly inhibited intestinal tumor formation in both models; reducing tumor incidence by 45% and tumor multiplicity by 60% in ApC1638<sup>f<sup>−</sup>−</sup> mice and tumor incidence and multiplicity by 50% in Muc<sup>−</sup>−<sup>/−</sup> mice (12). Mechanistic studies showed that BRBs inhibit tumor development in ApC1638<sup>f<sup>−</sup>−</sup> mice by suppressing β-catenin signaling and in Muc<sup>−</sup>−<sup>/−</sup> mice by reducing chronic inflammation (12). Intestinal cell proliferation was reduced in both mouse models by BRBs; however, mucus differentiation was not affected in either model (12). Feeding of BRBs at 5% and 10% of the diet to F-344 rats for 32 weeks after treatment of the rats with the carcinogen, azoxymethane, resulted in a reduction of adenocarcinoma multiplicity of 35% and 80%, respectively (13). In addition, dietary administration of BRBs has been shown to inhibit esophageal, oral cavity, and mammary tumorigenesis in animal models (11). In humans, BRBs have been shown to be well tolerated and exert protective effects in multiple phase I human clinical trials in patients with colorectal cancer (14). Barrett’s esophagus (15), and oral dysplasia (16, 17).

We recently reported that dietary BRBs hypomethylate p16 and Wnt signaling pathway inhibitor genes in colorectal adenocarcinomas (14). This effect was associated with reduced activity of the enzyme, DNA methylation methyltransferase 1 (DNMT1) in colorectal tumors (14). DNMT1 is chiefly responsible for DNA methylation homeostasis, whereas two other methyl transferases; i.e., DNMT3a and DNMT3b, primarily catalyze de novo hypermethylation (18). All three enzymes, DNMT1, DNMT3a, and DNMT3b, have been shown to be overexpressed in several tumor types, including colorectal cancer (14, 19). Interestingly, both sporadic and FAP-related colon adenocarcinomas have common, for example, p16 and MGMT, as well as different, for example, PAX3, tumor-suppressor genes that are methylated, and hypermethylation seems to be a general feature of both sporadic and FAP-related carcinomas (20, 21). In this study, we determined whether BRBs administered in the form of a rectal suppository and/or in combination with oral administration cause suppression of polyp development in subjects with FAP, and whether BRBs affect DNA methylation in tissues collected from these patients.

Materials and Methods

Freeze-dried BRB powder, placebo powder, and BRB suppositorys

The procedures for the preparation of BRB powder for use in clinical trials have been described in detail (11) and are given in Supplementary Materials and Methods. A 100-g sample of the berry powder was analyzed for content of nutritive and nonnutritive constituents, most with known chemopreventive potential, by Covance Laboratories and these constituents are listed in Supplementary Table S1. BRBs contain four major polyphenolic anthocyanins that are responsible for the color of the berries. Their content in the powder was: cyanidin-3-glucoside (278.5 mg/100 g dry weight [DW]), cyanidin-3-sambubioside (56.0 mg/100 g DW), cyanidin-3-rutinoside (1,790 mg/100 g DW), and cyanidin-3-xylorutinoside (853.5 mg/100 g DW), resulting in a total anthocyanin content of 2,978 mg/100 g DW. We have shown previously that the anthocyanins in BRBs are chemopreventive in a rat model of esophageal cancer (22).

The placebo agent was a purple colored powder that contained maltodextrin (75%) and dextrose (25%). Maltodextrin is a nonsweet saccharide mixture that is freely soluble in water. Dextrose is a water-soluble monosaccharide that is not as sweet as sugar. BRB suppositories were prepared by the Central Ohio Compounding Pharmacy using the BRB powder described above. Each BRB suppository contained 720-mg BRB powder mixed in wax.

Clinical trial

Patients with FAP with at least 5, ≥2-mm rectal polyps on baseline endoscopy and who met the other inclusion criteria were eligible to participate. The subjects were randomly assigned to one of two treatment arms. The investigators and patients were blinded to the oral treatment arm assignment (Supplementary Fig. S1). This study was approved by the Institutional Review Boards of the Cleveland Clinic and The Ohio State University Medical Center (Columbus, OH). Treatment Schema: Arm 1 (N = 7), 20 g of placebo powder administered as an oral placebo (maltodextrin/dextrose) slurry three times per day (60 g/d total), plus two BRB rectal suppositories (each containing 720-mg BRB powder) administered at bedtime. Arm 2 (N = 7): 20 g of BRB powder administered orally three times per day (60 g/d total), plus two BRB rectal suppositories administered at bedtime. Photographs of a coffee cup containing BRB powder in water and of the rectal suppositories are shown in Supplementary Fig. S2.

Inclusion criteria. Patients accrued to the trial met the following criteria: (i) A willingness and ability to give informed consent; (ii) ≥18 years of age; (iii) nonpregnant and nonlactating female subjects either surgically sterile, postmenopausal, or using adequate birth control; lactating and nonlactating female subjects either surgically sterile, postmenopausal, or using adequate birth control; (vi) negative urine pregnancy test (if applicable); (v) diagnosis of FAP with at least 5, ≥2-mm rectal polyps on baseline endoscopy; (vi) have an endoscopically accessible rectal segment; (vii) have not taken chronic NSAIDs or selective COX-2 inhibitors for 2 months before participation in the study and willing to remain off NSAIDs for study duration. Discontinuation of NSAIDs or selective COX-2 inhibitors required approval by the physician treating the potential study participant; (viii) willingness to travel to study center for initial study procedures and for the 36-week evaluation; and (ix) have adequate home freezer storage for a 9-week supply of the BRB formulations.

Exclusion criteria. Patients excluded from the trial had one of the following: (i) known allergies or hypersensitivity to berries, including BRBs; (ii) subjects taking NSAIDs or COX-2 inhibitors who could not be taken off the medication.
due to their clinical condition; (iii) pregnant or lactating subjects; (iv) active peptic ulcer disease; (v) any gastrointestinal problem that in the opinion of the investigator would affect absorption (including chronic use of antacids) or ability to consume the oral preparations of BRBs; (vi) clinically significant hepatic or renal dysfunction; (vii) diabetes mellitus; (viii) on baseline evaluations: AST > 1.5 × upper limit of normal, ALT > 1.5 × upper limit of normal, alkaline phosphatase > 1.5 × upper limit of normal, total bilirubin > 2 × upper limit of normal, serum creatinine or BUN > 1.5 × upper limit of normal.

Distribution of powders and suppositories and monitoring for toxicity and compliance. BRBs and placebo powders for oral consumption were packaged in hermetically sealed aluminum bags each containing 20 g of powder by the Central Ohio Compounding Pharmacy. Two-month supplies of either berry or placebo powder and of the BRB suppositories were sent by the Compounding Pharmacy to subjects in the trial. The subjects were instructed to store the suppositories in a freezer (−20°C) upon receipt, and to transfer the number of bags and suppositories required for one week’s use from their freezer to a refrigerator. Research personnel from the Central Ohio Compounding Pharmacy telephoned each subject at 7 to 10 days after randomization to the trial to evaluate compliance and adverse event monitoring. Subsequent evaluations were done at monthly intervals by phone. Subjects were given instructions on how to complete a diary to record the date and time the berry or placebo powder was consumed and also to record the date and time the two suppositories were inserted.

Polyp size, number, and tissue collection. Subjects underwent flexible sigmoidoscopy before study treatment (baseline) and at end of study (EOS) at 36 weeks (+ 10 days) after the initiation of study treatment. One endoscopist performed all endoscopic examinations (C.A. Burke). The size and number of rectal polyps between the anal verge and ileorectal anastomosis were counted at each visit and photo-documented. The size of each polyp was measured by using an open or closed biopsy forceps. The polyp burden was calculated as the sum of diameters of all adenomas ≥2 mm. Indigo carmine dye spray was used after polyp counting to distinguish normal mucosa from polyps. All polyps over 1 cm were removed at baseline and sent for local histologic assessment. Up to two polyps were removed for biomarker studies at baseline. Biopsies were obtained of normal rectal mucosa (>8) at baseline and end of study. All rectal polyps were harvested at the end of the study. Tissues were placed in 10% buffered formalin for histologic analysis. Any polyps removed at baseline were accounted for in the analysis. The difference in the number of ≥2-mm rectal polyps between baseline and end of study was compared in those subjects that completed 36 weeks of therapy (after adjustment for baseline polyp removal). All tissue specimens were classified histologically by a pathologist (W.L. Frankel) with interest in gastrointestinal diseases. All rectal polyps were classified as tubular adenoma (hereafter referred to as adenoma). Histologically confirmed paraffin-embedded rectal polyps were used for immunohistochemical analyses and for subsequent molecular analysis.

Laboratory analyses

Immunohistochemical staining and computer-assisted image analysis. Baseline and EOS adjacent normal tissue and adenomas were cut into 4-μm sections and placed on slides. Methods for staining and quantification of Ki-67, cMyc, p16, DNMT1, DNMT3b, or TUNEL are detailed in Supplementary Materials and Methods. The commercial sources of the antibodies are given in Supplementary Table S2.

Stained tissue was viewed and photographed at ×200 magnification with a bright-field microscope mounted with a high-resolution spot camera. The camera was interfaced with a computer containing a matrix frame grabber board and image analysis software (Simple PCI Imaging Systems; Compix Inc.) as described before (14).

Analysis of DNA methylation. DNA extraction and bisulfite conversion. Paraffin-embedded tissues were cut into 10-μm sections and DNA was extracted using a PicoPure DNA Kit (MDS Analytical Technologies). Extracted DNA was purified using the QiAquick PCR purification Kit (Qiagen). Of note, 500 ng of extracted DNA was bisulfite-converted using the EZ DNA Methylation Kit (Zymo Research) according to the manufacturer’s instructions.

MassARRAY. Bisulfite-converted DNA was amplified with primers (primer sequences are listed in Supplementary Table S3), the PCR products spotted on a 384-pad SpectroCHIP (Sequenom), and spectrally acquired on a MassARRAY analyzer. Methylation data of individual units (1–4 CpG sites/unit) were generated by EpiTyper software (Sequenom).

Pyrosequencing. Bisulfite-converted DNA was amplified and sequenced using the PyroMark LINE-1 Kit (Qiagen), which contains PCR primers and a sequencing primer provided by the company. PCR-cycling conditions were 95°C (30 s), 50°C (30 s), and 72°C (30 s) for 35 cycles. The PCR product was purified and methylation quantified using the PSQ HS 96 Pyrosequencing System (Pyrosequencing Inc.).

MBDCap-seq, mapping, and normalization for genome-wide methylation analysis. As will be noted below, adenomas from a total of 2 subjects treated with orally administered BRBs plus BRB suppositories and 1 subject treated with BRB suppositories only did not respond to berry treatment. These subjects had more rectal adenomas at the end of the trial than at baseline, and the adenomas were used for the MBDCap-seq assay to determine whether differences in the response of adenomas from nonresponders and responders might be due to differences in effects of the BRBs on genome-wide DNA methylation (23). Unfortunately, due to other uses, adjacent normal tissues from two of the nonresponders were not available for the MBDCap-seq assay. Therefore, adjacent normal tissues from 3 responders and 1 nonresponder as well as adenomas from 3 responders and 3 nonresponders were used in the assay.

Paraffin-embedded tissues were cut into 10-μm sections and placed on slides. Methods for staining and quantification of Ki-67, cMyc, p16, DNMT1, DNMT3b, or TUNEL are detailed in Supplementary Materials and Methods. The commercial sources of the antibodies are given in Supplementary Table S2.

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MBDCap-seq, mapping, and normalization for genome-wide methylation analysis. As will be noted below, adenomas from a total of 2 subjects treated with orally administered BRBs plus BRB suppositories and 1 subject treated with BRB suppositories only did not respond to berry treatment. These subjects had more rectal adenomas at the end of the trial than at baseline, and the adenomas were used for the MBDCap-seq assay to determine whether differences in the response of adenomas from nonresponders and responders might be due to differences in effects of the BRBs on genome-wide DNA methylation (23). Unfortunately, due to other uses, adjacent normal tissues from two of the nonresponders were not available for the MBDCap-seq assay. Therefore, adjacent normal tissues from 3 responders and 1 nonresponder as well as adenomas from 3 responders and 3 nonresponders were used in the assay.
MBDCap-seq, mapping, and normalization were detailed in Supplementary Materials and Methods. The methylated regions could be any length, but 8 kb was used because the majority of CpG islands are within 2 kb up- or downstream of the transcription start site (TSS), and CpG island shores are up to 2-kb distance relative to each gene’s CpG islands (24).

**Statistical analysis**

Adenoma number and burden (the sum of diameters of all adenomas ≥2 mm) as well as data from MassARRAY and immunohistochemical staining were compared by the Wilcoxon signed-rank test. All analyses were two-sided, and a P value of less than 0.05 was considered significant.

**Results**

**Effects of oral BRBs and BRB suppositories on the development of adenomas**

The mean age of patients was 48 years (30–67), including 7 men and 7 women. All patients were Caucasian (Supplementary Table S4). All adverse events of randomized subjects considered possibly, probably, or definitely related to study treatment are as follows: anal fissure (n = 2), bloating (n = 3), diarrhea (n = 5), flatulence (n = 2), nausea (n = 7), and rectal irritation (n = 6). In addition, 8 randomized subjects (total 24 patients were randomized) experienced difficulty retaining the rectal suppositories. When data from all 14 patients were combined, there was a decrease in the number (−3.5, P = 0.069) and burden (−8.5, P = 0.036) of rectal adenomas (Supplementary Table S5). No additional benefit on adenomas was noted in the group randomized to oral BRBs (Supplementary Table 5). Only 1 of 7 patients (#1–7) who received oral placebo and BRB suppositories had more adenomas. Of the 7 patients (#8–14) who received oral BRBs plus BRB suppositories, only 4 had reductions in adenoma number, 1 had no change in adenoma number, and 2 had more adenomas at the end of the study (Fig. 1A). Figure 1B indicates the decrease in polyp burden that parallels the regression in number of polyps (Fig. 1A). These data suggest that the BRB suppository seems sufficient to regress rectal polyps.

**BRBs decreased cellular proliferation in adenomas from responders**

We then determined whether BRB treatments (oral plus suppository and suppository only) led to different biomarker alterations in responders versus nonresponders. As indicated above, 3 patients with FAP had more rectal adenomas at the end of the BRB treatment than in the beginning (nonresponders). We determined whether this might be related to differences in the effects of BRBs on cell proliferation and apoptosis in the adenomas from nonresponders and responders. Ki-67 staining was significantly decreased in adenomas from responders but not from nonresponders (Fig. 2A). There was no significant change in TUNEL (Fig. 2B) and cMyc (Fig. 2C) staining in adjacent normal or adenoma tissues from responders or nonresponders.

**Effects of BRBs on DNA methylation in tissues from responders and nonresponders**

As noted previously (14), colorectal adenocarcinomas from patients with cancer who had consumed BRBs (60 g/d total) orally for at least 4 weeks had reduced levels of DNMT1 protein after berry treatment when compared with baseline. This observation was correlated with reduced methylation of p16 and multiple regulatory genes in the Wnt signaling pathway. We asked, therefore, whether BRBs administered as oral BRBs plus suppository and suppository only differentially affect DNMTs and the promoter methylation status of p16 and the Wnt pathway regulators, for example, SFRP2 and WIF1, in both adjacent normal tissues and adenomas from responders and nonresponders. As shown in Fig. 3A, BRBs significantly decreased DNMT1 protein expression in adenomas from responders. BRBs did
not change DNMT3b expression in adjacent normal tissues or in adenomas from responders or nonresponders (Fig. 3B). p16 protein expression was significantly increased in adjacent normal tissues from responders and nonresponders (Fig. 3C). p16 promoter methylation was significantly decreased in adjacent normal tissues and in adenomas from responders (Fig. 3D). However, BRBs did not affect promoter methylation of SFRP2 (Fig. 3E) and WIF1 (Fig. 3F), Wnt antagonists, in adjacent normal tissues or adenomas from responders or nonresponders.

**BRBs decreased levels of promoter methylation of other genes identified by MBDCap-seq**

We used MBDCap-seq to globally screen methylation changes induced by BRBs in 3 nonresponders (1 received suppository only, patient #1; and 2 received oral BRBs plus BRB suppository, patients #8 and #9) and in the 3 best responders (2 received suppository only, patients #2 and #3; and 1 received oral BRBs plus BRB suppository, patient #11). Adjacent normal tissues from 2 nonresponders were exhausted and were not available for MBDCap-seq assay. Genome-wide methylation patterns for adjacent normal tissues from 3 responders and 1 nonresponder as well as adenomas from 3 responders and 3 nonresponders are depicted in Fig. 4A. Methylation status increased immediately before the TSS and dropped at the TSS. It then increased in the gene bodies and then dropped at the transcription termination site (TTS). LINE1 global methylation was not significantly altered by BRBs in adjacent normal tissues or in adenomas from responders or nonresponders (Fig. 4B). We then looked into the numbers of regions being demethylated by BRBs. In general, more regions were demethylated in adenomas than in adjacent normal tissues in responders (Fig. 4C). Similarly, more TSSs were demethylated in adenomas than in adjacent normal tissues in responders (Fig. 4D).

In an attempt to determine whether BRB treatment might affect methylation differently in responders and nonresponders, we asked whether there are differences in the genes demethylated by BRBs in adenomas from responders and nonresponders. As shown in Fig. 5A, the TSSs of a higher number of genes were demethylated in adenomas from responders than from nonresponders; i.e., 1,358 versus 327. In addition, more genes were commonly demethylated by BRBs in adenomas from responders than nonresponders. For example, 27 genes were commonly demethylated in all 3 responders and there was no gene commonly demethylated in all 3 nonresponders; 292 genes were commonly demethylated in any 2 responders, whereas there were 30 genes commonly demethylated in any 2 nonresponders. Genes demethylated in all 3 and in any 2 responders (27 + 292 = 319), and in all 3 and in any 2 nonresponders (0 + 30 = 30) were used to make the pie chart to illustrate the numbers of genes uniquely and commonly demethylated in responders or nonresponders (Fig. 5A). There were 312 and 23 genes uniquely demethylated in adenomas from responders and nonresponders, respectively (Fig. 5A). Pathway analysis of the 312 and 23 genes indicated that there are no signaling pathways that were significantly altered (data not shown). Interestingly, there were 37 miRNAs uniquely demethylated in adenomas from responders; however, there was only one miRNA uniquely demethylated in adenomas from nonresponders (Fig. 5A). Predicted targets for the 37 miRNAs were used.

![Figure 2. BRBs decreased cellular proliferation in adenomas from all responders. Immunohistochemical staining of Ki-67 (A), TUNEL (B), and cMyc (C) in adjacent normal tissues (N) and adenomas (T) from 11 responders (R) and 3 nonresponders (NR) at baseline (Ba) and after 9 months (EOS) of BRB treatment from both protocols; BRB suppository only and oral BRBs plus BRB suppository. •, *P < 0.05.](image-url)
for pathway analysis and pathways with $P$ values <0.05 are presented in Fig. 4B. Interestingly, the Wnt pathway was the most significantly altered signaling pathway followed by axon guidance, TGF-$\beta$ signaling, focal adhesion, MAPK signaling, Notch signaling, tight junction, and insulin signaling (Fig. 5B).

We next asked the question whether there are differences in the responses of adjacent normal tissues and adenomas from responders to BRB treatment. Interestingly, there were no genes commonly demethylated by BRBs in adjacent normal tissues from all 3 or any 2 responders; however, 27 and 292 genes were commonly demethylated in adenomas from all 3 and any 2 responders, respectively (Fig. 6A). The numbers of genes demethylated in adenomas from all 3 and any 2 responders ($27 + 292 = 319$) and in adjacent normal tissues from any responder (= 120) were used to make the pie chart to illustrate the numbers of genes uniquely and commonly demethylated in adenomas and adjacent normal tissues from responders (Fig. 6A). There were 286 and 87 genes uniquely demethylated in adenomas and in adjacent normal tissues from responders, respectively (Fig. 6A). Pathway analysis of the 286 and 87 genes suggests that there were no pathways that were significantly altered (data not shown). Interestingly, there were 33 miRNAs uniquely demethylated in adenomas from responders; however, there were only three miRNAs uniquely demethylated in adjacent normal tissues from responders (Fig. 6A). Predicted targets for the 33 miRNAs were used for pathway analysis and pathways with $P$ values <0.05 are presented in Fig. 6B. Interestingly, the Wnt pathway is again the most significantly altered signaling pathway followed by axon guidance, endocytosis, adherens junction, focal adhesion, TGF-$\beta$ signaling, ErbB signaling, etc. (Fig. 6B).

**Discussion**

Although there were 3 nonresponders in this study, our data suggest that BRBs have the ability to regress rectal polyps in patients with FAP; in particular, BRB suppositories alone might be sufficient to suppress rectal polyp development (Fig. 1. Supplementary Table S5). In other clinical trials with oral administration of berries, BRBs have been
shown to be well tolerated and exert protective effects in patients with colorectal cancer (14) and Barrett’s esophagus (15), and strawberries regress mild dysplastic esophageal lesions that are precursors to squamous cell carcinoma (25). Accordingly, we anticipated that oral BRBs plus BRB suppositories could be more effective than suppositories alone in regressing polyps in patients with FAP. Surprisingly, however, our data suggest that BRB suppositories alone are sufficient to regress polyps as no additional benefit was noted in the group randomized to oral BRBs (Supplementary Table S5). However, with only 7 subjects in each arm, it is difficult to arrive at definitive conclusions and a more expanded study is necessary to draw final conclusions.

It is well known that FAP is caused by mutations in the APC gene. The severity of FAP is known to be influenced by the site of the APC mutation (26, 27). We asked whether nonresponders in this study have different somatic SNPs in the APC gene than responders. Forty SNPs in APC genes were assayed in adjacent normal and adenomas collected at baseline from all 14 patients. There was no significant difference in the 40 SNPs in the APC gene in responders versus nonresponders in adjacent normal tissues and adenomas (Supplementary Fig. S3).

We previously reported that an average of 4 weeks of BRB consumption decreased promoter methylation of $p16$ and of Wnt pathway regulator tumor-suppressor genes in biopsies collected from patients with human colorectal cancer (14). Therefore, we decided to investigate whether BRB-induced promoter DNA demethylation might be different in responders and nonresponders. Interestingly, our data showed that there were more TSSs commonly demethylated in adenomas from responders than nonresponders (Fig. 5A). It is possible that resistance of these adenomas to BRB treatment is due to decreased sensitivity of their responses to BRB-induced DNA demethylation.

Interestingly, BRBs demethylated approximately 10 times fewer regions and TSSs in adjacent normal tissues from responders than in adenomas (1,358 vs. 120, Fig. 6A).
replication in normal tissues is low and two rounds of cell replication are required to achieve a heritable demethylation effect; first round produces semi-demethylated DNA and second round produces fully-demethylated DNA (28). The observation that the berries demethylated more regions in adenomas than in adjacent normal tissues is likely due to differences in rates of cell replication in the two tissues.

A previous study in our laboratory found that the promoter regions of p16 and Wnt regulator genes in colorectal tumors were demethylated by oral consumption of BRBs (14). In contrast, in the present study, p16 promoter methylation was decreased in adjacent normal tissues and in adenomas from responders (Fig. 3D). However, BRBs exerted no significant effects on promoter methylation of SFRP2 and WIF1, Wnt regulators, in either adjacent normal tissues or in adenomas from responders and nonresponders (Fig. 3E and F). Although promoter methylation of examined Wnt regulators was not changed by BRB treatment (Fig. 3E and F), miRNAs regulating Wnt pathway were uniquely demethylated by BRBs in adenomas from responders (Figs. 5A and 6A). These results suggest that BRBs regulate the Wnt pathway through demethylation of miRNAs in adenomas from patients with FAP. These miRNAs also regulate other pathways associated with the development of colorectal cancer, for example, TGFβ, MAPK, Notch, Tight junction, insulin signaling, and so forth. Thus, BRBs may regulate multiple signaling pathways associated with the development of colorectal cancer through their effects on miRNAs. Finally, our results suggest that berry suppositories might be an alternative to Celecoxib for the treatment of patients with FAP.

Disclosure of Potential Conflicts of Interest
G. Stoner has ownership interest (including patents) in BerriProducts, Inc., Corvallis, Oregon. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: C.A. Burke, T.H.-M. Huang, G.D. Stoner
Development of methodology: C.A. Burke
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L.-S. Wang, C.A. Burke, H. Hasson, W.L. Frankel
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Cancer Prevention Research

A Phase Ib Study of the Effects of Black Raspberries on Rectal Polyps in Patients with Familial Adenomatous Polyposis

Li-Shu Wang, Carol A. Burke, Henrietta Hasson, et al.


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