Supplemental Figure 1

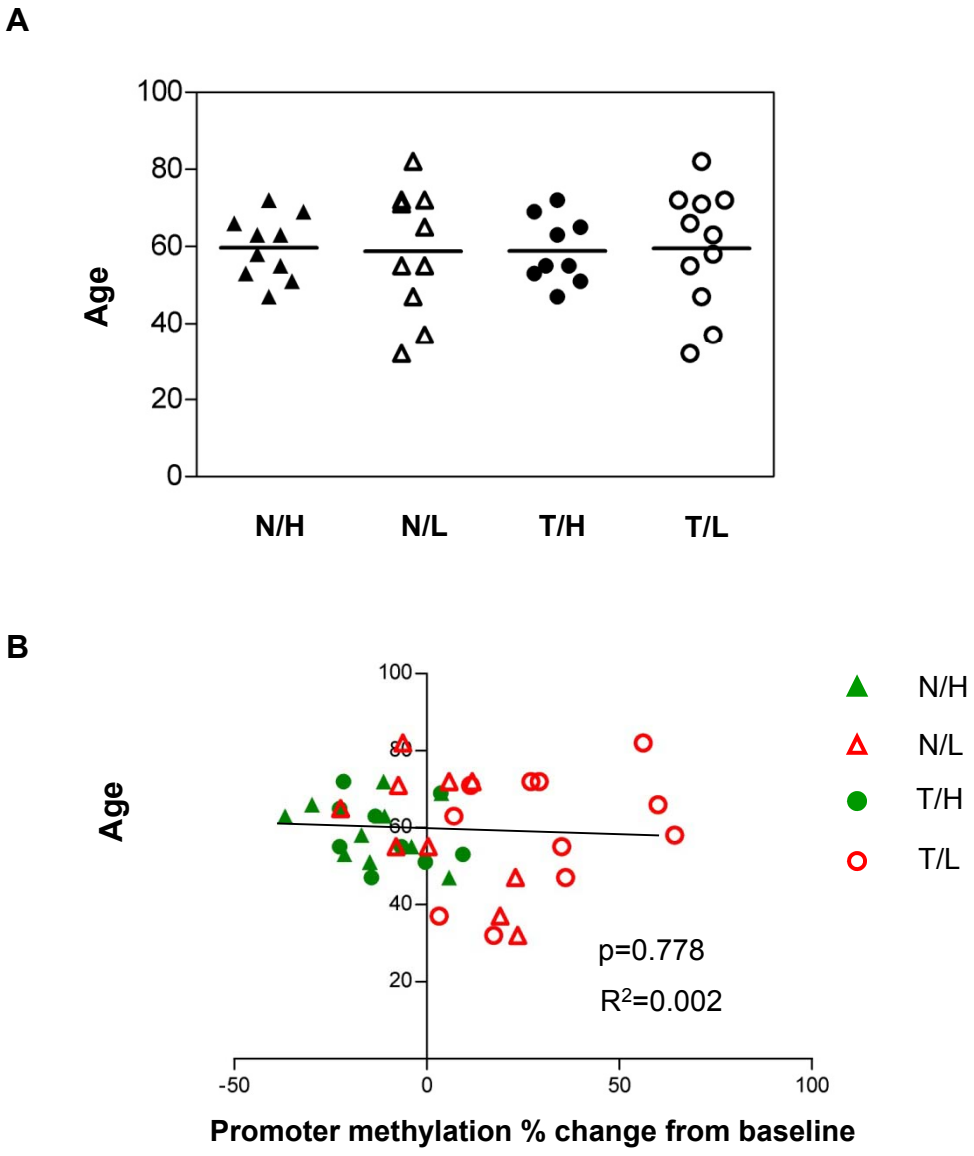

Fig. S1. Average age in high and lose dose berry groups, and correlation between berry-induced promoter methylation changes and age. (A) There was no difference of average age in N/H, N/L, T/H, T/L. They were all around 60 years old. (B) Berry-induced methylation changes of all 5 genes combined (*SFRP2*, *PAX6a*, *p16*, *SFRP5,* and *WIF1*) were not age-dependent.

Supplemental Methods

Black raspberries.

The freeze-dried berries were pulverized into a fine powder which represented approximately 1:10 w/w equivalent of fresh BRBs. The powder was shipped frozen to the Ohio State University where it was kept at -20ºC until packaged for use in the trial. A sample of berry powder was shipped to Covance Laboratories in Madison, Wisconsin to assay for levels of the nutrients listed in Supplemental Table 2, and for potential contamination with fungicides, pesticides and herbicides. The anthocyanin content of the berries was determined in the laboratory of Dr. Steven Schwartz.

After the berry powder was analyzed for nutrient/chemical content, it was vacuum packed and sealed in 20g quantities in bags composed of layered nylon, linear low-density polyethylene and aluminum by Central Compounding Pharmacy in Worthington, OH. This composition provided an excellent barrier to moisture and oxygen while maintaining flexibility. The bags were taken to the Ohio State University Comprehensive Cancer Center where they were kept frozen until distributed to patients accrued to the trial. Patients were instructed to keep the berry powder in their freezer at home during use in the trial.

Measurement of berry anthocyanins in colorectal tissues and urine.

A. Sample preparation. Three hundred μL of 5% formic acid in methanol was added to 15-30 mg of frozen adjacent normal or colorectal tumor tissues. The tissues were sonicated on ice and centrifuged at 16,000 x g for 1 min. The pellet was discarded. The supernatant was transferred to a glass vial, dried under nitrogen, and the dried pellet dissolved in 200 μL of 80% aqueous / 20% methanol (each containing 5% formic acid).

Finally, the mixture was centrifuged at 16,000 x g for 6 min and the supernatant transferred into a glass vial for liquid chromatography. All samples were stored at –80°C prior to anthocyanin analysis.

Urine samples were thawed and 6 mL of urine applied to a C18 Sep Pak Plus cartridge (Waters Corp.) previously conditioned with methanol and 5% (v/v) formic acid in water. Urine applied to the cartridge was rinsed with 6 mL of 5% formic acid and eluted with 3 mL of 5% (v/v) formic acid in methanol. The eluent was dried under nitrogen, redissolved in 200 μL 80% aqueous / 20% methanol (each containing 5% formic acid) and centrifuged for 1 min in a Minicent centrifuge at 10,000 rpm. Twenty μL of supernatant was injected for HPLC-tandem mass spectrometry (MS/MS) analysis.

B. HPLC –MS/MS analysis of anthocyanins. Separation of tissue and urinary anthocyanins was conducted on a Symmetry C18 column (4.6 × 75 mm, 3.5 μm; Waters Corp.) using an Alliance 2695 HPLC system coupled with a Quattro Ultima triple quadrupole mass spectrometer (Waters Corp.) via an electrospray probe operated in positive mode as described (22). Monitored anthocyanins were *m/z* 595 → 287 (cyanidin-3-rutinoside), *m/z* 727 → 287 (cyanidin-3-xylosylrutinoside), *m/z* 449 → 287 (cyanidin-3-glucoside) and *m/z* 581 → 287 (cyanidin-3-sambubioside).

C. Standards and calibration curves. Cyanidin-3-glucoside obtained from Indofine Chemical Co., Inc. was used as the standard for quantification. It was dissolved in deionized-distilled water containing 5% formic acid to a concentration of 2.85 mmol/L and, thereafter, diluted (0.0285-2.85 μmol/L) to make a standard curve (R^2 = 0.99). All levels of anthocyanins analyzed in tissue and urine samples fell within the standard curve range and are expressed as cyanidin glucoside equivalents in pmol/mL urine or fmol/mg

tissue. Total anthocyanins recovered from tissue and urine samples were calculated by summing the concentrations of individual anthocyanin peaks.

Analysis of DNA methylation.

A. 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). 500 ng of extracted DNA was bisulfite-converted using the EZ DNA Methylation kit (Zymo Research) according to the manufacturer's instructions.

B. MassARRAY. bisulfite-converted DNA was amplified with primers (primer sequences are listed in Supplemental Table 3), the PCR products spotted on a 384-pad SpectroCHIP (Sequenom), and spectrally acquisited on a MassARRAY analyzer. Methylation data of individual units (1-4 CpG sites per unit) were generated by EpiTyper software (Sequenom).

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

Immunohistochemical staining and computer-assisted image analysis.

Pre- and post-treatment adjacent normal and colorectal tumor biopsies were cut into 4 μm sections and placed on slides. The slides were placed in a 60°C oven for 1 hr, cooled, deparaffinized, and rehydrated through xylenes and graded ethanol solutions to

water. All slides were treated for 5 min with a 3% H_2O_2 solution in water to block endogenous peroxidase. Antigens were retrieved by placing the slides in a vegetable steamer in Dako Target Retrieval Solution for 25 min, after which they were cooled for 15 min. The slides were then placed on a Dako Autostainer for automated staining with primary antibodies to either ß-catenin, E-cadherin, c-Myc, Ki-67, TUNEL, p16, CD105 or DNMT1 for 1 hr at room temperature. The commercial sources of the antibodies are given in Supplemental Table 4. Slides were then stained with their respective secondary antibody. The ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (#S7101, Chemicon, Temecula, CA) was used for TUNEL staining following the manufacturers' instructions.

Stained tissues were viewed and photographed at 200x 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 (1). We were assisted by a pathologist (Dr. Wendy Frankel) in the selection of stained areas within adjacent normal and tumor tissues for quantification of specific antigens. For all antigens, up-to 30 whole crypts of normal epithelium and 30 fields of tumor from the three pieces of adjacent normal and tumor biopsies collected at each time point per patient were analyzed and the staining intensities quantified by image analysis software. Quantification of Ki-67 staining in normal crypts was done in the proliferative region; i.e., approximately the lower $1/3^{rd}$ of each crypt. Nuclear but not cytoplasmic ß-catenin was quantified.

Reference

1. Wang LS, Hecht SS, Carmella SG, *et al*. Anthocyanins in black raspberries prevent esophageal tumors in rats. Cancer Prev Res 2009;2:84-93.

Supplemental Tables

Supplemental Table 1. BRB-derived anthocyanins in urine and colorectal tissues before and after 1-to-9 wks of BRB treatment

	Pre-treatment Mean ± S.D. (Range)	Post-treatment Mean ± S.D. (Range)	
Urine (pmol/mL)	0.0 ± 0.0 (0.0-0.0)	423.9 ± 429.3 (56.2-1822.1)	
Adjacent normal (fmol/mg)	0.0 ± 0.0 (0.0-0.0)	299.9 ± 754.9 (1.7-2011.5)	p=0.21
Adenocarcinoma (fmol/mg)	0.0 ± 0.0 (0.0-0.0)	55.4 ± 60.8 (2.2-109.2)	

Supplemental Table 2. Some potential chemopreventive agents in BRBs[*]

Components	mg/100g dry weight[†]
Minerals	
Calcium	217.00
Selenium	<3.00
Zinc	2.40
Vitamins	
vitamin A from carotene	98.95
vitamin E nature	16.00
β-carotene	0.06
α-tocopherol	10.48
γ-tocopherol	11.05
Folate	0.15
Sterols	
β-sitosterol	102.00
campesterol	5.50
Simple phenols	
ellagic acid	100.00
ferulic acid	<5.00
ρ-coumaric acid	8.34
Quercetin	42.45
Anthocyanins (complex phenols)	
cyanidin-3-glucoside	200.00
cyanidin-3-sambubioside	180.00
cyanidin-3-rutinoside	2002.00
cyanidin-3-xylosylrutinoside	400.00

[*] Data from crop year 2004.

[†] Components reported in mg/100g dry weight, except selenium in μg/100g, and vitamin A from carotene and vitamin E nature in IU/100g

Supplemental Table 3. List of primer sequences for MassARRAY analysis

Gene	Forward (3'-5')	Reverse (3'-5')
CDKN2A	aggaagagagGGTTAGTGTAGGTTTTATTTTTTT	cagtaatacgactcactatagggagaaggctCTTCTCAATAACTTCCTATTCATAC
PAX6a	aggaagagagGAAGGTTAAAGTAGGGGTTGGAGT	cagtaatacgactcactatagggagaaggctCCTACCCCAAAATTTAAATATCAATAA
SFRP2	aggaagagagGGTTAGGTTTTTTTGTTTGTTGTTT	cagtaatacgactcactatagggagaaggctAAAAAACTAATCACTACTTTCTAAATC
SFRP5	aggaagagagTAGGGAGTTTTGGGGAGAAA	cagtaatacgactcactatagggagaaggctCCTAACCCCAACTCCAAAAC
WIF1	aggaagagagGGGAATAGTTTTGGTTGAGGG	cagtaatacgactcactatagggagaaggctCAACAAACACAAAAAAATACTCCAA

Supplemental Table 4. Vender information and dilution for primary antibodies

	Vender	Dilution
β-catenin	BD Transduction labs	1:5000
c-Myc	Santa Cruz Biotechnology, Inc	1:100
CD105	Lab Vision Products	1:100
DNMT1	LifeSpan Biosciences	1:100
E-cadherin	Dako	1:100
Ki-67	Dako	1:150
p16	MTM Laboratories AG	1:20