Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

[Jessica Citronberg] Date

Effects of Ginger Supplementation on Markers of Apoptosis, Proliferation, and Differentiation in the Normal-Appearing Colonic Mucosa of Patients at High Risk for Colorectal Cancer: Results from a Pilot, Randomized, Controlled Trial

By

Jessica Citronberg
Master of Public Health

Department of Epidemiology

_____

[Roberd M. Bostick, M.D., M.P.H.]
Thesis Advisor

Effects of Ginger Supplementation on Markers of Apoptosis, Proliferation, and Differentiation in the Normal-Appearing Colonic Mucosa of Patients at High Risk for Colorectal Cancer: Results from a Pilot, Randomized, Controlled Trial

By

Jessica Citronberg

B.S., University of Michigan, 2007

Thesis Advisor: Roberd M. Bostick, M.D., M.P.H.

An abstract of
A thesis submitted to the Faculty of the
Rollins School of Public Health of Emory University
in partial fulfillment of the requirements for the degree of
Master of Public Health
in Epidemiology
2012

Abstract

Effects of Ginger Supplementation on Markers of Apoptosis, Proliferation, and Differentiation in the Normal-Appearing Colonic Mucosa of Patients at High Risk for Colorectal Cancer: Results from a Pilot, Randomized, Controlled, Trial

By Jessica Citronberg

Background: To estimate the effects of ginger on apoptosis, proliferation, and differentiation in the normal human colorectal mucosa, we measured the expression of cell cycle markers in the normal-appearing colon mucosa of patients at increased for CRC in a pilot, randomized, double-blinded, placebo-controlled, clinical trial.

Methods: A total of 20 patients were treated with either 2.0 g (eight 250 mg capsules) of encapsulated ginger (standardized to 5%-gingerols) or placebo daily for 28 days. Overall expression and distributions of Bax, Bcl-2, p21, hTERT and MIB-1 in colorectal crypts in biopsies of normal-appearing colon mucosa were detected and measured using automated immunohistochemistry and quantitative image analysis. Percent changes in biomarker expression over the treatment period were calculated, categorized, assigned a score and then summed to construct a "cell-cycle score."

Results: Image analysis measurement intra-rater reliability was ≥ 0.90 for all biomarkers. In the ginger group relative to the placebo group, Bax expression decreased 15.6% ($p = 0.81$) along the full length of the crypts, 6.6% ($p = 0.38$) in the upper 40% (differentiation zone) of the crypts, and 21.7% ($p = 0.77$) in the lower 60% (proliferative zone) of the crypts; however, there was a 19% increase ($p = 0.80$) in the proportion of the expression of Bax in the upper 40% relative to the whole crypt. While p21 and Bcl-2 expression remained relatively unchanged, hTERT expression along the full length of crypts decreased by 41.2% ($p = 0.05$); the estimated treatment effect on hTERT expression was slightly more pronounced in the canonical differentiation zone of crypts (-47.9%; $p = 0.04$). In the ginger group relative to the placebo group, MIB-1 expression decreased along the entire crypt, upper 40% of crypts, and lower 60% of crypts by 16.9% ($p = 0.39$), 46.8% ($p = 0.39$), and 15.3% ($p = 0.41$), respectively.

Conclusions: These preliminary results 1) suggest that ginger may reduce proliferation in the normal-appearing colorectal epithelium and increase apoptosis and differentiation relative to proliferation—especially in the proliferative zone of the crypts, and 2) support a full-scale clinical trial to further investigate these results.

Effects of Ginger Supplementation on Markers of Apoptosis, Proliferation and Differentiation in the Normal-Appearing Colonic Mucosa of Patients at High Risk for Colorectal Cancer: Results from a Pilot Randomized, Controlled Trial

By

Jessica Citronberg

B.S., University of Michigan, 2007

Thesis Committee Chair: Roberd M. Bostick, M.D., M.P.H.

A thesis submitted to the Faculty of the
Rollins School of Public Health of Emory University
in partial fulfillment of the requirements for the degree of
Master of Public Health
in Epidemiology
2012

ACKNOWLEDGEMENTS

I sincerely thank Dr. Roberd Bostick, for his outstanding mentorship, insightful advice, and continued encouragement, support, and guidance. I would also like to thank Dr. Suzanna Zick for training opportunities and support. Additionally, I would like to thank Joyce Kong for providing "biomarker scoring" training and Thomas Ahern for providing statistical analyses advice. Finally, I would like to thank my family and friends for their continuous support in all my academic endeavors.

Table of Contents

BACKGROUND

Colorectal Cancer

Colorectal cancer is the second leading cause of cancer-related deaths in the United States. Estimates predict that 141,210 new cases of colorectal cancer (CRC) will develop in 2011, resulting in 49,380 deaths (1). The incidence of CRC continues to increase and despite improvements in detection and treatment, CRC mortality has declined only modestly in recent years. The prevailing strategy to address colorectal cancer has been through secondary preventions and while the benefits of screening have been well established, there is also substantial evidence to support increased efforts for primary prevention through lifestyle modification.

Colorectal cancer is usually observed in one of three specific patterns: sporadic, inherited, or familial. The strongest known risk factors for colorectal cancer include autosomally dominant familial adenomatosis polyposis (FAP) and hereditary non-polyposis colon cancer (HNPCC) genetic syndromes. However, these conditions account for only 5% of cases. Of the remaining cases, 30% have a family history of the disease and the remaining 65% are thought to be "sporadic."

The incidence of colorectal cancer increases with age; about 90% of colorectal cancer cases occur in people of age 50 or older (2). CRC occurs with approximately equal frequency in men and women, however, rectal cancer occurs up to twice as often in men as in women (3). Colorectal cancer rates vary by race and ethnicity. In the United States, black men and women have the highest colorectal cancer incidence and mortality rates.

International and migration studies point to the predominance of lifestyle and diet in colorectal cancer etiology, with roughly 70 percent of CRC being attributable to

environmental factors (4, 5). Colorectal cancer appears to be highly correlated with Western lifestyle and diet; however, specific dietary factors that prevent and promote colorectal carcinogenesis are disputed and not well established.

Dietary factors believed to adversely influence colorectal carcinogenesis include red and processed meats, while fruit, vegetables, fiber, calcium, and vitamin D are thought to protect against the development of colorectal cancer (2, 6, 7). Energy balance may be a central factor in the etiology of colon cancer and ties together with other risk factors such as BMI, physical activity, and total energy intake. Non-dietary risk factors associated with lower risk of colorectal cancer include the use of estrogen replacement therapy among women and use of anti-inflammatory drugs (8).

CRC can arise from at least two different pathways: the chromosomal instability pathway (CIN) and the microsatellite instability (MSI) pathway. The CIN pathway is characterized by mutations in oncogenes and tumor suppressor genes, such as *K*-ras and APC, and accounts for 80-85% of sporadic colorectal carcinomas. The MSI pathway accounts for the remaining 15% of sporadic colorectal cancers and is characterized by defects in DNA mismatch repair genes, which in turn, lead to mutations in microsatellite sequences (9).

The development of colorectal neoplasia occurs through a series of genetic steps that typically correspond to the histological progression from normal colonic epithelium to adenoma to carcinoma. The molecular biology of CRC is characterized by aberrant activity of several pathways including apoptosis, proliferation, cell cycle regulation, and signal transduction.

The colonic epithelium undergoes an almost constant and normal renewal of the superficial epithelium, which is achieved through cell proliferation, differentiation, and

apoptosis. The epithelium contains about 10^7 crypts, which are the main morphologic units of the colorectal mucosa. Colonocyte proliferation occurs at the base of the crypt, where stem cells undergo asymmetrical replicative divisions to produce proginator cells. The newly divided cells differentiate and mature during their migration to the surface, where they are replaced by a new generation of cells about every 3 – 6 days (3). Mature cells undergo apoptosis and exfoliate to the lumen; additionally, some cells within the crypts, such as damaged cells or stem cells at the bottom of the crypt, also undergo apoptosis (10).

The earliest phases of colorectal carcinogenesis begin in the normal colonic mucosa with a disorder of cell replication and renewal and are followed by the subsequent appearance of aberrant crypt foci, clusters of enlarged crypts possessing phenotypic and molecular alterations (11, 12).

Apoptosis, which is tightly controlled by various proteins, helps to establish a natural balance between cell death and cell renewal by destroying excess, damaged cells. Apoptosis is characterized by cytoplasmic fragmentation and nuclear condensation (13) and may be initiated through mitochondrial (i.e., intrinsic) or death-receptor (i.e., extrinsic) pathways. The key regulators of apoptosis are the Bcl-2 family proteins (e.g., the pro-apoptotic proteins Bak and Bax, and the anti-apoptotic proteins Bcl-2 and Bcl-xl)(14).

Bax is responsible for the mitochondrial damage that leads to caspase activation (15-17) and its absence can render cells highly resistant to apoptosis triggered by a number of agents including radiation, chemoprevention, and growth factor deprivation (18, 19). As such, tumor cells may acquire resistance to apoptosis by over-expression of anti-apoptotic proteins or by down-regulation or mutation of pro-apoptotic proteins. Bcl-2, which is located on the cytoplasmic face of the mitochondrial outer membrane, the nuclear envelope,

and the endoplasmic reticulum, inhibits the activation of Bax following a death signal by preventing the release of cytochrome c from the mitochondria (20, 21).

The apoptosis signaling systems have been known to provide promising targets for the development of novel anticancer agents (22). There is substantial evidence that markers of apoptosis, including Bax and Bcl-2 expression, represent viable candidates for modifiable biomarkers of risk for colorectal neoplasms. Previous studies have shown Bcl-2 protein expression in various premalignant lesions, implying that abnormal expression of Bcl-2 may lead to the accumulation of long-living cells, which ultimately result in tumor development (23, 24). Thus, measurement of both Bax and Bcl-2 in colon crypts provides a good indicator of apoptosis.

The regulation of telomerase activity in human cells also plays a significant role in the development of cancer. Human telomeres undergo progressive shortening with each cell division, and the critical shortening of telomeres associated with cellular aging triggers a signal for cells to stop dividing. Telomerase, a reverse transcriptase enzyme that adds telomeric-repeated sequences to the ends of human chromosomes, prevents progressive shortening of telomeres with cell division, most likely giving tumor cells a growth advantage over normal cells (25). Telomerase is repressed in most somatic cells, except proliferating progenitor cells and activated lymphocytes, and is found in approximately 90% of malignant tumors (26). Deregulation of telomerase expression in somatic cells may be involved in carcinogenesis by maintaining telomere length and subsequently preventing cancer cells from replicative senescence (27). hTERT, the catalytic subunit of telomerase, is expressed only in proliferative cells of colon crypts and its expression in primitive colon crypt cells is more reflective of long-term proliferative activity than "snapshot" proliferative indicators, such as

the S-phase markers PCNA and Ki-67. Most cancer cells express hTERT and are telomerase positive, making it a useful cancer-cell detecting marker.

The Ki-67 protein (also known as MKI67) is a cellular marker for proliferation. During interphase, the Ki-67 antigen can be exclusively detected within the cell nucleus, whereas in mitosis, most of the protein is relocated to the surface of the chromosomes. MIB-1/Ki-67 is found throughout the cell cycle (G1, S, G2, and M phases), but not in resting (G0) cells. The Ki-67 antigen is considered to be a good marker of the mitotic index and the fraction of dividing cells (28, 29). In normal crypts, MIB-1/Ki-67 positivity is only seen in the lower one-third of the crypt. However, dysplastic crypts show a reversed pattern in which the upper third of the crypt and mucosal surface are stained (30).

Aberrant proliferative indexes precede and accompany colorectal tumorigenesis progression in rodent models and are thought to be a consequence of exposure to carcinogenic agents (31). Animal experimental evidence and preliminary evidence in humans suggest that proliferation abnormalities, which include hyperproliferation and an upward shift in the proliferation zone of normal colonic mucosa, are reversible biomarkers that may act as precursors for colorectal tumorigenesis (32-34). As such, cell proliferation may be considered as a valid intermediate end-point for colorectal cancer. We hypothesized that hTERT expression in colorectal crypts better reflects average, long-term proliferative activity than do "snapshot" proliferative indicators, such as the S-phase markers PCNA (proliferating cell nuclear antigen) and MIB-1, which demonstrate rapid, large responses to short-term physiologic stimuli.

Inhibition of cell cycle progression is another important strategy for controlling cancer cell growth as derangements of the cell cycle can cause hyperproliferation and contribute to the malignant phenotype of tumor cells. The cell cycle consists of four main

stages: G1, the phase in which the cell prepares for DNA synthesis; S, the synthesis phase in which DNA is replicated; G2, the phase in which the cell prepares to divide and M, the mitotic (dividing) phase (Figure 1). Molecular control of cellular proliferation is regulated by three main groups of proteins: cyclin-dependent kinases (CDKs), cyclins and CDK inhibitors. Cell cycle progression is supported by the interaction between cyclin-dependent kinases (CDK) and cyclin. Alternatively, cyclin kinase inhibitors (CKIs) halt cell cycle progression and inhibit the activity of CDK complexes. In response to signals indicating DNA damage, the Cip/Kip family of Cdk inhibitors (including p21 and p27) bind to a broad range of Cdk-cyclin complexes, subsequently shutting off the cell cycle at multiple points.

p21 is a cyclin-dependent kinase inhibitor that plays an important role in cell cycle regulation by integrating genotoxic insults into growth arrest and apoptotic signaling pathways which ultimately determine cell fate (35). The activation of p21 blocks G1-S transition by inhibiting Cdk2-Cyclin E/A, leading to the arrest of the cell cycle. p21 is one of the major transcription targets of p53 and is subsequently responsible for p53-dependent cell cycle arrest (36-38). Furthermore, p21 is a potent inducer of differentiation in intestinal colonocytes, and its expression is known to be down-regulated during the early stages of colon tumorigenesis (39). Abnormalities in p21 expression has been linked to carcinogenesis (40) and p21 loss is observed in 79% of colon cancer tumors (41, 42). As such, p21 is a viable marker of differentiation and its expression may be considered an intermediate biomarker of risk for colorectal cancer.

Ginger and CRC

Dietary patterns may account for the larges differences seen in CRC rates globally and epidemiologic studies suggest that fruit and vegetable consumption is inversely associated with colorectal cancer risk (43, 44). This inverse association may be attributed to

biologically active secondary plant metabolites, which include phenolic compounds and carotenoids (45).

Ginger root (*Zingiber officinale*), a rhizomatous perennial plant, is the most commonly consumed spice in India and is used in traditional oriental medicine to treat gastrointestinal ailments, such nausea, dyspepsia and vomiting, arthritis, cardiovascular disease, and metabolic disease (46, 47). Ginger consists of both volatile oil components and non-volatile pungent phenol components. The pungent group, which includes gingerols, paradols, zingerone, and shogaols, are presumed to be responsible for ginger's putative therapeutic effects (Figure 2).

There is strong biological plausibility, as well as animal experimental evidence for protection against colorectal cancer by ginger supplementation, which has antioxidant, anti-inflammatory, anti-angiogenesis, and anti-atherosclerotic properties. The chemopreventive properties of ginger and its constituents have been demonstrated in both *in vitro* and animal studies. For example, [6]-gingerol, a constituent of ginger root, inhibited colorectal carcinogenesis *in vitro* by targeting leukotrine A4 hydrolase (48), and by increasing apoptosis through up-regulation of NAG-1 and down-regulation of cyclin D1 (49). Several additional *in vitro* and animal studies found that ginger potentiates apoptotic indices in different human cell lines through various signaling pathways (50-54).

In vitro and animal studies also suggest that ginger and its constituents may act as chemopreventive agents by reducing COX-2 expression (55-57), increasing immune function (58, 59), lowering the activity of microbial enzymes (beta-glucuronidase and mucinase) (46), and blocking angiogenic signals that supply blood to tumor cells (60, 61). More recently, a randomized controlled trial found that ginger supplementation decreased prostaglandin E_2 in the colonic mucosa of individuals at normal risk for CRC (62).

Despite the wealth of basic science studies demonstrating mechanistic support for ginger as a potential chemopreventive agent against CRC, several rodent studies found that ginger extract did not inhibit carcinogen-induced mouse bladder or colorectal tumorigenesis (63, 64).

Evidence suggests that ginger and its constituents may act as chemopreventive agents by inducing apoptosis, modulating signals in cells that cause growth and division, and providing anti-oxidant and anti-inflammatory protection. However, the exact mechanisms whereby ginger may modulate colorectal cancer risk remain unclear. While research efforts have focused on the effects of phytochemicals on signaling cascades associated with induction of cancer cell death and inhibition of cancer cell proliferation, the specific molecular and cellular targets need to be identified. Identification of molecular and cellular targets associated with the suppression of cell malignancy will provide a better understanding of the anticancer mechanisms of phytochemicals and may also be useful for determining the efficacy of other chemopreventive agents. However, there are no *in vivo* human studies measuring the effect of ginger on apoptotic, proliferative, and differentiation markers in the normal colon mucosa.

The purpose of this study was to estimate the effect of 2.0 g of ginger supplementation on markers of apoptosis, differentiation, and proliferation in the normal-appearing colonic mucosa of people at increased risk for developing colorectal cancer in a pilot, randomized, double-blinded, placebo-controlled, clinical trial.

INTRODUCTION

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in the United States. Estimates predict that 141,210 new cases of colorectal cancer will develop in

2011, resulting in 49,380 deaths (1). Reduced differentiation and deregulated cell cycle control provide the underlying platform for colon tumorigenesis; therefore, markers of cell cycle function, proliferation, and differentiation in the colorectal epithelium may serve as intermediate phenotypic biomarkers of risk for colorectal cancer and may be modifiable by dietary components.

International and migration studies point to the predominance of lifestyle and diet in colorectal cancer etiology, with roughly 70 percent of CRC being attributable to environmental factors (4, 5). The long latency period in CRC cancer provides a lengthy window of opportunity for intervention by chemopreventive agents. Furthermore, chemoprevention is an attractive option for reducing CRC risk, given that diet plays an important role in colorectal carcinogenesis.

Epidemiologic studies suggest that diets rich in fruit and vegetable intake and lower in red and processed meats are associated with lower risk of several malignancies, including colorectal cancer (65). Dietary constituents, including plant-derived phenolic compounds, may act as chemopreventive agents by affecting molecular events in the initiation, promotion and progression stages of carcinogenesis (66).

Ginger root (*Zingiber officinale*) and its main phenolic constituents (gingerols, paradols, zingerone, and shogaols) (Figure 2) have anti-oxidant (67-72), anti-inflammatory (56, 73-75), and anti-carcinogenic properties (47, 76-79). Ginger root can interfere with several cell signaling pathways that are important in the early development of cancer (76).

For example, ginger potentiated apoptotic indexes in different human cell lines *in vitro* through various signaling pathways (50-53). Additionally, several lines of evidence suggest that [6]-gingerol is effective in suppressing the transformation, hyperproliferation, and inflammatory processes that initiate and promote tumorigenesis (55, 60, 80, 81). Despite

its anticancer activity *in vitro*, the exact molecular mechanisms by which ginger exerts its chemopreventive effects are not fully understood. Furthermore, there are no reported human *in vivo* investigations of the effects of ginger on apoptotic, proliferative, and differentiation markers in the normal colonic mucosa.

The purpose of this study was to estimate the effect of 2.0 g of ginger supplementation on a marker of cell differentiation (p21waf1/cip1), two markers of apoptosis (Bax and Bcl-2), and two markers of cell proliferation (MIB-1/Ki-67 and hTERT) in the normal-appearing colonic mucosa of people at increased risk for developing colorectal cancer in a pilot, randomized, double-blinded, placebo-controlled, clinical trial. We used telomerase expression, as indicated by hTERT in colon crypt cells, as a marker of long-term proliferative activity, and the S-phase marker MIB-1 as a "snapshot," or short-term proliferative indicator. We hypothesized that ginger supplementation would increase differentiation, decrease proliferation, and increase apoptosis within the normal-appearing colorectal epithelium of people at increased risk for developing colorectal cancer.

METHODS

Participants

Participant recruitment and flow is depicted in Figure 3. Participants were recruited from the surrounding community of Ann Arbor, MI through fliers posted around the University of Michigan, advertisements in local newspapers, and word-of-mouth between June 2009 and January 2010. Eligible participants were healthy male and female volunteers 18 years and older who were considered to be at increased risk for colorectal cancer. Increased CRC risk was defined as an individual who either had a first degree relative with colorectal cancer under the age of 60 at diagnosis or who had a previous adenomatous polyp

or early (Dukes A, B or C) colon cancer resected. With the exception of curative surgery for small lesions, such as endoscopically removed cancers, eligible subjects were at least one year post-treatment for cancer. Exclusion criteria included lactose intolerance, a current diagnosis of peptic ulcer disease, gastrointestinal bleeding from gastric or duodenal ulcers, gastrin secreting tumors, a known allergy to ginger, supplements use/therapies that could obscure the ability to detect anti-inflammatory effects, and pregnant or lactating women. Additionally, individuals with hereditary non-polyposis colon cancer or familial adenomatous polyposis (HNPCC/FAP), inflammatory bowel disease, or coagulopathy/hereditary hemorrhagic disorders were excluded. Over the 6-month recruitment period, 42 people were assessed for eligibility, of whom 21 (50%) met all eligibility criteria and were randomized (11 to placebo and 10 to 2.0 g ginger).

Participants were asked to avoid all foods containing ginger within the 14 days prior to drug administration. This was confirmed by having participants complete a food checklist to verify that they were not consuming any ginger-rich foods such, as ginger ale, or Japanese food. All of the participants were reimbursed for their time.

The study was approved by the University of Michigan Institutional Review Board. All study procedures were administered at the University of Michigan Clinical Research Unit (MCRU) after the participant gave written, informed consent.

Ginger Intervention

The ginger product used in this study was manufactured by Pure Encapsulations. Pure Encapsulation's ginger (*Zingiber officinale* radix) powder was processed using Good Manufacturing Procedures (GMP). Each capsule contained 250 mg dry extract of ginger root [10:1 (v/v) extraction solvent (ethanol 50%): root] standardized to 5%-gingerols. On the basis of high performance liquid chromatography (HPLC) analysis, a 250 mg capsule of

ginger extract (from both batches) contained 5.38 mg (2.15%) 6-gingerol, 1.80 mg (0.72%) 8-gingerol, 4.19 mg (1.78%) 10-gingerol, and 0.92 mg (0.37%) 6-shogaol. Gingerol and shogaol content was verified by an independent laboratory using appropriate HPLC techniques (Integrated Biomolecule Corporation).

The study was conducted using two batches (ZO/06006 and ZO/07006) of ginger powder extract, both of which were tested for gingerol and shogaol content. The 2.0 g dose used in the study was chosen based on the highest tolerated amount of ginger extract in a phase I dose escalation study in healthy volunteers (82). Placebo consisted of lactose powder. Ginger powder and lactose were placed into identical opaque red capsules. Placebo and ginger capsules were assembled, stored, and dispensed by the Investigational Drug Service of the University of Michigan (U of M IDS). The participants were instructed to take four 250 mg capsules twice daily with food and to bring any unused capsules to the final (28 day) study visit.

Randomization, allocation, and blinding

Eligible participants were randomized to one of two groups: placebo or ginger extract (2.0 g). The randomization code was computer-generated by the study biostatistician. The randomization code was kept by the University of Michigan (U of M IDS), which assigned the next available randomization number as the study team informed them of eligible participants. Study participants and all study personnel who assessed outcomes, worked with study data, or administered tests or questionnaires were unaware of the randomization list or treatment assignment.

Adherence and assessment of blinding

Participants were assessed for adherence by a research coordinator through weekly telephone calls, self-report, and pill counts at the end of the study. Adherence was defined as

taking the capsules within four hours of the agreed upon time, twice daily. Participants were classified as adherent if the weekly monitoring suggested that 70% or more of the doses were taken as prescribed. Blinding was assessed by asking the participants during their final visit which treatment they believed they received ("ginger," "placebo," or "do not know").

Toxicity assessment

Participants were assessed for toxicity by direct questioning in person, by email, or by telephone at weekly intervals. The National Cancer Institute (NCI) Common Toxicity Scale V 3.0 (Regulatory Affairs Branch, Cancer Therapy Evaluation program, Division of Cancer Treatment, Diagnosis, and Centers, NCI; ref. 29) was used to quantify toxicity.

Flexible sigmoidoscopy and tissue collection

Participants underwent two flexible sigmoidoscopies, one at baseline and the second within 24 hours of the last ginger/placebo dose on day 28. Participants did not have to be fasting for their visits and did not take a bowel cleansing preparation or enema. Participants were, however, asked to evacuate their bowel as close to the procedure as possible. Participants were placed in a left lateral decubitus position and a flexible sigmoidoscope was passed at least 15 cm from the anal sphincter. Four tissue samples were taken by opening and pressing the biopsy forceps perpendicular to the mucosal surface with mild pressure. Each biopsy specimen was taken approximately 2 cm or more from other biopsy sites in distal sigmoid colonic mucosa that had no visual appearance of trauma or recent biopsy.

Tissue handling and disposition

The biopsies were immediately placed in phosphate buffered saline, reoriented under a dissecting microscope, and transferred to 10% normal buffered formalin followed by transfer to 70% ethanol 24 hours after initial placement in formalin. The paraffin blocks, each containing four biopsies, were then cut into 3-μm-thick sections, with each level 40 μm

apart. Five slides with three biopsy levels each were processed and stained within 7 days of being cut, yielding a total of 15 biopsy levels per patient.

Immunohistochemistry protocol

Slides were immunohistochemically processed using a labeled streptavidin-biotin method for Bax, Bcl-2, p21, hTERT, and MIB-1 as summarized in Figure 4. Slides were not counterstained. After staining, the slides were coverslipped with a Leica CV5000 Coverslipper (Leica Microsystems, Inc., IL). Baseline and follow-up biopsy slides were included in same staining batch. Additionally, each staining batch included both positive and negative control slides.

A quantitative image analysis method (scoring) was used to evaluate the expression of the biomarkers in the colon crypts, as described previously (83). The basic scoring method (Figure 5) used to describe and quantify various characteristics of the labeled antigens in the colon crypts was an image analysis scoring procedure for antigens that were labeled with a wide range of intensities in gradient distributions along the crypt axis. The unit of analysis was the "hemicrypt," defined as one half of a crypt bisected from crypt base to colon lumen. A "scorable" hemicrypt was defined as an intact hemicrypt that extended from the muscularis mucosa to the colon lumen. Hemicrypts were manually traced by a trained technician and divided by the software into a number of segments corresponding in width to an average normal crypt epithelial cell. Overall hemicrypt- and segment-specific optical signal densities were then calculated by the software and stored into a Microsoft Access database along with various dimensional parameters of the hemicrypt. All images were obtained at 200× magnification and stored as 16-bit grayscale 1,600 × 1,200 pixel images.

The goal was to analyze a minimum of 16 hemicrypts on each of two biopsies, for a total of 32 hemicrypts. Blinded subsets of previously analyzed slides were resubmitted to the

technician during the study to assess intra-reader reliability, which was found to range from 0.92 to 0.98 throughout.

Statistical methods and sample size

Balance between treatment groups on baseline characteristics was tested using independent sample t-tests for continuous variables and Pearson's chi square and Fisher's exact tests, as appropriate, for categorical variables. Slide "scoring" reliability was analyzed using intra-class correlation coefficients.

The mean optical density of Bax, Bcl-2, MIB-1, hTERT, and p21 labeling in normal colon crypts was calculated for each patient at baseline and 28 day follow-up by summing all the densities from all analyzed crypts from the biopsy specimens and dividing by the number of crypts analyzed (this measure indicates the overall rate of proliferation, apoptosis, or differentiation of colorectal crypt epithelial cells). The crypt differentiation compartment was defined *a priori* as the upper 40% of the crypts, and the crypt proliferation compartment was defined as the bottom 60% of the crypts. Measures of the within-crypt distributions of the biomarkers were calculated for each patient by taking the mean of the biomarker densities in the upper 40% of crypts and dividing it by the biomarker densities in the entire crypt (Φ_h).

Primary analyses were based on assigned treatment at the time of randomization, regardless of adherence status (intent-to-treat analysis). Treatment effects were evaluated by assessing the differences in biomarker concentration from baseline to the 28 day follow-up visit between the ginger and placebo group by a repeated-measures linear mixed effects model, as implemented using a Proc MIXED effects model. The model included the intercept, indicators for treatment group and visit (baseline and follow-up), and a treatment by visit interaction term. Since optical density is measured in arbitrary units, to provide

perspective on the magnitude of the treatment effects we also calculated relative effects, defined as: [treatment group follow-up mean/treatment group baseline mean]/[placebo follow-up mean/placebo baseline mean]. The relative effect provides a conservative estimate of the proportional change in the treatment group relative to that in the placebo group, and its interpretation is somewhat analogous to that of an odds ratio (e.g., a relative effect of 2.0 would mean that the proportional change in the treatment group was two times that in the placebo group). Since the treatment groups were balanced on risk factors at baseline, no adjustment was made for other covariates in the primary intent-to-treat analyses.

To assess the effects of ginger supplementation on cellular functioning, two cell cycle summary scores were created; summary scores included Bax, Bcl-2, p21 and one marker of proliferation (either hTERT or MIB-1). Scores for each biomarker were based on the percent change in biomarker expression over the treatment period ([participant biomarker at follow-up – participant biomarker at baseline]/participant biomarker at baseline). Percent changes were divided into seven equal interval categories, which were determined *a priori*, (≤-75%, -75% to -45.1%, -45% to -15.1%, -15% to 15%, 15.1% to 45%, 45.1% to 75% and ≥75%) and corresponded to a score ranging from -3 to 3. The combined cell cycle score was created for each participant by summing the interval scores of each individual biomarker (Bcl-2, hTERT and MIB-1 were included with a negative sign as increases in these biomarkers are generally thought to be associated with greater risk for tumorigenesis). As such, positive scores reflect higher levels of apoptosis and/or differentiation relative to proliferation while negative scores reflect the opposite balance.

Statistical analyses were performed using SAS 9.2 statistical software (SAS Institute Inc.). A P value ≤ 0.05 (two-sided) was considered statistically significant. In addition to analyzing cell cycle scores and overall mean changes in biomarker expression, ratios

comparing changes within and between each of the biomarkers were tested. Given that a total of 42 tests were conducted, at least two would be expected to be significant by chance alone at the 0.05 level of significance.

RESULTS

Characteristics of study participants

Treatment groups did not differ significantly on characteristics measured at baseline (Table 1). The mean age of participants was 51 years, 35% were male, 75% were white, and 50% had a first degree relative with colorectal cancer under the age 60 at the time of diagnosis. One participant withdrew from the study after randomization as they were found to not to be at increased risk for CRC. Nine (90%) participants in the ginger group reported an adverse event over the course of the study compared to four participants in the placebo group ($p = 0.06$; Table 1a). No toxicities greater than NCI Common Toxicity Criteria (v. 3.0) Grade I were reported. GI symptoms, which included bloating, gas, nausea, and heartburn, were the most commonly reported adverse event, occurring in 70% of individuals in the ginger group and 30% of individuals in the placebo group ($p = 0.18$).

Over the four week trial period, participants in the ginger and placebo groups respectively took 98% and 95% of the pills administered at trial onset.

Effects of ginger on the separate and relative expressions of Bax, Bcl-2, p21, hTERT and MIB-1 in normal colorectal crypts

At baseline, there were no statistically significant differences between treatment groups in Bax, Bcl-2, p21, hTERT or MIB-1 levels (Table 2). There were also no statistically significant changes in the overall crypt expression of Bax or Bcl-2 after four weeks of treatment, although there was a suggestion that the mean biomarker levels decreased slightly.

Bax expression in the ginger group decreased by 15.6% ($p = 0.78$) along the full length of the crypts, and increased by 20% ($p = 0.13$) in the Φ_h of crypts, relative to the placebo group. There were no statistically significant treatment effects on the expression of Bcl-2 in the crypts overall or in the Φ_h of crypts.

After four weeks of treatment, the Bax/Bcl-2 ratio decreased 26.6% ($p = 0.62$) along the entire length of the crypts and increased 16.7% ($p = 0.58$) in the Φ_h of crypts, relative to the placebo group.

p21 expression in the ginger group decreased by 18.2% ($p = 0.43$) along the full length of the crypts, 16.9% ($p = 0.45$) in the upper 40% of crypts, and 53.2% ($p = 0.73$) in the lower 60% of crypts, relative to the placebo group; the p21 labeling index Φ_h increased by 5.7% ($p = 0.33$) in the ginger group relative to the placebo group.

The estimated relative treatment effects on MIB-1 expression along the entire crypt, the upper 40% of crypts, and the lower 60% of crypts were decreases of 16.9% ($p = 0.39$), 46.8% ($p = 0.39$), and 15.3% ($p = 0.41$), respectively. Additionally, MIB-1 expression in the Φ_h of crypts decreased by 35.5% ($p = 0.60$) in the ginger group relative to the placebo group.

In the ginger group, hTERT expression along the full length of crypts statistically significantly decreased by 41.2% ($p = 0.05$), relative to the placebo group; the treatment effect on hTERT expression was slightly more pronounced in the canonical differentiation zone of crypts (-47.9%; $p = 0.04$).

Following four weeks of treatment, the p21/hTERT ratio increased 34.6% ($p = 0.34$) along the entire length of the crypt, 39% ($p = 0.32$) in the upper 40% of crypts, and 14.2% ($p = 0.18$) in the Φ_h of crypts. In the ginger group, the p21/MIB-1 ratio decreased by 23.3% ($p = 0.69$) along the entire length of the crypts relative to the placebo group. The

treatment effect was more pronounced in the upper 40% of crypts (73.6%; p = 0.55). Furthermore, the p21/MIB-1 ratio in the Φ_h of crypts increased 192% (p = 0.38) in the ginger group relative to the placebo group. In the ginger group, the Bax/hTERT ratio increased 82% (p = 0.22) along the entire length of the crypts and 25.6% (p = 0.05) in the Φ_h of crypts, relative to the placebo group. After four weeks of treatment, the Bax/MIB-1 ratio remained relatively unchanged along the entire length of the crypts (-4.9%; p = 0.96), while increasing 127% (p = 0.48) in the upper 40% of crypts and 168% (p = 0.37) in the Φ_h of crypts, relative to the placebo group.

Effects of ginger on the cell cycle score

The effects of ginger supplementation on the "cell cycle scores" are summarized in Table 3. On one hand, there was relatively no treatment effect on the cell cycle score that included MIB-1 as the proliferation marker. On the other hand, the estimated treatment effect on the cell cycle score that included the long-term proliferation hTERT was a 74% increase in the cell cycle score in the ginger group relative to the placebo group ([-0.33 vs. -1.30]; p=0.35).

DISCUSSION

Our results suggest that ginger supplementation may reduce proliferation in the crypts of the normal-appearing colorectal epithelium and increase apoptosis and differentiation relative to proliferation—especially in the proliferative zone of crypts of individuals at increased risk for CRC. These findings are consistent with previous studies which have suggested that the chemopreventive properties of ginger may lie in its ability to regulate cell function and viability.

In vitro and animal studies also suggest that ginger and its constituents may act as chemopreventive agents by reducing COX-2 expression (55-57), increasing immune function (58, 59), lowering the activity of microbial enzymes (beta-glucuronidase and mucinase) (46), and blocking angiogenic signals that supply blood to tumor cells (60, 61).

In the present study, expression of proliferation markers decreased in response to ginger supplementation, a finding that is consistent with those from animal models (84, 85) and *in vitro* (61, 86-89) studies. Our findings also indicate that the strongest treatment effect was in hTERT expression, which is consistent with previous reports that found that ginger inhibited hTERT and c-Myc expression in human non-small lung cancer cells (90). While the estimated treatment effect was not as strong on MIB-1, the effect was more pronounced in the upper sections of the colorectal crypts, suggesting that ginger decreases proliferation in the parts of the colorectal crypts most exposed to bowel lumen carcinogens.

Although we hypothesized that ginger supplementation would increase apoptosis and differentiation, results from the study indicated that ginger reduced Bax and p21 expression. Our findings are not consistent with previous *in vitro* (49-53) and animal studies (48, 91), although similar null results were reported in several rodent models (63, 64) and *in vitro* studies of HT-29 and Caco-2 colon tumor cell lines (49, 92). A possible explanation for this finding is that the biomarkers used may not have been the best measures of cellular differentiation and apoptosis in normal colorectal crypts. However, p21 is a potent inducer of differentiation in intestinal colonocytes, and its expression is known to be down-regulated during the early stages of colon tumorigenesis (39). Abnormalities in p21 expression has been linked to carcinogenesis (40) and p21 loss is observed in 79% of colon cancer tumors (41, 42). As such, p21 is a viable marker of differentiation and its expression may be considered an intermediate biomarker of risk for colorectal cancer. Additionally, there is

substantial evidence that markers of apoptosis, including Bax and Bcl-2 expression, are plausible candidates for treatable biomarkers of risk for colorectal neoplasms.

Failure to delete cells with accumulated genetic and epigenetic changes via apoptosis is an important step in colon carcinogenesis that may lead to adenoma development subsequent neoplasia. Pro- or anti-apoptotic tendencies in the normal colon mucosa are reflected by the expression of Bax and Bcl-2 proteins, respectively. Previous studies have shown Bcl-2 protein expression in various premalignant lesions, implying that abnormal expression of Bcl-2 may lead to the accumulation of long-living cells, and subsequent tumor development (23, 24). Thus, measuring both of these proteins in colon crypts most likely provides a good indicator of apoptosis.

Furthermore, even if cell differentiation and apoptotic markers are valid intermediate biomarkers of risk for colorectal cancer, interpretation of both marker expression and influence of ginger supplementation on marker expression may differ based upon stage of carcinogenesis progression. Chemopreventive agents whose influences are confined to a specific stage of colorectal tumorigenesis could be missed using the current study population. For example, ginger may influence p21 expression in individuals with a family history of CRC (and those without prior adenomas or CRC), but this finding would be obscured in the current study population, which also includes individuals with a previously resected adenomatous polyp or CRC. Additionally, the relatively short length of the study may not have been long enough for ginger to produce any measurable changes within the colonic mucosa.

There are several additional possible explanations for these finding, including chance—especially considering the small sample size—and non-transferability of *in vitro* and animal model results to human models; all previous studies were either *in vitro* or animal

experimental studies and the chemopreventive effects of ginger observed in these studies may not necessarily translate to decreased CRC risk in humans. Additionally, previous studies focused on the effect of ginger on tumor cells, rather than normal cells. As such, ginger's anti-carcinogenic effects may be confined to active cancer cell lines or may be expressed differently in normal human cells.

While both the Bax/Bcl-2 ratio and p21 expression decreased in the treatment group, relative to the placebo group, our results suggest that ginger "normalized" Bax and p21 expression in the luminal zone. Animal studies and preliminary evidence in humans suggest that an upward shift in the proliferative zone of normal colonic mucosa is a precursor for colon neoplasia (33, 93). Furthermore, patients with a history of sporadic adenoma (94-101) and those with a family history of colorectal cancer (99, 102, 103) were found to have an increased proliferation rate and an extension of the proliferation zone to include the luminal zone. Thus, "normalization" of the luminal compartment of the crypt may be an integral mechanism by which ginger suppresses the initiation of colorectal carcinogenesis in individuals at increased risk for CRC.

Moreover, given that apoptosis and proliferation occur through tightly regulated processes, decreased apoptosis may simply be a reflection of decreased proliferation. Our results indicated a positive correlation between Bax and both hTERT and MIB-1. The positive correlation between apoptosis and proliferation indices suggests that apoptosis may reflect not only cell death, but also proliferation activity. This finding, which is in agreement with previous studies using rodent models (104, 105), suggests that a link exists between the two pathways and may, in part, be explained by cell cycle sequence: apoptosis primarily occurs in the late G1 and G2 phases, but not in the G0 and M phases (106).

This trial emphasizes the importance of randomization in cancer chemoprevention trials. In the placebo group, we observed a decrease in Bax expression and an increase in both hTERT and MIB-1 expression after 4 weeks of follow-up. The causes for the time-related influences producing these decreases in the placebo group are unknown; however, they may have been due to: underlying biological processes in individuals at increased risk for CRC, participants developing recurrent polyps during the study period, laboratory drift, and/or chance. In clinical trials, such extraneous temporal influences are presumed to occur equally across all treatment groups; therefore, change in the placebo group is "subtracted" from any change in an active treatment group to yield the true treatment effect. Thus, in this trial, without a placebo-control group, the treatment effect on apoptotic markers would have been overestimated while treatment effect on markers of proliferation would have been underestimated.

Additionally, this trial highlights the importance of studying all major cell cycle functions when assessing the impact of certain chemopreventive agents. The processes of apoptosis, differentiation, and proliferation are intricately correlated; research focusing strictly on one or two phenotypic cell markers will most likely not provide enough information to accurately describe the status of key systems within colonocytes whose malfunction can lead to the development of colorectal cancer.

This study has several limitations and strengths. Proliferation, differentiation, and apoptosis markers are not proven biomarkers of risk for colon cancer; however, substantial basic science literature supports an important role for cell cycle functioning in colon carcinogenesis.

Given that the endpoints investigated were intermediate markers of risk, this study cannot prove that ginger-mediated reductions in proliferation will translate to actual

reductions in colon cancer risk. The study design also provides neither estimates of the rapidity of a response to ginger nor a determination of whether an effect would be apparent with prolonged use. Furthermore, given the small sample size of the pilot, randomized, controlled trial, the study had limited statistical power; thus, our findings may have been due to chance. Also, the small size did not allow us to conduct additional subgroup analyses by risk type (e.g., family history, previous adenoma, previous CRC). On the other hand, this study, to the best of our knowledge, is the first randomized, double-blinded, placebo-controlled trial to have assessed the effects of ginger on apoptosis, proliferation, and differentiation markers in the normal-appearing colorectal epithelium, there was high protocol adherence by study participants, immunohistochemical staining was automated, and, via the use of novel quantitative image analysis procedures, biopsy analysis reliability was high.

In summary, these preliminary results 1) suggest that ginger may reduce proliferation in the crypts of normal-appearing colorectal epithelium and increase apoptosis and differentiation relative to proliferation–especially in the proliferative zone of crypts, and 2) support a full-scale clinical trial to further investigate these results.

TABLES AND FIGURES

Table 1. Selected baseline characteristics of participants

Characteristics	Ginger n=10	Placebo n=10	P[†]
Sex, No. (%)			
Men	4 (40)	3 (30)	0.64
Women	6 (60)	7 (70)	
Race, No. (%)			
White	8 (80)	7 (70)	0.38
Other*	2 (20)	3 (30)	
Age, mean (SD), years	51.1 (11.7)	50.8 (14.6)	0.95
Reason for being high risk for CRC[‡], No. (%)			
First degree relative	4 (40)	6 (60)	0.47
Previous adenoma	6 (60)	6 (60)	
Previous CRC	1 (10)	0 (0)	

*African American, Asian, Pacific Islander, American Indian/Alaskan Native

[†] Independent sample t-test or Pearson's Chi-square, as appropriate

[‡] CRC=Colorectal cancer; 1st degree relative must have had a diagnosis of colorectal cancer before the age of 60; Prior colorectal cancer must have been fully excised and either Duke's A or B; Values add up to >100% due to participants having several reasons for being at high risk for colorectal cancer

Table 1a. Adverse Events (AE) Reported by Participants

AE	Ginger n=10	Placebo n=10	P[†]
Participants reporting any AE, No. (%)	9 (90)	4 (40)	0.06
GI[‡]	7 (70)	3 (30)	0.18
Other	3 (30)	1 (10)	0.58

[†]: Chi-Sqaure or Fisher's Exact Test, as appropriate

[‡] Symptoms include: bloating, urgency, gas, nausea, heartburn, sores in mouth & anorexia

[††]: Other includes: allergic reaction, nose bleed, skin rash

Table 2. Changes in biomarkers of apoptosis, proliferation and differentiation in colorectal crypts

Biomarker	n	Baseline		4-week Follow-up		Absolute Treatment Effect†			Relative Treatment Effect‡
		Mean	SD	Mean	SD	Mean	SD	P*	
Bax									
Placebo	10	658	333	520	391	N/A	N/A	N/A	N/A
Luminal		240	129	187	158	N/A	N/A	N/A	N/A
Proliferative		391	211	310	224	N/A	N/A	N/A	N/A
Ratio of Luminal/Total		0.37	0.07	0.35	0.05	N/A	N/A	N/A	N/A
Ginger	9	585	232	390	164	-56	194	0.78	**0.844**
Luminal		213	109	155	62	-5	79	0.95	**0.934**
Proliferative		348	138	216	106	-50	117	0.67	**0.783**
Ratio of Luminal/Total (Φh)		0.36	0.08	0.40	0.06	0.06	0.04	0.14	**1.190**
Bcl-2									
Placebo	10	539	278	501	255	N/A	N/A	N/A	N/A
Luminal		67	80	59	53	N/A	N/A	N/A	N/A
Proliferative		498	219	439	212	N/A	N/A	N/A	N/A
Ratio of Luminal/Total		0.11	0.06	0.11	0.05	N/A	N/A	N/A	N/A
Ginger	10	648	236	570	185	-40	152	0.80	**0.947**
Luminal		54	27	48	21	2	32	0.95	**1.013**
Proliferative		592	223	520	173	-43	131	0.75	**0.997**
Ratio of Luminal/Total (Φh)		0.09	0.03	0.09	0.03	0.50	0.03	0.87	**1.028**
p21									
Placebo	10	355	141	388	148	N/A	N/A	N/A	N/A
Luminal		318	117	360	146	N/A	N/A	N/A	N/A
Proliferative		37	50	28	22	N/A	N/A	N/A	N/A
Ratio of Luminal/Total (Φh)		0.91	0.10	0.93	0.06	N/A	N/A	N/A	N/A
Ginger	10	311	124	278	103	-66	82	0.43	**0.818**
Luminal		286	121	269	100	-59	77	0.45	**0.831**
Proliferative		25	30	9	6	-7	20	0.73	**0.468**
Ratio of Luminal/Total (Φh)		0.90	0.11	0.97	0.02	0.05	0.05	0.33	**1.057**
hTERT									
Placebo	10	2,054	769	2,715	1,114	N/A	N/A	N/A	N/A
Luminal		661	286	896	454	N/A	N/A	N/A	N/A
Proliferative		1,347	467	1,760	630	N/A	N/A	N/A	N/A
Ratio of Luminal/Total (Φh)		0.32	0.04	0.32	0.04	N/A	N/A	N/A	N/A
Ginger	10	2,651	1,084	2,059	683	-1,253	589	0.05	**0.588**
Luminal		911	405	643	208	-502	223	0.04	**0.521**
Proliferative		1,668	667	1,369	478	-713	359	0.06	**0.628**
Ratio of Luminal/Total (Φh)		0.34	0.04	0.32	0.06	-0.02	0.03	0.57	**0.951**
MIB-1									
Placebo	10	1,388	452	1,571	598	N/A	N/A	N/A	N/A
Luminal		45	51	72	92	N/A	N/A	N/A	N/A
Proliferative		1,343	420	1,498	514	N/A	N/A	N/A	N/A
Ratio of Luminal/Total (Φh)		0.03	0.03	0.04	0.03	N/A	N/A	N/A	N/A
Ginger	10	1,432	536	1,346	284	-268	305	0.39	**0.831**
Luminal		68	68	58	43	-37	42	0.39	**0.532**
Proliferative		1,363	496	1,287	263	-231	275	0.41	**0.847**
Ratio of Luminal/Total (Φh)		0.04	0.04	0.04	0.03	-0.01	0.02	0.60	**0.645**

† Absolute Treatment Effect is the absolute change from baseline to follow-up in the treatment group minus the absolute change from baseline to follow-up in the placebo group from mixed model

‡ Relative Treatment Effect is defined as: (treatment group follow-up/treatment group baseline)/(placebo follow-up/placebo baseline)

* P values for difference between each treatment group and placebo group from mixed model

Table 2. Changes in biomarkers of apoptosis, proliferation and differentiation in colorectal crypts (Cont'd)

Biomarker	n	Baseline		4-week Follow-up		Absolute Treatment Effect†			Relative Treatment Effect‡
		Mean	SD	Mean	SD	Mean	SD	P*	
Bax/Bcl-2 Ratio									
Placebo	9	1.40	0.74	1.38	1.19	N/A	N/A	N/A	N/A
Luminal		5.32	2.82	4.88	3.91	N/A	N/A	N/A	N/A
Proliferative		0.96	0.60	0.97	0.91	N/A	N/A	N/A	N/A
Ratio of Luminal/Total		4.16	2.01	3.93	1.74	N/A	N/A	N/A	N/A
Ginger	9	0.99	0.47	0.72	0.36	-0.26	0.51	0.62	**0.734**
Luminal		4.82	3.12	3.47	1.56	-0.92	1.95	0.64	**0.783**
Proliferative		0.64	0.27	0.44	0.25	-0.21	0.38	0.60	**0.685**
Ratio of Luminal/Total (Φh)		4.64	1.84	5.12	2.00	0.71	1.23	0.58	**1.167**
Bax/hTERT Ratio									
Placebo	9	0.37	0.25	0.22	0.19	N/A	N/A	N/A	N/A
Luminal		0.43	0.29	0.25	0.25	N/A	N/A	N/A	N/A
Proliferative		0.33	0.23	0.20	0.17	N/A	N/A	N/A	N/A
Ratio of Luminal/Total (Φh)		1.16	0.16	1.11	0.21	N/A	N/A	N/A	N/A
Ginger	9	0.23	0.08	0.25	0.25	0.17	0.13	0.22	**1.820**
Luminal		0.25	0.11	0.29	0.24	0.22	0.15	0.17	**1.997**
Proliferative		0.22	0.07	0.22	0.25	0.14	0.13	0.27	**1.734**
Ratio of Luminal/Total (Φh)		1.04	0.15	1.25	0.23	0.26	0.12	0.05	**1.256**
Bax/MIB-1 Ratio									
Placebo	9	0.48	0.23	0.32	0.20	N/A	N/A	N/A	N/A
Luminal		81.19	156.40	15.44	14.50	N/A	N/A	N/A	N/A
Proliferative		0.49	0.24	0.33	0.22	N/A	N/A	N/A	N/A
Ratio of Luminal/Total (Φh)		71.55	143.30	20.03	19.60	N/A	N/A	N/A	N/A
Ginger	9	0.46	0.24	0.29	0.11	-0.01	0.13	0.96	**0.961**
Luminal		42.25	66.75	18.26	29.01	41.76	57.31	0.48	**2.272**
Proliferative		0.49	0.26	0.31	0.12	-0.02	0.14	0.89	**0.937**
Ratio of Luminal/Total (Φh)		24.60	31.16	18.43	19.24	45.35	49.71	0.37	**2.676**
p21/hTERT Ratio									
Placebo	10	0.18	0.07	0.16	0.07	N/A	N/A	N/A	N/A
Luminal		0.53	0.21	0.47	0.23	N/A	N/A	N/A	N/A
Proliferative		0.03	0.05	0.02	0.02	N/A	N/A	N/A	N/A
Ratio of Luminal/Total (Φh)		2.92	0.54	2.92	0.32	N/A	N/A	N/A	N/A
Ginger	10	0.13	0.08	0.15	0.08	0.05	0.05	0.34	**1.346**
Luminal		0.37	0.26	0.45	0.18	0.14	0.14	0.32	**1.390**
Proliferative		0.02	0.01	0.01	0.00	0.00	0.02	0.86	**0.734**
Ratio of Luminal/Total (Φh)		2.69	0.36	3.07	0.50	0.38	0.28	0.18	**1.142**
p21/MIB-1 Ratio									
Placebo	10	0.28	0.12	0.26	0.11	N/A	N/A	N/A	N/A
Luminal		57.13	129.18	15.52	17.56	N/A	N/A	N/A	N/A
Proliferative		0.03	0.03	0.02	0.02	N/A	N/A	N/A	N/A
Ratio of Luminal/Total (Φh)		192.44	422.80	53.55	53.07	N/A	N/A	N/A	N/A
Ginger	10	0.25	0.16	0.21	0.21	-0.03	0.08	0.69	**0.867**
Luminal		26.76	51.35	12.62	13.91	27.46	44.53	0.55	**1.736**
Proliferative		0.02	0.02	0.01	0.00	0.00	0.01	0.80	**0.539**
Ratio of Luminal/Total (Φh)		73.76	89.08	59.90	65.26	125.05	139.20	0.38	**2.918**

† Absolute Treatment Effect is the absolute change from baseline to follow-up in the treatment group minus the absolute change from baseline to follow-up in the placebo group from mixed model

‡ Relative Treatment Effect is defined as: (treatment group follow-up/treatment group baseline)/(placebo follow-up/placebo baseline)

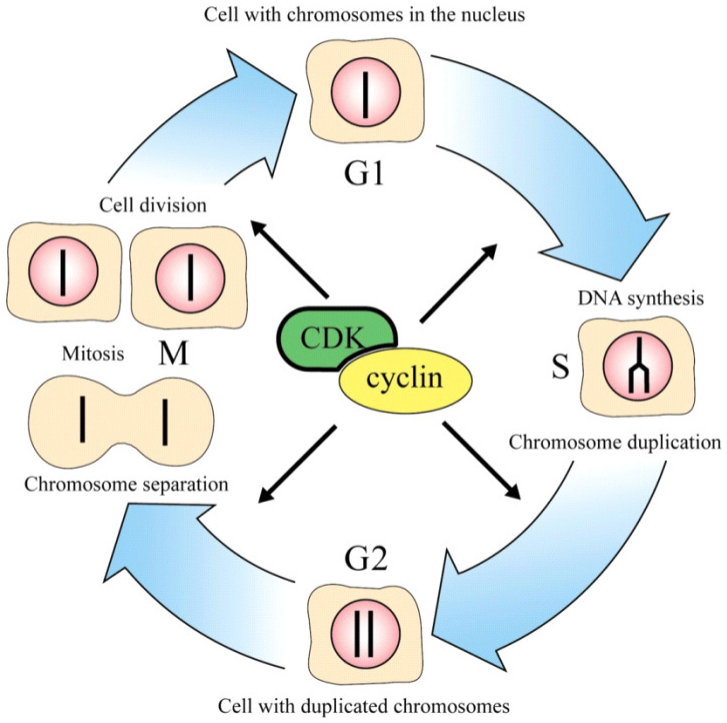
* P values for difference between each treatment group and placebo group from mixed model

Table 3. Changes in cell cycle score

	n	Treatment Mean	SD
Cell Cycle Score (w/ MIB-1)			
Placebo	9	-0.70	2.63
Ginger	9	-0.78	3.31
Cell Cycle Score (w/hTERT)			
Placebo	9	-1.30	2.87
Ginger	9	-0.33	1.22

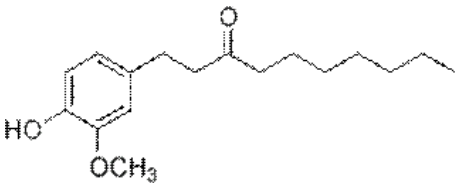
* P-value is based on an independent samples t-test

Figure 1. Schematic of a cell cycle

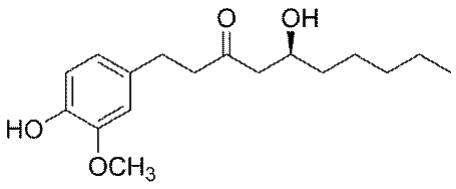


"Physiology or Medicine for 2001 - Press Release". Nobelprize.org. 20 Apr 2012

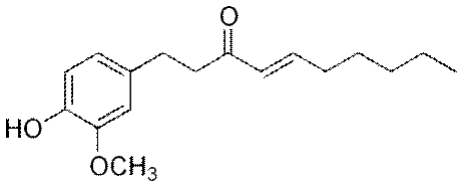
Figure 2. Chemical structure of major ginger phenolics



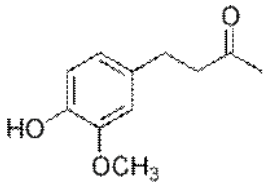
Paradols



Gingerols



Shogaols



Zingerone

Figure 3. Flow diagram of a trial of ginger supplementation over 4 weeks on markers of apoptosis, proliferation and differentiation in the normal-appearing colorectal mucosa of individuals at increased risk for CRC.

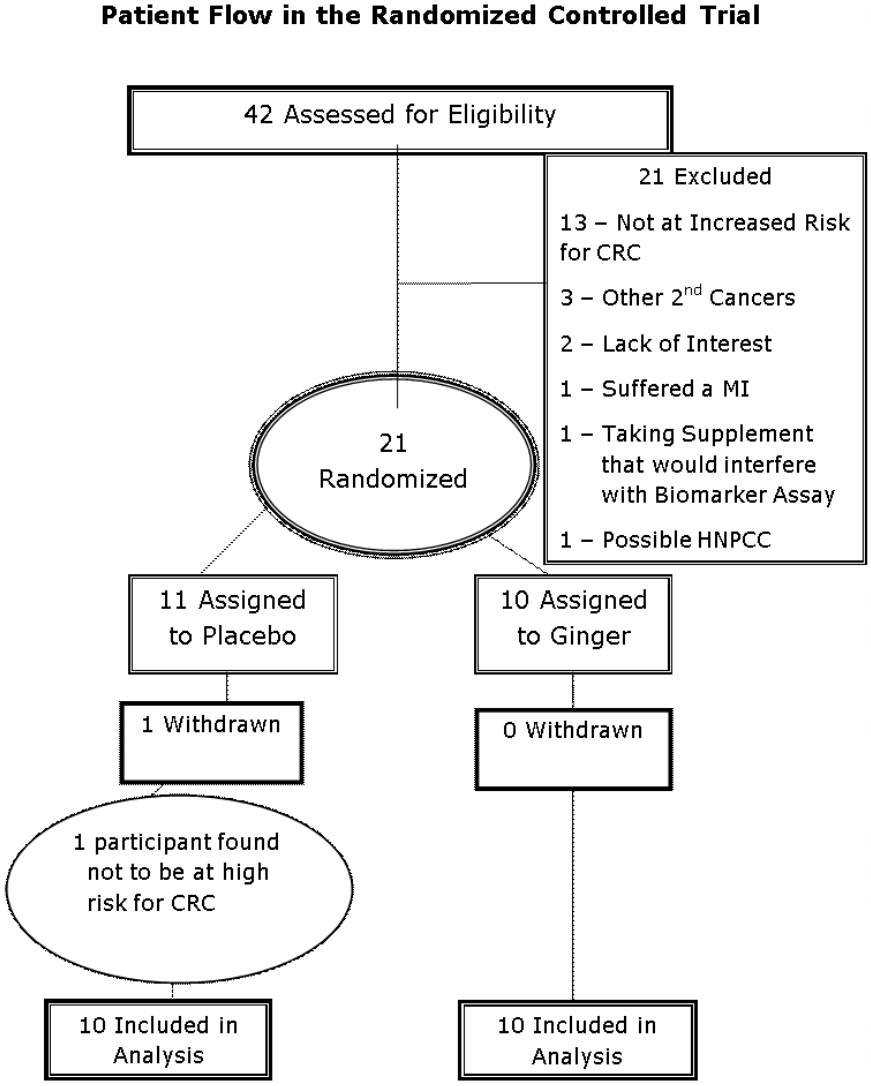


Figure 4. Summary of biomarker immunochemical protocols and images (at 200x magnification) of colon crypts immunohistochemically processed for: Bax, apoptotic marker; Bcl-2, anti-apoptotic marker; p21, differentiation marker; MIB-1, proliferation marker (short term); hTERT, proliferation marker (long term)

Antibody	Clone	Host	Manufacturer	Dilution	Catalog
Bax	NA	R-pAb	Dako	1:250	A3533
Bcl-2	100	M-mAb	Santa Cruz	1:250	SC-509
p21 (#02)	SX118	M-mAb	Dako	1:50	M7202
hTERT	C-term	R-mAb	Epitomics	1:50	1531-1
Ki-67 (MIB-1)	MIB-1	Mouse	Dako	1:350	M7240

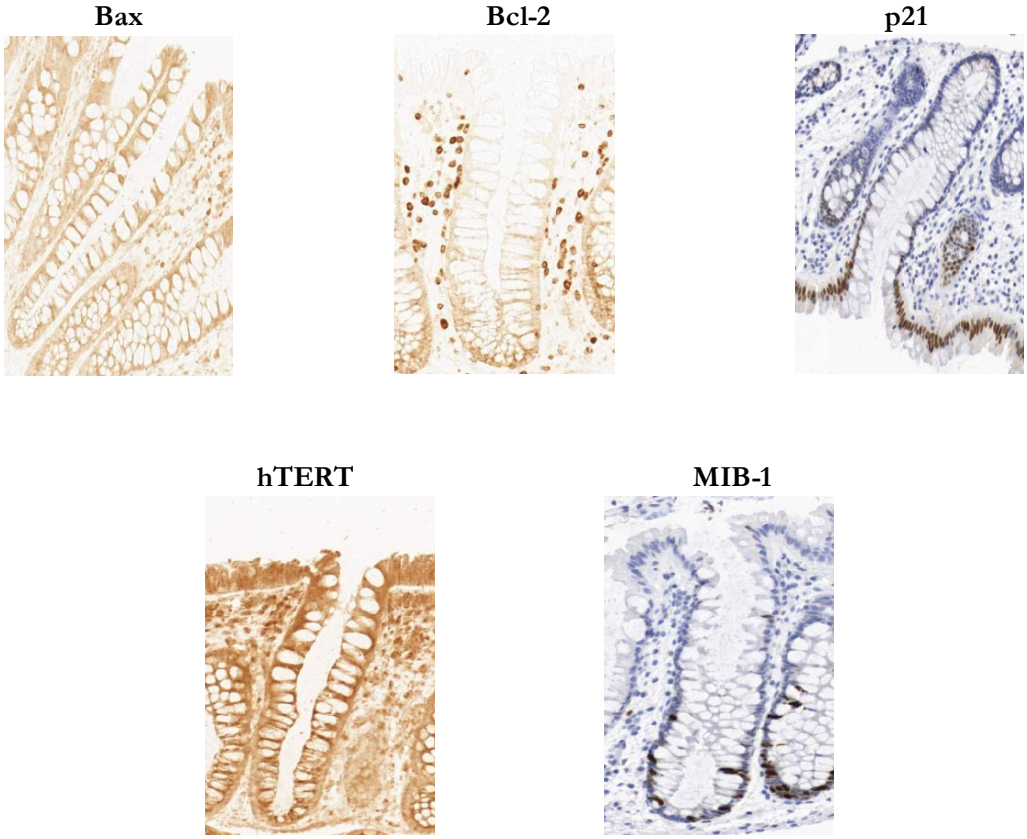
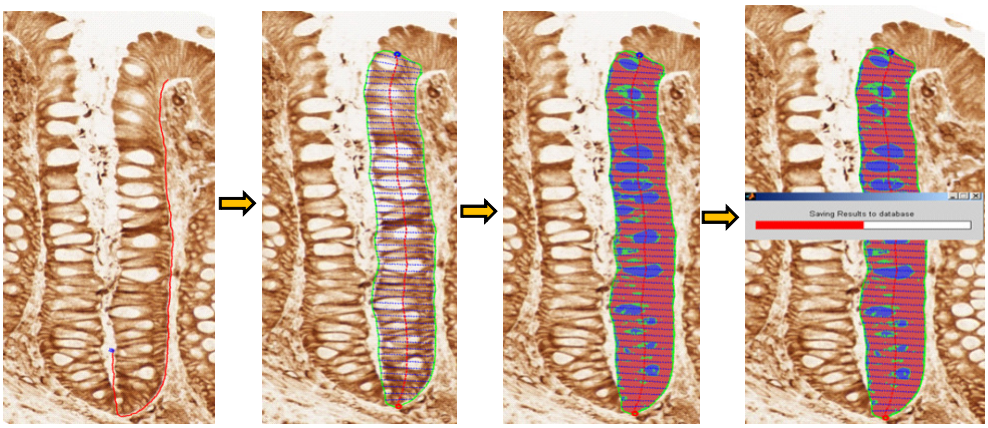


Figure 5. Quantitative image analysis using Aperio Scanscope and CellularEyes software



CONCLUSIONS, PUBLIC HEALTH IMPLICATIONS, AND FUTURE DIRECTIONS

Early proliferative and apoptotic changes in the normal colorectal mucosal crypt epithelium appear to precede or at least accompany the development of adenomas or cancer and, thus, may have value as predictive or diagnostic markers. Although the literature suggested that decreases in apoptosis accompanied by increases in proliferation typically precede and promote colorectal carcinogenesis initiation, their expression was positively correlated in the present study. However, little or no research has been done in the normal-appearing human colonic mucosa and, as such, associations suggested by the literature may only be translatable to tissue undergoing progressive or advanced stages of colorectal carcinogenesis. Our results further suggest the possibility that apoptosis may reflect not only cell death, but also proliferation activity; however, this was not directly addressable by our study design and requires further investigation.

Our study emphasizes the importance of using multiple biomarkers to provide a net summary of cell cycle functioning as one protein or even several proteins that take part in one pathway of carcinogenesis will most likely not provide enough information to accurately describe the status of key systems within colonocytes whose malfunction can lead to the development of colorectal cancer.

Our results suggested that ginger may reduce proliferation in the crypts of normal-appearing colorectal epithelium and increase apoptosis and differentiation relative to proliferation—especially in the proliferation zones of crypts. Thus, our results support conducting a full-scale clinical trial of ginger on cell cycle markers in the normal-appearing colorectal mucosa in patients at increased risk for colorectal cancer. If such a trial were successful, then a trial of ginger and colorectal adenoma recurrence would be supported.

Future biomarker endpoint studies should provide a net summary of the major cell cycle characteristics including proliferation, differentiation, and apoptosis when investigating the potential effectiveness of chemopreventive agents. In addition, studies are needed to elucidate the mechanisms by which ginger may modulate apoptotic and proliferative indices in the normal-appearing human colonic mucosa.

REFERENCES

1. American Cancer Society. Cancer Facts and Figures. Atlanta, Ga: American Cancer Society; 2011.

2. Potter JD, Slattery ML, Bostick RM, Gapstur SM. Colon cancer: a review of the epidemiology. Epidemiol Rev 1993;15(2):499-545.

3. Potter JD. Colorectal cancer: molecules and populations. J Natl Cancer Inst 1999;91(11):916-32.

4. McMichael AJ, Giles GG. Cancer in migrants to Australia: extending the descriptive epidemiological data. Cancer Res 1988;48(3):751-6.

5. Ziegler RG, Devesa SS, Fraumeni JF, Jr. Epidemiologic patterns of colorectal cancer. Important Adv Oncol 1986:209-32.

6. Santarelli RL, Pierre F, Corpet DE. Processed meat and colorectal cancer: a review of epidemiologic and experimental evidence. Nutr Cancer 2008;60(2):131-44.

7. Gandini S, Boniol M, Haukka J, Byrnes G, Cox B, Sneyd MJ, et al. Meta-analysis of observational studies of serum 25-hydroxyvitamin D levels and colorectal, breast and prostate cancer and colorectal adenoma. Int J Cancer 2011;128(6):1414-24.

8. Chan AT, Giovannucci EL. Primary prevention of colorectal cancer. Gastroenterology 2010;138(6):2029-2043 e10.

9. Moran A, Ortega P, de Juan C, Fernandez-Marcelo T, Frias C, Sanchez-Pernaute A, et al. Differential colorectal carcinogenesis: Molecular basis and clinical relevance. World J Gastrointest Oncol 2010;2(3):151-8.

10. Hambly RJ, Saunders M, Rijken PJ, Rowland IR. Influence of dietary components associated with high or low risk of colon cancer on apoptosis in the rat colon. Food Chem Toxicol 2002;40(6):801-8.

11. Siu IM, Robinson DR, Schwartz S, Kung HJ, Pretlow TG, Petersen RB, et al. The identification of monoclonality in human aberrant crypt foci. Cancer Res 1999;59(1):63-6.

12. Lipkin M. Proliferative changes in the colon. Am J Dig Dis 1974;19(11):1029-32.

13. Kerr JF, Winterford CM, Harmon BV. Apoptosis. Its significance in cancer and cancer therapy. Cancer 1994;73(8):2013-26.

14. Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer 2002;2(9):647-56.

15. Cheng EH, Wei MC, Weiler S, Flavell RA, Mak TW, Lindsten T, et al. BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. Mol Cell 2001;8(3):705-11.

16. Zong WX, Lindsten T, Ross AJ, MacGregor GR, Thompson CB. BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. Genes Dev 2001;15(12):1481-6.

17. Wang X. The expanding role of mitochondria in apoptosis. Genes Dev 2001;15(22):2922-33.

18. Fletcher JI, Huang DC. Controlling the cell death mediators Bax and Bak: puzzles and conundrums. Cell Cycle 2008;7(1):39-44.

19. Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, et al. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science 2001;292(5517):727-30.

20. Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, et al. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science 1997;275(5303):1129-32.

21. Gross A, Jockel J, Wei MC, Korsmeyer SJ. Enforced dimerization of BAX results in its translocation, mitochondrial dysfunction and apoptosis. EMBO J 1998;17(14):3878-85.

22. Hu W, Kavanagh JJ. Anticancer therapy targeting the apoptotic pathway. Lancet Oncol 2003;4(12):721-9.

23. Lu QL, Abel P, Foster CS, Lalani EN. bcl-2: role in epithelial differentiation and oncogenesis. Hum Pathol 1996;27(2):102-10.

24. Nakamura T, Nomura S, Sakai T, Nariya S. Expression of bcl-2 oncoprotein in gastrointestinal and uterine carcinomas and their premalignant lesions. Hum Pathol 1997;28(3):309-15.

25. van Diest PJ, Brugal G, Baak JP. Proliferation markers in tumours: interpretation and clinical value. J Clin Pathol 1998;51(10):716-24.

26. Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, et al. Specific association of human telomerase activity with immortal cells and cancer. Science 1994;266(5193):2011-5.

27. Horikawa I, Barrett JC. Transcriptional regulation of the telomerase hTERT gene as a target for cellular and viral oncogenic mechanisms. Carcinogenesis 2003;24(7):1167-76.

28. Cattoretti G, Becker MH, Key G, Duchrow M, Schluter C, Galle J, et al. Monoclonal antibodies against recombinant parts of the Ki-67 antigen (MIB 1 and MIB 3) detect proliferating cells in microwave-processed formalin-fixed paraffin sections. J Pathol 1992;168(4):357-63.

29. McCormick D, Chong H, Hobbs C, Datta C, Hall PA. Detection of the Ki-67 antigen in fixed and wax-embedded sections with the monoclonal antibody MIB1. Histopathology 2002;41(3A):173-8.

30. Sheikh RA, Min BH, Yasmeen S, Teplitz R, Tesluk H, Ruebner BH, et al. Correlation of Ki-67, p53, and Adnab-9 immunohistochemical staining and ploidy with clinical and histopathologic features of severely dysplastic colorectal adenomas. Dig Dis Sci 2003;48(1):223-9.

31. Bostick RM, Fosdick L, Lillemoe TJ, Overn P, Wood JR, Grambsch P, et al. Methodological findings and considerations in measuring colorectal epithelial cell proliferation in humans. Cancer Epidemiol Biomarkers Prev 1997;6(11):931-42.

32. Newmark HL, Lipkin M. Calcium, vitamin D, and colon cancer. Cancer Res 1992;52(7 Suppl):2067s-2070s.

33. Bostick RM, Potter JD, Fosdick L, Grambsch P, Lampe JW, Wood JR, et al. Calcium and colorectal epithelial cell proliferation: a preliminary randomized, double-blinded, placebo-controlled clinical trial. J Natl Cancer Inst 1993;85(2):132-41.

34. Rozen P. An evaluation of rectal epithelial proliferation measurement as biomarker of risk for colorectal neoplasia and response in intervention studies. Eur J Cancer Prev 1992;1(3):215-24.

35. Weinberg WC, Denning MF. P21Waf1 control of epithelial cell cycle and cell fate. Crit Rev Oral Biol Med 2002;13(6):453-64.

36. Gartel AL, Serfas MS, Tyner AL. p21--negative regulator of the cell cycle. Proc Soc Exp Biol Med 1996;213(2):138-49.

37. Gartel AL, Tyner AL. Transcriptional regulation of the p21((WAF1/CIP1)) gene. Exp Cell Res 1999;246(2):280-9.

38. el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, et al. WAF1, a potential mediator of p53 tumor suppression. Cell 1993;75(4):817-25.

39. Besson A, Dowdy SF, Roberts JM. CDK inhibitors: cell cycle regulators and beyond. Dev Cell 2008;14(2):159-69.

40. Nosho K, Yamamoto H, Adachi Y, Endo T, Hinoda Y, Imai K. Gene expression profiling of colorectal adenomas and early invasive carcinomas by cDNA array analysis. Br J Cancer 2005;92(7):1193-200.

41. Ogino S, Nosho K, Shima K, Baba Y, Irahara N, Kirkner GJ, et al. p21 expression in colon cancer and modifying effects of patient age and body mass index on prognosis. Cancer Epidemiol Biomarkers Prev 2009;18(9):2513-21.

42. Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D. p21 is a universal inhibitor of cyclin kinases. Nature 1993;366(6456):701-4.

43. Voorrips LE, Goldbohm RA, van Poppel G, Sturmans F, Hermus RJ, van den Brandt PA. Vegetable and fruit consumption and risks of colon and rectal cancer in a prospective cohort study: The Netherlands Cohort Study on Diet and Cancer. Am J Epidemiol 2000;152(11):1081-92.

44. Terry P, Giovannucci E, Michels KB, Bergkvist L, Hansen H, Holmberg L, et al. Fruit, vegetables, dietary fiber, and risk of colorectal cancer. J Natl Cancer Inst 2001;93(7):525-33.

45. Gali-Muhtasib HU, Younes IH, Karchesy JJ, el-Sabban ME. Plant tannins inhibit the induction of aberrant crypt foci and colonic tumors by 1,2-dimethylhydrazine in mice. Nutr Cancer 2001;39(1):108-16.

46. Manju V, Nalini N. Effect of ginger on bacterial enzymes in 1,2-dimethylhydrazine induced experimental colon carcinogenesis. Eur J Cancer Prev 2006;15(5):377-83.

47. Manju V, Nalini N. Chemopreventive efficacy of ginger, a naturally occurring anticarcinogen during the initiation, post-initiation stages of 1,2 dimethylhydrazine-induced colon cancer. Clin Chim Acta 2005;358(1-2):60-7.

48. Jeong CH, Bode AM, Pugliese A, Cho YY, Kim HG, Shim JH, et al. [6]-Gingerol suppresses colon cancer growth by targeting leukotriene A4 hydrolase. Cancer Res 2009;69(13):5584-91.

49. Lee SH, Cekanova M, Baek SJ. Multiple mechanisms are involved in 6-gingerol-induced cell growth arrest and apoptosis in human colorectal cancer cells. Mol Carcinog 2008;47(3):197-208.

50. Keum YS, Kim J, Lee KH, Park KK, Surh YJ, Lee JM, et al. Induction of apoptosis and caspase-3 activation by chemopreventive [6]-paradol and structurally related compounds in KB cells. Cancer Lett 2002;177(1):41-7.

51. Miyoshi N, Nakamura Y, Ueda Y, Abe M, Ozawa Y, Uchida K, et al. Dietary ginger constituents, galanals A and B, are potent apoptosis inducers in Human T lymphoma Jurkat cells. Cancer Lett 2003;199(2):113-9.

52. Lee E, Surh YJ. Induction of apoptosis in HL-60 cells by pungent vanilloids, [6]-gingerol and [6]-paradol. Cancer Lett 1998;134(2):163-8.

53. Pan MH, Hsieh MC, Kuo JM, Lai CS, Wu H, Sang S, et al. 6-Shogaol induces apoptosis in human colorectal carcinoma cells via ROS production, caspase activation, and GADD 153 expression. Mol Nutr Food Res 2008;52(5):527-37.

54. Hsu YL, Chen CY, Hou MF, Tsai EM, Jong YJ, Hung CH, et al. 6-Dehydrogingerdione, an active constituent of dietary ginger, induces cell cycle arrest and apoptosis through reactive oxygen species/c-Jun N-terminal kinase pathways in human breast cancer cells. Mol Nutr Food Res 2010;54(9):1307-17.

55. Kim SO, Kundu JK, Shin YK, Park JH, Cho MH, Kim TY, et al. [6]-Gingerol inhibits COX-2 expression by blocking the activation of p38 MAP kinase and NF-kappaB in phorbol ester-stimulated mouse skin. Oncogene 2005;24(15):2558-67.

56. Lantz RC, Chen GJ, Sarihan M, Solyom AM, Jolad SD, Timmermann BN. The effect of extracts from ginger rhizome on inflammatory mediator production. Phytomedicine 2007;14(2-3):123-8.

57. Nonn L, Duong D, Peehl DM. Chemopreventive anti-inflammatory activities of curcumin and other phytochemicals mediated by MAP kinase phosphatase-5 in prostate cells. Carcinogenesis 2007;28(6):1188-96.

58. Liu H, Zhu Y. [Effect of alcohol extract of Zingben officinale rose on immunologic function of mice with tumor]. Wei Sheng Yan Jiu 2002;31(3):208-9.

59. Jiang H, Xie Z, Koo HJ, McLaughlin SP, Timmermann BN, Gang DR. Metabolic profiling and phylogenetic analysis of medicinal Zingiber species: Tools for authentication of ginger (Zingiber officinale Rosc). Phytochemistry 2006;67(15):1673-85.

60. Kim EC, Min JK, Kim TY, Lee SJ, Yang HO, Han S, et al. [6]-Gingerol, a pungent ingredient of ginger, inhibits angiogenesis in vitro and in vivo. Biochem Biophys Res Commun 2005;335(2):300-8.

61. Brown AC, Shah C, Liu J, Pham JT, Zhang JG, Jadus MR. Ginger's (Zingiber officinale Roscoe) inhibition of rat colonic adenocarcinoma cells proliferation and angiogenesis in vitro. Phytother Res 2009;23(5):640-5.

62. Zick SM, Turgeon DK, Vareed SK, Ruffin MT, Litzinger AJ, Wright BD, et al. Phase II study of the effects of ginger root extract on eicosanoids in colon mucosa in people at normal risk for colorectal cancer. Cancer Prev Res (Phila) 2011;4(11):1929-37.

63. Bidinotto LT, Spinardi-Barbisan AL, Rocha NS, Salvadori DM, Barbisan LF. Effects of ginger (Zingiber officinale Roscoe) on DNA damage and development of urothelial tumors in a mouse bladder carcinogenesis model. Environ Mol Mutagen 2006;47(8):624-30.

64. Dias MC, Spinardi-Barbisan AL, Rodrigues MA, de Camargo JL, Teran E, Barbisan LF. Lack of chemopreventive effects of ginger on colon carcinogenesis induced by 1,2-dimethylhydrazine in rats. Food Chem Toxicol 2006;44(6):877-84.

65. Giovannucci E, Willett WC. Dietary factors and risk of colon cancer. Ann Med 1994;26(6):443-52.

66. Surh YJ. Cancer chemoprevention with dietary phytochemicals. Nat Rev Cancer 2003;3(10):768-80.

67. Krishnakantha TP, Lokesh BR. Scavenging of superoxide anions by spice principles. Indian J Biochem Biophys 1993;30(2):133-4.

68. Ahmed RS, Seth V, Banerjee BD. Influence of dietary ginger (Zingiber officinales Rosc) on antioxidant defense system in rat: comparison with ascorbic acid. Indian J Exp Biol 2000;38(6):604-6.

69. Banerjee S, Bueso-Ramos C, Aggarwal BB. Suppression of 7,12-dimethylbenz(a)anthracene-induced mammary carcinogenesis in rats by resveratrol: role of nuclear factor-kappaB, cyclooxygenase 2, and matrix metalloprotease 9. Cancer Res 2002;62(17):4945-54.

70. Sekiwa Y, Kubota K, Kobayashi A. Isolation of novel glucosides related to gingerdiol from ginger and their antioxidative activities. J Agric Food Chem 2000;48(2):373-7.

71. Kuo JM, Yeh DB, Pan BS. Rapid photometric assay evaluating antioxidative activity in edible plant material. J Agric Food Chem 1999;47(8):3206-9.

72. Reddy AC, Lokesh BR. Studies on spice principles as antioxidants in the inhibition of lipid peroxidation of rat liver microsomes. Mol Cell Biochem 1992;111(1-2):117-24.

73. Grzanna R, Lindmark L, Frondoza CG. Ginger--an herbal medicinal product with broad anti-inflammatory actions. J Med Food 2005;8(2):125-32.

74. Jolad SD, Lantz RC, Chen GJ, Bates RB, Timmermann BN. Commercially processed dry ginger (Zingiber officinale): composition and effects on LPS-stimulated PGE2 production. Phytochemistry 2005;66(13):1614-35.

75. Jolad SD, Lantz RC, Solyom AM, Chen GJ, Bates RB, Timmermann BN. Fresh organically grown ginger (Zingiber officinale): composition and effects on LPS-induced PGE2 production. Phytochemistry 2004;65(13):1937-54.

76. Shukla Y, Singh M. Cancer preventive properties of ginger: a brief review. Food Chem Toxicol 2007;45(5):683-90.

77. Ahmad N, Katiyar SK, Mukhtar H. Antioxidants in chemoprevention of skin cancer. Curr Probl Dermatol 2001;29:128-39.

78. Katiyar SK, Agarwal R, Mukhtar H. Inhibition of tumor promotion in SENCAR mouse skin by ethanol extract of Zingiber officinale rhizome. Cancer Res 1996;56(5):1023-30.

79. Surh Y. Molecular mechanisms of chemopreventive effects of selected dietary and medicinal phenolic substances. Mutat Res 1999;428(1-2):305-27.

80. Bode AM, Ma WY, Surh YJ, Dong Z. Inhibition of epidermal growth factor-induced cell transformation and activator protein 1 activation by [6]-gingerol. Cancer Res 2001;61(3):850-3.

81. Lee HS, Seo EY, Kang NE, Kim WK. [6]-Gingerol inhibits metastasis of MDA-MB-231 human breast cancer cells. J Nutr Biochem 2008;19(5):313-9.

82. Zick SM, Djuric Z, Ruffin MT, Litzinger AJ, Normolle DP, Alrawi S, et al. Pharmacokinetics of 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol and conjugate metabolites in healthy human subjects. Cancer Epidemiol Biomarkers Prev 2008;17(8):1930-6.

83. Fedirko V, Bostick RM, Flanders WD, Long Q, Shaukat A, Rutherford RE, et al. Effects of vitamin D and calcium supplementation on markers of apoptosis in normal colon mucosa: a randomized, double-blind, placebo-controlled clinical trial. Cancer Prev Res (Phila) 2009;2(3):213-23.

84. Khater DS. The influence of ginger as a chemopreventive agent on proliferation and apoptosis in chemically induced oral carcinogenesis. Nature and Science 2010;8(11):44-51.

85. Lu J, Guan S, Shen X, Qian W, Huang G, Deng X, et al. Immunosuppressive activity of 8-gingerol on immune responses in mice. Molecules 2011;16(3):2636-45.

86. Murakami A, Takahashi D, Kinoshita T, Koshimizu K, Kim HW, Yoshihiro A, et al. Zerumbone, a Southeast Asian ginger sesquiterpene, markedly suppresses free radical generation, proinflammatory protein production, and cancer cell proliferation accompanied by apoptosis: the alpha,beta-unsaturated carbonyl group is a prerequisite. Carcinogenesis 2002;23(5):795-802.

87. Harliansyah MN, Ngah WZW, Yusof YAM Antiproliferative, antioxidant and apoptosis effects of *Zingiber offincinale* and 6-gingerol on HepG2 cells. Asian J Biochem 2007;2(6):421-426.

88. Yagihashi S, Miura Y, Yagasaki K. Inhibitory effect of gingerol on the proliferation and invasion of hepatoma cells in culture. Cytotechnology 2008;57(2):129-36.

89. Seo EY LH, Kim WK. Effect of [6]-Gingerol on Inhibition of Cell Proliferation in MDA-MB-231 Human Breast Cancer Cells. Korean J Nutr 2005;38(8):656-662.

90. Tuntiwechapikul W, Taka T, Songsomboon C, Kaewtunjai N, Imsumran A, Makonkawkeyoon L, et al. Ginger extract inhibits human telomerase reverse transcriptase and c-Myc expression in A549 lung cancer cells. J Med Food 2010;13(6):1347-54.

91. Lee E, Park KK, Lee JM, Chun KS, Kang JY, Lee SS, et al. Suppression of mouse skin tumor promotion and induction of apoptosis in HL-60 cells by Alpinia oxyphylla Miquel (Zingiberaceae). Carcinogenesis 1998;19(8):1377-81.

92. Yodkeeree S, Sung B, Limtrakul P, Aggarwal BB. Zerumbone enhances TRAIL-induced apoptosis through the induction of death receptors in human colon cancer cells: Evidence for an essential role of reactive oxygen species. Cancer Res 2009;69(16):6581-9.

93. Wargovich MJ, Baer AR. Basic and clinical investigations of dietary calcium in the prevention of colorectal cancer. Prev Med 1989;18(5):672-9.

94. Bleiberg H, Buyse M, Galand P. Cell kinetic indicators of premalignant stages of colorectal cancer. Cancer 1985;56(1):124-9.

95. Paganelli GM, Santucci R, Biasco G, Miglioli M, Barbara L. Effect of sex and age on rectal cell renewal in humans. Cancer Lett 1990;53(2-3):117-21.

96. Paganelli GM, Biasco G, Santucci R, Brandi G, Lalli AA, Miglioli M, et al. Rectal cell proliferation and colorectal cancer risk level in patients with nonfamilial adenomatous polyps of the large bowel. Cancer 1991;68(11):2451-4.

97. Biasco G, Brandi G, Renga M, Paganelli GM, Tomassetti P, Calabrese C. Is there a relationship between hypergastrinemia and colorectal cancer risk? Rectal cell proliferation in Zollinger-Ellison syndrome. Am J Gastroenterol 1995;90(8):1365-6.

98. Ponz de Leon M, Roncucci L, Di Donato P, Tassi L, Smerieri O, Amorico MG, et al. Pattern of epithelial cell proliferation in colorectal mucosa of normal subjects and of patients with adenomatous polyps or cancer of the large bowel. Cancer Res 1988;48(14):4121-6.

99. Lipkin M, Uehara K, Winawer S, Sanchez A, Bauer C, Phillips R, et al. Seventh-Day Adventist vegetarians have a quiescent proliferative activity in colonic mucosa. Cancer Lett 1985;26(2):139-44.

100. Stadler J, Yeung KS, Furrer R, Marcon N, Himal HS, Bruce WR. Proliferative activity of rectal mucosa and soluble fecal bile acids in patients with normal colons and in patients with colonic polyps or cancer. Cancer Lett 1988;38(3):315-20.

101. Terpstra OT, van Blankenstein M, Dees J, Eilers GA. Abnormal pattern of cell proliferation in the entire colonic mucosa of patients with colon adenoma or cancer. Gastroenterology 1987;92(3):704-8.

102. Lipkin M, Blattner WE, Fraumeni JF, Jr., Lynch HT, Deschner E, Winawer S. Tritiated thymidine (phi p, phi h) labeling distribution as a marker for hereditary predisposition to colon cancer. Cancer Res 1983;43(4):1899-904.

103. Gerdes H, Gillin JS, Zimbalist E, Urmacher C, Lipkin M, Winawer SJ. Expansion of the epithelial cell proliferative compartment and frequency of adenomatous polyps in the colon correlate with the strength of family history of colorectal cancer. Cancer Res 1993;53(2):279-82.

104. Reid S, Ritchie A, Boring L, Gosling J, Cooper S, Hangoc G, et al. Enhanced myeloid progenitor cell cycling and apoptosis in mice lacking the chemokine receptor, CCR2. Blood 1999;93(5):1524-33.

105. Traver D, Akashi K, Weissman IL, Lagasse E. Mice defective in two apoptosis pathways in the myeloid lineage develop acute myeloblastic leukemia. Immunity 1998;9(1):47-57.

106. Scopa CD, Tsamandas AC, Zolota V, Kalofonos HP, Batistatou A, Vagianos C. Potential role of bcl-2 and ki-67 expression and apoptosis in colorectal carcinoma: a clinicopathologic study. Dig Dis Sci 2003;48(10):1990-7.

APPENDICES

Table 4. Scoring System

Percent Change	Score
75.1% +	3
45.1% to 75%	2
15.1% to 45%	1
-15% to 15%	0
-15.1% to - 45%	-1
- 45.1% to - 75%	-2
-75.1% +	-3

Table 5. Percent Changes in Biomarker

Bax

Placebo

Obs	Overall % Change	% Change - Proliferative	% Change - Luminal
1	-76.6851	-69.678	-83.995
4	-6.1153	40.144	-22.6887
5	-24.1109	-26.544	-13.9134
6	-3.8298	-11.606	10.5565
8	-6.1324	-6.692	-8.3847
10	25.6195	15.681	36.8486
12	-33.2974	-37.432	-30.981
15	-47.8036	-56.801	-39.5768
16	-67.7536	-70.771	-65.5333
19	55.7937	115.095	-5.2052

Ginger

Obs	Overall % Change	% Change - Proliferative	% Change - Luminal
2	-60.5387	-69.1853	-34.54
3	3.3467	30.3244	-20.635
7	-55.0314	-65.7319	-41.08
9	-47.3875	-48.9597	-44.104
11	91.6143	95.7815	72.471
13	-29.5447	-34.8181	-21.222
14	-60.1017	-63.148	-57.369
17	-59.1112	-60.8732	-59.929
18	31.417	-6.0343	127.412

Bcl-2

Placebo

Obs	Overall % Change	% Change - Proliferative	% Change - Luminal
1	16.6016	15.4056	22.675
4	-39.6621	-35.8266	-69.518
5	-14.3313	-16.9545	45.576
6	-21.2638	-14.881	-36.102
8	-5.0336	-2.7381	-27.85
10	-23.6945	-26.381	7.268
12	54.3947	71.8997	-48.516
15	47.9038	42.1572	163.608
16	-10.8936	-16.2428	59.224
19	-36.655	-38.1768	-31.718

Ginger

Obs	Overall % Change	% Change - Proliferative	% Change - Luminal
2	-25.1084	-27.0728	11.293
3	-12.8986	-12.3187	-19.578
7	2.3018	-5.388	111.938
9	16.8458	13.2226	71.251
11	54.4186	63.7133	-4.731
13	17.3564	18.2099	7.7
14	-50.7436	-50.9599	-49.352
17	-8.0107	-9.2899	4.929
18	-35.2962	-29.0089	-73.746

p21

Placebo

Obs	Overall % Change	% Change - Proliferative	% Change - Luminal
1	-16.074	31.45	-20.281
4	-34.751	-80.086	-36.785
5	-0.519	-13.894	-4.678
6	-26.303	-72.449	-15.12
8	-22.403	43.583	-30.98
10	107.515	341.653	105.07
12	32.714	138.47	44.397
15	45.361	-17.421	63.121
16	80.775	26.209	126.109
19	-24.606	-78.56	-23.261

Ginger

Obs	Overall % Change	% Change - Proliferative	% Change - Luminal
2	-2.43	-81.597	30.357
3	-66.245	-69.717	-70.195
7	-0.468	666.08	-11.84
9	-55.657	-80.772	-61.614
11	330.345	-81.681	586.363
13	-10.493	15.01	-5.55
14	51.342	-65.129	74.403
17	-6.625	-32.535	-4.955
18	-20.888	25.808	-24.392

hTERT

Placebo

Obs	Overall % Change	% Change - Proliferative	% Change - Luminal
1	-4.991	2.996	-23.678
4	35.911	40.554	30.629
5	-7.894	-8.943	-4.432
6	30.299	25.991	36.055
8	22.101	28.344	8.292
10	-1.021	0.608	-5.857
12	57.093	43.057	93.945
15	136.317	144.136	119.615
16	44.683	29.803	84.422
19	46.422	44.731	51.77

Ginger

Obs	Overall % Change	% Change - Proliferative	% Change - Luminal
2	-25.1862	-29.9821	-15.4253
3	-31.5683	-21.9594	-44.2267
7	-21.3741	-19.6699	-23.5856
9	-78.7245	-81.4456	-74.8867
11	92.5536	84.7362	99.5917
13	-24.2766	-6.6999	-48.8758
14	-10.165	-13.3945	-6.0031
17	-58.3284	-55.3443	-63.2353
18	50.615	51.6526	44.3314

Mib-1

Placebo

Obs	Overall % Change	% Change - Proliferative	% Change - Luminal
1	-20.107	-20.119	-15.26
4	15.869	15.508	44.52
5	-6.242	-6.449	3.27
6	-7.335	-5.412	-39.31
8	6.971	4.223	189.55
10	-7.004	-14.115	255.39
12	59.923	64.493	-42.3
15	18.906	15.179	5282.32
16	-15.511	-8.922	-85.28
19	145.087	128.435	783.36

Ginger

Obs	Overall % Change	% Change - Proliferative	% Change - Luminal
2	-38.298	-38.601	31.58
3	-41.086	-39.78	-88.31
7	7.189	-0.891	646.43
9	10.181	17.28	-46.37
11	0.874	-0.868	47.71
13	167.258	149.688	5056.11
14	-17.236	-13.691	-56.24
17	16.386	17.399	3.6
18	-31.923	-27.333	-84.28

Table 6. Biomarker Scores

Bax		Bcl-2		p21		hTERT		Mib-1		Bax +p21 - Bcl-2 - hTERT		Bax +p21 - Bcl-2 - Mib-1	
Placebo		Placebo		Placebo		Placebo		Placebo		Placebo		Placebo	
Obs	**Score - overall**	**Obs**	**Score - overall**	**Obs**	**Score - overall**	**Obs**	**Score - overall**	**Obs**	**Score - overall**	**Cell Cycle Score (w/hTERT)**		**Cell Cycle Score (w/Mib-1)**	
1	-3	**1**	1	**1**	-1	**1**	0	**1**	-1		-5		-4
4	0	**4**	-1	**4**	-1	**4**	1	**4**	1		-1		-1
5	-1	**5**	0	**5**	0	**5**	0	**5**	0		-1		-1
6	0	**6**	-1	**6**	-1	**6**	1	**6**	0		-1		0
8	0	**8**	0	**8**	-1	**8**	1	**8**	0		-2		-1
10	1	**10**	-1	**10**	3	**10**	0	**10**	0		5		5
12	-1	**12**	2	**12**	2	**12**	2	**12**	2		-3		-3
15	-2	**15**	2	**15**	2	**15**	3	**15**	1		-5		-3
16	-2	**16**	0	**16**	3	**16**	1	**16**	-1		0		2
19	2	**19**	-1	**19**	-1	**19**	2	**19**	3		0		-1
										Total	-1.3	Total	-0.7
Ginger		Ginger		Ginger		Ginger		Ginger		Ginger		Ginger	
Obs	**Score - overall**	**Obs**	**Score - overall**	**Obs**	**Score - overall**	**Obs**	**Score - overall**	**Obs**	**Score - overall**	**Cell Cycle Score (w/hTERT)**		**Cell Cycle Score (w/Mib-1)**	
2	-2	**2**	-1	**2**	0	**2**	-1	**2**	-1		0		0
3	0	**3**	0	**3**	-2	**3**	-1	**3**	-1		-1		-1
7	-2	**7**	0	**7**	0	**7**	-1	**7**	0		-1		-2
9	-2	**9**	1	**9**	-2	**9**	-3	**9**	0		-2		-5
11	3	**11**	2	**11**	3	**11**	3	**11**	0		1		4
13	-1	**13**	1	**13**	0	**13**	-1	**13**	3		-1		-5
14	-2	**14**	-2	**14**	2	**14**	0	**14**	-1		2		3
17	-2	**17**	0	**17**	0	**17**	-2	**17**	1		0		-3
18	1	**18**	-1	**18**	-1	**18**	2	**18**	-1		-1		2
										Total	-0.333333333	Total	-0.777777778