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Dan Chen Date

Colorectal Epithelial Cell Proliferation and Risk for Incident, Sporadic Colorectal Adenomatous Polyps

By

Dan Chen

Master of Public Health

Global Epidemiology

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Roberd “Robin” M. Bostick, M.D., M.P.H.

Faculty Thesis Advisor

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Dan Chen

B.S., Tsinghua University, 2009

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Abstract

Colorectal Epithelial Cell Proliferation and Risk for Incident, Sporadic Colorectal Adenomatous Polyps

By Dan Chen

Background: It is hypothesized that colorectal epithelial cell proliferation kinetics are altered in the normal mucosa of patients at increased risk for colon cancer, the second leading cause of cancer deaths in the United States; however, there are no reports of well-conducted observational epidemiologic studies that have investigated this hypothesis.

Objective: To assess whether colorectal epithelial cell proliferation in the normal-appearing colorectal mucosa may be a valid, potentially modifiable biomarker of risk for colorectal neoplasms.

Methods: We conducted a pilot, colonoscopy-based case-control study (30 cases, 50 controls) of incident, sporadic colorectal adenoma. Cell proliferation was measured using immunohistochemistry for MIB1 (epitope of Ki-67). The labeling index (LI), the indicator of overall proliferation, was calculated as the proportion of labeled cells in the crypt; an LI_{b60} and LI_{t40} were also calculated to indicate the degree of proliferation in the upper 40% of the crypts (differentiation zone) and the lower 60% of the crypts (proliferative zone), respectively. A distributional index (Φ_h) to indicate expansion of the proliferative zone into the differentiation zone was calculated as the proportion of labeled cells in the crypts that were in the upper 40% of

the crypts. Cases and controls were compared using analysis of covariance and logistic regression.

Results: In the adenoma cases relative to the controls, the LI, LI_{b60}, LI_{t40}, and Φ_h were proportionately lower by 17% (p = 0.03), 17% (p = 0.02), 28% (p = 0.08) and 28% (p = 0.33), respectively; the corresponding crude odds ratios (OR) and 95% confidence intervals were 0.39 (0.15, 1.05), 0.50 (0.19, 1.31), 0.62 (0.25, 1.54), and 0.88 (0.35, 2.17). The inverse associations tended to be stronger with adjustment for other risk factors, such as calcium and total fat intakes. The Φ_h was 36% higher (p = 0.05) among those with total calcium consumption above the mean.

Conclusion: Opposite to our hypotheses, these preliminary data suggest that lower cell proliferation as indicated by MIB1 expression in the normal colonic mucosa may be associated with increased risk of incident, sporadic colorectal adenoma as well as with modifiable risk factors thought to decrease risk for colorectal neoplasms.

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Tabel of Contents

Illustration

List of Tables

Chapter I. Literature Review

1. Colorectal Cancer and Descriptive Epidemiology

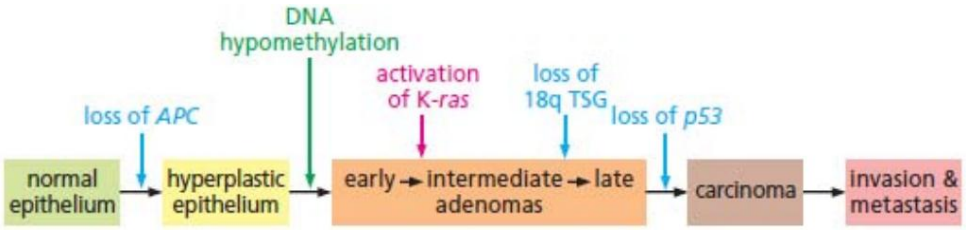
Colorectal cancer (cancer of the colon and rectum) is the third most common incident cancer the second leading cancer killer in the United States (1). In 2007 (the most recent year for which statistics are available), 142,672 Americans were diagnosed with colorectal cancer, including 72,755 men and 69,917 women and 53,219 people in the United States died from colorectal cancer, including 27,004 men and 26,215 women (2). Colon cancer affects men and women approximately equally, but rectal cancer frequency can be up to twice as high in men as in women (3, 4). Patterns of incidence rates vary between countries, with the highest rates in Australia, New Zealand, Europe, and North America, whereas the lowest rates are in Africa and South-Central Asia (5). The colorectal cancer incidence rate stayed relatively unchanged during the past 30 years, while the mortality rate decreased modestly, particularly in females (6). The United States is the only country with decreasing incidence rates in both males and females in the most recent time period, which largely reflects detection and removal of precancerous lesions through colorectal cancer screening (7, 8). While colorectal cancer death rates have been decreasing in several Western countries (8), largely resulting from improved treatment and increased awareness and early detection (7), rates continue to increase in many countries with more limited resources and health infrastructure, particularly in Central and South America and Eastern Europe (8).

2. Molecular Basis of Colon Carcinogenesis

The molecular basis of colon carcinogenesis is a multi-step process involving genetic alteration of *APC*, *K-ras*, a tumer suppressor gene in chromosome 18q, and *p53* (Figure 1)(9). There are at least two not necessarily entirely mutually exclusive major pathways driving this process. The first, the “APC pathway”, accounts for familial adenomatous polyposis (FAP) and approximately 80% of sporadic cancers. In FAP, the affected person is both with an inactivating

mutation in one allele of the "patheway gatekeeper" *APC* tumor suppressor gene, and has only to acquire an inactivation of the second allele to begin development of a colonic neoplasm; whereas, in the sporadic patient, inactivation of both alleles must be acquired, either through somatic mutation or epigenetic phenomena, predominantly the former.

Figure 1. The adenoma-carcinoma sequence (from Weinberg, R.A. (9))



The second pathway, the "Mismatch Repair (MMR) Pathway" accounts for hereditary non-polyposis colon cancer (HNPCC) and approximately 15% of sporadic cancers. In HNPCC, the affected person is born with an inactivating mutation in one of the mismatch repair genes, predominantly *MSH2* and *MLH1*. The sporadic patient in this pathway must acquire an inactivation, either through mutation or epigenetic silencing, predominantly the latter. The protein product of the "geteway" gene, *APC*, functions to degrade β-catenin which is both pro-proliferative and regulates E-cadherin, a calcium-dependent cell adhesion molecule necessary for colon crypt structure and function. Progressive alteration of cell proliferation and cell adhesion from the normal colon crypt to adenoma to carcinoma is a hallmark of colon carcinogenesis. When β-catenin is not degraded by APC, both c-myc and, further downstream, cyclin D1, are up-regulated, promoting entry of colonocytes into the proliferative phase of the cell cycle, with the net effect being increased proliferation (direct effect) and , indirectly, decreased differentiation and apoptosis.

The DNA mismatch repair (MMR) genes serve to repair mismatches in paired DNA strands post relication (9). When MMR genes are impaired mismatches in other genes can be propagated and progressively expanded, eventually hampering the ability of the affected genes to function

properly. Genes that characteristically become impaired as a result of deficient mismatch repair in MMR pathway colon carcinogenesis are *bax* and *TGFβ RII*. Impairment of bax, a pro-apoptotic regulator, causes a decrease in apoptosis, while impairment of *TGFβ RII* results in increased proliferation. The net result, as for the APC pathway, is increased proliferation and decreased differentiation and apoptosis.

3. Risk Factors for Colorectal Neoplasms

Age

Age is a strong risk factor for colorectal cancer (CRC). Diagnosis with CRC is rare before the age of 40, with peak incidence around 60 years (10). Age-specific CRC incidence rates begin to rise during the fifth decade of life (11). In a prospective cohort study of 75,266 Medicare enrollees, researchers observed that proximal colon cancers occurring most frequently among elderly patients, and the incidence of colorectal cancer increased from 1.59 cases per 1000 in those 65-69 years to 3.87 cases per 1000 in those 85 years and older; an almost two-fold increase in incidence (12). Furthermore, the incidence rates increased with age for all anatomic locations (rectum, distal colon, and proximal colon) (12). Similarly, adenomatous polyps have been shown to also increase with age.

Family History of Colorectal Cancer

Family history of colorectal cancer is positively associated with sporadic colorectal cancer risk. About 30% of sporadic colorectal cancer cases have a history of the disease in a first degree relative (13), which is associated with a 2- to 3-fold increased risk of colorectal cancer (14, 15). Moreover, having a history of CRC in a first degree relative younger than 40 years of age is associated with a 5-fold increase in risk of the disease (15). Furthermore, having two relatives of any age with CRC is associated with a 6-fold increase in risk (14, 15).

Obesity/BMI

Obesity is strongly and consistently associated with an increased risk of colorectal cancer. A recent meta-analysis of 28 cohort studies found a statistically significant 3% increase in CRC risk per 1 kg/m^2 increase in BMI. As with physical activity, a more consistent association and a larger increase in risk were found for colon cancer than for rectal cancer, or for colorectal cancer as a whole (16). It is also should be pointed out that BMI may not be an ideal measurement of the adiposity in humans for CRC risk prediction becasue: 1) fat is not distributed equally around the body; 2) there are two patterns of fat stores in the human body (peripheral/abdominal) that are largely determined by genetic factors; and 3) the size of intra-abdominal fat stores influences several hormone systems and predicts the risk of chronic diseases better than overall indicators of body fatness, such as BMI or subcutaneous fat measures (16).

Energy Intake and Physical Activity

Physical activity has consistently been shown to have an inverse association with CRC. Several large cohort studies and meta-analyses found a 20–29% reduction of colon cancer risk in individuals with high levels of physical activity compare to sedentary individuals (17, 18). However, no association was observed for rectal cancer. There are several, likely complimentary, mechanisms by which physical activity may protect against colorectal carcinogenesis: 1) stimulation of colon peristalsis resulting in reduced gut transit time and thus less carcinogen contact time with the colon epithelium); 2) reduction in insulin resistance; 3) favorable effects on the immune system; 4) effects on endogenous steroid hormone metabolism; and 5) reduction in body fatness (16).

Inflammation and Nonsterodial Anti-Inflammatory Drugs

In response to a range of toxic and pathogenic challenges, lymphocytes infiltrating into colorectal epithelium can release proinflammatory cytokines. Continual release of

proinflammatory cytokines may lead to increased generation of genotoxic compounds, such as reactive oxygen species (ROS), in the colorectal epithelium and can cause chronic inflammation, which has been reported to play a major role in colorectal tumorigenesis (19). Multiple observational studies and randomized clinical trials found that regular use of anti-inflammatory drugs (NSAIDs), such as aspirin and other NSAIDs, reduces the risk of colorectal neoplasms (20-22). The anti-carcinogenic effects of NSAIDs are thought to be largely through COX-2 inhibition which can cause gastrointestinal bleeding and renal failure. Randomized clinical trials (20, 21, 23, 24) have shown a decreased risk of colorectal adenoma recurrence in subject who was given aspirin or selective COX-2 inhibitors such as celecoxib and rofecoxib.

Tobacco

Cigarette and pipe smoking, especially long-term and with early onset, is linked to the development of colorectal neoplasms (25, 26). Compared to non-smokers, smokers had a greater number of colorectal polyps, and large adenomas were associated with long-time smoking (27-29). One of the proposed mechanisms for an association between tobacco smoking and colorectal neoplasms is that smoking may affect methylation of the MLH1 promoter region resulting in decreased or absent MLH1 expression and deficient DNA repair (26). Moreover, tobacco smoke contains many carcinogens, including polycyclic hydrocarbons, nitrosamines, heterocyclic amines, and other blood-borne carcinogens that may cause DNA mutations (e.g., APC gene) (30). When DNA repair mechanisms are altered, colonocytes may become more susceptible to mutations that may lead to neoplastic changes (31).

Alcohol

The epidemiologic evidence on an association of alcohol consumption with colorectal cancer is not consistent. Although the majority of the observational epidemiologic studies found a positive association between alcohol consumption and colorectal neoplasms, most of them

yielded statistically nonsignificant results. In a large, pooled analysis of 8 cohort studies (4,600 CRC cases and 475,000 participants), the group that had the highest alcohol consumption (≥45 g/day) was at 41% higher CRC risk (RR = 1.41, 95% CI: 1.16–1.72) (32). Giovanucci et al. also reported a positive association between alcohol intake and the risk of colon cancer in groups with lower consumption of folate and methionine; however, this association disappeared in people who had high levels of these nutrients in their diet (33, 34). The mechanism by which alcohol may increase the risk of CRC is not yet fully understood. A plausibal explanation may be that alcohol (ethanol) is metabolized into acetaldehyde, which degrades folate and may result in irregular DNA methylation (35, 36). Alcohol may also inhibit DNA repair and function as a solvent for other carcinogenic molecules, thus enhancing their penetrations into colonocytes (15, 16). Lastly, alcohol consumption may interact with tobacco smoking (37-39).

Postmenopausal Hormone Use in Women

Data on postmenopausal hormone use and colorectal cancer in women are not entirely consistent: nine studies reported decreased risk with hormone replacement therapy (HRT) use (39-47), two studies were null (48, 49), and one found an increased risk (50). Longer use of HRT is probably associated with lower risk, but more studies are needed to confirm this (3). In a randomized clinical trial, treatment with estrogen and progestin considerably reduced invasive colorectal cancer risk (hazard ratio = 0.56; 95% CI: 0.38–0.51) (51). In addition, a recent case-control study found that conjugated estrogen with progestin is more strongly associated than estrogen alone with risk for MSI-low and MSI-stable, but not MSI-high colorectal tumors (52). Moreover, an inverse association of HRT use with colorectal adenomas was also found (53, 54).

Total Dietary Fat

The association between dietary fat and risk of CRC remains inconsistent. While ecologic and older studies, which did not properly adjust for total energy intake, suggested a positive

association between dietary fats and incidence of CRC, more recent studies that do properly adjust for energy intake generally report a null association between dietary fat and CRC (16, 35, 55). These analyses suggest that there may be no energy-independent association between dietary fat intake and colorectal cancer. However, dietary fat is the largest source of energy and contributes to a high energy intake and obesity. In addition, it is difficult to disentangle the contribution of specific nutrients in the diet. Therefore, dietary fat may appear to be associated with colorectal cancer due to its contribution to the energy intake and obesity that are related to colorectal cancer risk (56).

Dietary Fiber

The substantial epidemiologic evidence on the inverse association between dietary fiber and colorectal cancer risk is not consistent despite strong biologic plausibility and a substantial body of epidemiologic literature. A large meta-analysis of 20 cohort studies found a 10% decrease in risk per 10g of dietary fiber per day (RR = 0.90, 95% CI: 0.84–0.97) with an apparent dose-response association (57). However, a pooled analysis of 13 prospective cohort studies (8,081 colorectal cancer cases and 730,000 participants) found a statistically non-significant 6% decreased risk for those with the highest intake of dietary fiber after adjusting for other risk factors (RR = 0.94, 95% CI: 0.86–1.03) (58). The results of epidemiologic studies of a dietary fiber – CRC association are inconsistent, probably because of the heterogeneous nature of fiber itself, issuing with measurement of fiber intake (3) and the presence of other compounds in fiber rich foods. Intervention studies are much less consistent with the hypothesis that dietary fiber reduces colorectal cancer risk. Three randomized clinical trials that tested the effect of high-fiber diets did not show the reduction in colorectal adenoma recurrence (59-61). However, the results of the randomized trials should be interpreted with caution, as the intervention was relatively short term (3–5 years) and was done in the patients who already had neoplastic changes such as adenoma in their colons.

Folate

Folate is a generic term referring to the naturally-occurring family of water-soluble B-group vitamins and is essential for normal DNA repairing, synthesis, and methylation.

Epidemiologic data suggest an inverse association between folate intake and incident CRC, however, data remain inconclusive and may rely more on the timing and dose of folate interventions (62). A recent meta-analysis of 7 cohort and 9 case-control studies observed lower CRC risk with higher dietary folate consumption (RR = 0.75, 95% CI: 0.64-0.89; OR = 0.76，95% CI: 0.60-0.96) (63). Conversely, a 20-years descriptive, population-based study in Chile found that folate fortification of foods was associated with an increase in CRC risk. The highest relative risk of CRC was observed among the age groups 45-64 years (RR: 2.6; CI: 2.58-2.93) and 65-79 years (RR: 2.9; CI: 2.86-3.25) (64). Recently, a randomized clinical trial of folic acid supplementation found no reduction in colorectal adenoma recurrence, but statistically significant increases in the occurrence of multiple adenomas (RR = 2.32, 95% CI: 1.23–4.35) and large adenomas (RR = 1.67, 95% CI: 1.00–2.80) (65). Similar findings were seen in animal studies. After establishing microscopic neoplastic foci in the colon, high folic acid doses as well as a folate intervention promoted, rather than suppressed, colon carcinogenesis (66). Recently, it was proposed that folate may play a dual role in carcinogenesis: it may act as a preventive agent during the early stages of carcinogenesis in individuals with a low folate status, and it may promote carcinogenesis during the later stages of tumorigenesis, especially if administered at very high doses (67, 68). Also, the form of folate (natural folate in food vs. synthetic folic acid in supplements) may play an important role in cancer prevention.

Vitamin D

Vitamin D is a group of fat-soluble pro-hormones. The active form of vitamin D is 1α,25-$(OH)_2$-vitamin D. People acquire vitamin D from two sources: cutaneous synthesis after

exposures to UVB (about 90%) and diet (69). The hypothesis that vitamin D plays a role in preventing cancer was first initiated by the observation in the 1930s of an inverse correlation between cancer risk and sunlight exposure (70). Garland firstly proposed the hypothesis that vitamin D status accounted for an inverse association between solar ultraviolet-B exposure and risk of colon cancer (71).

Based on recent biological and epidemiologic evidence, vitamin D is a promising dietary chemopreventive agent. Animal and in-vitro studies show that vitamin D and vitamin D analogues regulate cell proliferation, differentiation, and apoptosis; promote bile acid degradation and xenobiotic metabolism; and influences growth factor signaling, cell adhesion, DNA repair, angiogenesis, inflammation, and immune function (72-75). In human cell lines from the colon and other organs, vitamin D increases expression of enzymes involved in antioxidant responses, thereby decreasing oxidative stress in the colorectal epithelium (76-78), inhibits proliferation, induces differentiation, and promots apoptosis (79-85). Some epidemiologic studies suggest that vitamin D is associated with lower risk for colorectal cancer (86-89) and adenoma (90-93). In studies that investigated dietary vitamin D intake without considering exposure to UVB light, the association between vitamin D intake and colorectal adenoma/cancer was not consistent. This inconsistency between these studies can be explained by misclassification of actual vitamin D exposure, which leads to an underestimation of the association. In those few studies that assessed the main form of circulating vitamin D, 25-(OH)-vitamin D, an inverse association was observed between 25-(OH)-vitamin D levels and colorectal cancer (94-96) or adenomas (90, 91). The results of these studies suggest that circulating vitamin D level is a better marker of vitamin D exposure than indirect estimates of vitamin D exposure based solely on a diet. However, the use of circulating 25-(OH)-vitamin D levels as vitamin D exposure has its own complications due to seasonal variations in vitamin D levels and assay sensitivity/variability that should be kept in mind during data analysis.

Calcium

Calcium is an element that is essential for living organisms with multiple functions in the body, including as the bone structure and a second messenger in intracellular signaling; and as a modulator of cell proliferation and differentiation. Calcium homeostasis is controlled by three hormones: vitamin D, parathyroid hormone (PTH), and calcitonin (97).

The protective effects of calcium against colorectal neoplasms are supported by a large amount of evidence; however, its exact anticarcinogenic effects are not clear. Proposed mechanisms of calcium against colorectal cancer include protection of colonocytes against bile acids and fatty acids (98, 99), direct effects on cell cycle regulation (100), promotion of colonocyte differentiation (101, 102), and modulation of E-cadherin and β-catenin expression via the calcium-sensing receptor CaSR (100, 103, 104). Further, there is some evidence that extracellular calcium activates protein kinase C, which is associated with the differential induction of p21 in the intestinal epithelium (100). Calcium may also act as an oxidative stress and DNA damage reducing agent in the colon. In the colon lumen, bile acids damage cell membranes through an oxidative mechanism (105), provoking an inflammatory response and causing DNA damage (106), and calcium can bind the free bile acids rendering them inert (99). Further investigations are needed to understand the role of calcium in colon carcinogenesis.

Inverse associations for calcium and colorectal adenoma have been consistently observed in observational studies: two cohort studies (93, 107), seven case-control studies (90, 108-113), four case-control/cohort studies nested in randomized clinical trials (92, 114-116)， and two cross-sectional studies (37, 117). Several clinical trials found reduced colorectal adenoma recurrence with calcium supplementation (118-120). The calcium-adenoma association appears to be nearing causal status, but requires some additional large clinical trials and mechanistic confirmation.

4. Colorectal Adenomatous Polyps

Colorectal cancer results from colorectal mucosa progressing through multiple genetic transformations, which can be initiated by an inherited condition, an external stimulus, or a combination of the two (121). Adenomatous polyps are an agreed upon precursor to colorectal cancer. The transformation from normal mucosa to adenomas is accompanied by increasing rates of cellular proliferation and decreases in cellular apoptosis (121). Most colorectal cancers (70% to 90%) are developed from adenomas. The prevalence of adenomatous polyps is approximately 30% in the middle-aged, and around 50% in elderly persons; however, less than 1% of all adenomas develop into cancer. The likelihood of an adenomatous polyp transforming into a cancer depends on several characteristics, such as size, histologic features, and appearance of the lesion (19, 122).

Nowadays, colorectal adenomatous polyps are well-established precursors to most colorectal cancers. The most reliable method for diagnosing colorectal adenomas is the colonoscopy, which is labor intensive, expensive, and poorly tolerated by patients.

5. Role of Cell proliferation in the Development of Colorectal Adenoma

Cell proliferation can be defined as a process involving a sequential pattern of repeating changes in gene expression leading to the physical division of the cells (123). Increased proliferation may increase the rate of DNA damage and decrease the rate of repair, thus facilitating colon carcinogenesis. Hyperproliferation in the colorectal mucosa is thought to be a phenotypic biomarker of risk for colorectal neoplasms, and may be modulated by multiple interacting genetic, epigenetic, and environmental factors. Traditionally, in human studies there are two basic measurements of colorectal epithelial cell proliferation kinetics, one to indicate the rate of proliferation of colon crypt epithelial cells and the second to indicate the distribution of proliferating cells within the colon crypts (124). Hyperproliferation of the colorectal mucosa with

a shift of the proliferative zone to the upper portion of the crypt is thought to be an early step of a complex transition from normal mucosa to adenoma to carcinoma (125, 126). There have been two large clinical trials of calcium and colorectal epithelial cell proliferation (127, 128) and smaller trials (reviewed in ref. (124)). One large full-scale clinical trial found no evidence for a reduction in the overall proliferation level, but a marked statistically significant shift of the colon crypt proliferative zone downwards (127). Five out of eight small studies found a decrease in proliferation at the top of the crypt relative to the entire crypt, two studies reported an increase in the LI of the upper crypt compartments, and one study reported no change (reviewed in ref.(124)).

6. Biomarkers of Risk for Colorectal Neoplasms

There are no currently accepted, modifiable ("treatable"), pre-neoplastic biomarkers of risk for colorectal neoplasms that would be analogous to markers of risk for ischemic heart disease, such as lipid profiles. With the advent of biological measurements are markers of risk for ischemic heart disease 30 – 50 years ago, plausible preventive interventions – both lifestyle and pharmacologic – could be readily investigated, response to preventive treatment could be monitored, and, subsequently, with individual and population control of the "biomarker", mortality rates from the disease began a dramatic 67% decline which continues taday.

Based on recent advances in understanding molecular basis of colon carcinogenesis, researchers developed a panel of plausivle, reliable biomarkers that describe molecular phenotypes, including cell proliferation, differentiation, and apoptosis, of the normal-appearing colorectal epithelium. A marker of a cell in a proliferative phase is the MIB1 epitope of Ki-67 (129); an informative long-term indicator of proliferation is hTERT, a catalytic subunit of telomerase (130); and a marker of a cell that can no longer proliferate and is differentiated is p21 (131). Detection of expression of inhibitors (bcl-2) and promoters (bax) of apoptosis can be

readily demonstrated in characteristic gradients in crypts of normal colon tissue (132), and a general indicator of apoptosis is CK-18 (cytokeratin-18) (133).

To the author's knowledge, there is limited literature addressing the validity of cell proliferation of the normal-appearing colorectal epithelium, as a biomarker of risk for colorectal adenomas in case-control study. To address this, we conducted a case-control study of incident, sporadic colorectal adenoma in which we measured the expression of MIB1 and the distribution of MIB1 expression within the crypts of the normal-appearing colorectal mucosa and estimated their associations with colorectal adenoma and known risk factors for colorectal neoplasms.

Hypothesis

I hypothesize that subjects with a higher MIB1 expression in the whole normal colorectal crypt are at higher risk of colorectal adenoma and that the association is modified by risk factors for colon neoplasms.

I hypothesize that subjects with a higher MIB1 expression in the bottom 60% of the crypt are at higher risk of colorectal adenoma and that the association is modified by risk factors for colon neoplasms.

I hypothesize that subjects with a higher MIB1 expression in the top 40% of the crypt are at higher risk of colorectal adenoma and that the association is modified by risk factors for colon neoplasms.

I hypothesize that subjects with a larger shift expension of the proliferative zone to the upper portion of the crypt are at higher risk of colorectal adenoma and that the association is modified by risk factors for colon neoplasms.

Chapter II. Manuscript

Colorectal Epithelial Cell Proliferation and Risk for Incident, Sporadic Colorectal Adenomatous Polyps

By Dan Chen

Abstract

Background: It is hypothesized that colorectal epithelial cell proliferation kinetics are altered in the normal mucosa of patients at increased risk for colon cancer, the second leading cause of cancer deaths in the United States; however, there are no reports of well-conducted observational epidemiologic studies that have investigated this hypothesis.

Objective: To assess whether colorectal epithelial cell proliferation in the normal-appearing colorectal mucosa may be a valid, potentially modifiable biomarker of risk for colorectal neoplasms.

Methods: We conducted a pilot, colonoscopy-based case-control study (30 cases, 50 controls) of incident, sporadic colorectal adenoma. Cell proliferation was measured using immunohistochemistry for MIB1 (epitope of Ki-67). The labeling index (LI), the indicator of overall proliferation, was calculated as the proportion of labeled cells in the crypt; an LI_{b60} and LI_{t40} were also calculated to indicate the degree of proliferation in the upper 40% of the crypts (differentiation zone) and the lower 60% of the crypts (proliferative zone), respectively. A distributional index (Φ_h) to indicate expansion of the proliferative zone into the differentiation zone was calculated as the proportion of labeled cells in the crypts that were in the upper 40% of the crypts. Cases and controls were compared using analysis of covariance and logistic regression.

Results: In the adenoma cases relative to the controls, the LI, LI_{b60}, LI_{t40}, and Φ_h were proportionately lower by 17% (p = 0.03), 17% (p = 0.02), 28% (p = 0.08) and 28% (p = 0.33), respectively; the corresponding crude odds ratios (OR) and 95% confidence intervals were 0.39 (0.15, 1.05), 0.50 (0.19, 1.31), 0.62 (0.25, 1.54), and 0.88 (0.35, 2.17). The inverse associations tended to be stronger with adjustment for other risk factors, such as calcium and total fat intakes. The Φ_h was 36% higher (p = 0.05) among those with total calcium consumption above the mean.

Conclusion: Opposite to our hypotheses, these preliminary data suggest that lower cell proliferation as indicated by MIB1 expression in the normal colonic mucosa may be associated with increased risk of incident, sporadic colorectal adenoma as well as with modifiable risk factors thought to decrease risk for colorectal neoplasms.

1. Introduction

Colorectal cancer is the third most common incident cancer and the second most common cause of cancer death in the U.S. in men and women combined (1). It is a multi-factorial disease that appears to be the result of interacting lifestyle and genetic factors. The adenoma is a fairly reliable biomarker of colorectal cancer risk, and removal of this polyp reduces risk of cancer development (4). However, screening procedures for adenoma are costly, labor intensive, and poorly tolerated by patients. Pre-neoplastic biomarkers or profiles of biomarkers of risk for colorectal neoplasms will help address these challenges. However, there are no currently accepted, modifiable ("treatable"), pre-neoplastic biomarkers of risk for colorectal neoplasms that would be analogous to markers of risk for ischemic heart disease, such as lipid profiles. Hyperproliferation in the colorectal mucosa is thought to be a phenotypic biomarker of risk for colorectal neoplasms, and may be modulated by multiple interacting genetic, epigenetic, and environmental factors.

To the author's knowledge, there is limited literature addressing the validity of cell proliferation in the normal-appearing colorectal epithelium as a reliable biomarker of risk for colorectal adenomas. All of the published earlier studies, which used [^{3}H]thymidine ([^{3}H]dThd) (126, 134) or bromodeoxyuridine (BrdUrd) (135-137) as a marker of cell proliferation, suggested that hyperproliferation and an upwards expansion of the proliferative zone may be a common feature in cases with colorectal adenoma or cancer. However, all of these previous studies but one had very small sample sizes and all used convenience samples and were not true case-control studies with unbiased selection of cases and controls or ascertainment of potential confounding or effect modifying variables. To address the limitations in the previous literature, we conducted a case-control study of incident, sporadic colorectal adenoma in which we measured the expression of the MIB1 epitope of Ki-67 (an indicator of cells in or around the S-phase of the cell cycle (129) within the crypts of the normal-appearing colorectal mucosa and estimated associations of

parameters of its expression with colorectal adenoma and known and suspected risk factors for colorectal neoplasms.

2. Methods

Study Design

The Markers of Adenomatous Polyps II (MAP II, 2002) study is a pilot case-control study designed to investigate potential biomarkers of risk for incident, sporadic colorectal adenomas. Subjects were recruited from people scheduled for elective outpatient colonoscopy at Consultants in Gastroenterology, a large gastroenterology practice in Columbia, South Carolina (138). Subjects were 30 to 74 years old, of both sexes and all races, English-speaking, and capable of providing informed consent. Specific exclusion criteria included a history of previous colorectal adenomas, history or findings consistent with familial adenomatous polyposis (FAP) or hereditary nonpolyposis colon cancer syndromes, inflammatory bowel disease, bowel resection, history of cancer other than nonmelanoma skin cancer, and medical contraindication to colorectal mucosal biopsies (medically unstable, bleeding disorders, and cannot stop warfarin or aspirin), and polyethylene glycol colon-cleansing preparations.

Over a 5-month period, 351 patients were identified for recruitment; 232 (76%) of these agreed to participate in the study and 205 (51 cases and 154 controls) met final eligibility criteria and were included in the study. Due to limited tissue and financial resources, biopsies from only 80 subjects (30 cases and 50 controls) were processed for MIB1 expression and used for the analysis reported here.

Data on medical history, family history of cancer, diet, lifestyle, and anthropometrics were collected using mailed questionnaires, including a modified Willett Food Frequency Questionnaire, before the colonoscopy visit and knowledge of case-control status.

Biopsy Specimen Processing and Immunohistochemical Staining

One millimeter thick biopsy specimens were taken from the mucosa of a valve or fold in the rectum 10 cm above the level of the external anal aperture. The biopsies were then immediately placed in normal saline and transferred to an on-site dissecting microscope where they were immediately examined and reoriented. The biopsies were then immediately placed in 10% normal buffered formalin, left undisturbed for at least six hours, and transferred to 70% ethanol 24 hours after being placed in formalin. The biopsy specimens were embedded in paraffin blocks within two weeks of the biopsy procedure, cut and stained within another four weeks, and analyzed within another four weeks. Five slides with four section levels each taken 40 microns apart were prepared for Ki-67 antigen, yielding a total of 20 levels. Heat-mediated antigen retrieval (AR) was used to break the protein cross-links formed by formalin to uncover the epitope. Immunohistochemical (IHC) staining was done using a LSAB (Labeled Streptavidin Biotin) method on the DAKO Automated stainer (DAKO Corp., Carpinteria, CA). The Autostainer was programmed for MIB1 /Ki-67 antibody (DAKO Corp., catalog no. M7240, dilution 1:350), and TBS buffer (DAKO S1968). The slides were not counterstained. After staining, the slides were automatically coverslipped with glass coverslips with a Leica CV5000 Coverslipper (Leica Microsystems, Inc., IL) and placed in opaque slide folders. In each staining batch of slides, positive and negative control slides were included. Tonsil was used as a control tissue. The control tissues were fixed, embedded, and cut in the same manner as the patient's tissue. The negative and the positive control slides were treated identically to the patient's slides except that antibody diluent was used rather than primary antibody on the negative control slide.

Image analysis (Scoring)

The unit of analysis was the hemicrypt, defined as one side of a colon crypt bisected from lumen to base, which, in order to be eligible for analysis, had to be intact from the muscularis mucosae (bottom of the crypt) to the lumen (opening of the crypt). An average of 16 to 20 hemicrypts on each of two out of the three biopsies was scored for each set of slides. Hemicrypts with cell loss >2 as artifact from handling or cutting cannot be used. An image of each scorable hemicrypt was captured with a digital light microscope camera.

MIB1 expression, detected by immunohistochemical staining, was measured by counting labeled and unlabeled cells. An unlabeled cell was defined as a cell with a blue nucleus. A labeled cell was defined as a cell with a nucleus that was light brown in color. This was distinguished from any background stain that occasionally occurred. Whenever there was any doubt about whether a cell was unlabeled or labeled, it was scored as unlabeled. Labeled cells in this study were coded in "2". Unlabeled cells were coded as "1". Once a scorable crypt was located, the biopsy specimen number, slide number, section level number, and crypt number were entered into a scoring data entry program. The crypt was always counted under 400× magnifications. Cells within a crypt were counted by beginning at the top right of the crypt and continuing down and around to the top left.

For scoring reliability and quality assurance, slide sets from 10% of the subjects were randomly selected by the statistical team, blinded, and resubmitted to the scorer for rescoring(139).

Statistical Analysis

The overall cell proliferation rate, as indicated by the labeling index (LI) (see Formula 1), was calculated for each biopsy specimen by dividing the total number of labeled cells (LC) in the crypts by the total number of cells (TC) in the crypts and multiplying by 100% (127). We were

also interested in the cell proliferation rate in the bottom 60% of the crypt (LI_{b60}) (see Formula 2) as well as the cell proliferation rate in the top 40% of the crypt (LI_{t40}) (see Formula 3). A measure of the distribution of proliferating cells in the crypt, as indicated by the distributional index (Φ_h) (see Formula 4), was calculated on each specimen by dividing the number of labeled cells counted in the top 40% of the crypt (LC_{t40}) by the total number of labeled cells counted (LC) and multiplying by 100%. The natural logarithm transformation was used to improve normality. Before transformation, both the LI_{t40} and Φ_h were adjusted by adding 0.05 to the numerator.

$$LI = \frac{LC}{TC} \times 100\% \quad \textit{Formula 1}$$

$$LI_{b60} = \frac{LC_{b60}}{TC_{b60}} \times 100\% \quad \textit{Formula 2}$$

$$LI_{t40} = \frac{LC_{t40}}{TC_{t40}} \times 100\% \quad \textit{Formula 3}$$

$$\Phi_h = \frac{LC_{t40}}{LC} \times 100\% \quad \textit{Formula 4}$$

TC: total number of cells.

TC_{b60}: the number of cells in the bottom 60% of the crypt.

TC_{t40}: the number of cells in the top 40% of the crypt.

LC: total number of labeled cells.

LC_{b60}: the number of labeled cells in the bottom 60% of the crypt

LC_{t40}: the number of labeled cells in the top 40% of the crypt.

Statistical analyses were done using SAS 9.2 statistical software (© 2002-2008 by SAS Insti-tute). The subset of MAP II study population (30 cases and 50 controls) for whom slides were immunohistochemically processed for MIB1 were assessed for comparability using the t test for continuous variables and χ^2 test for categorical variables as appropriate. The correlation

among MIB1 expression measurements within each patient was not taken into account in order to ensure their normality. Mean proportional differences were calculated as the model-predicted mean MIB1 expression for cases minus that for controls divided by the mean for controls. Statistical significance of these measurements differences was evaluated by t test.

Potential confounders were evaluated based on biological plausibility and whether the variable of interest was associated with the exposure based on existing epidemiologic, medical, and basic science literature. Potential confounders considered in this analysis included age, sex, body mass index (BMI), physical activity, family history of colo-rectal cancer in a first-degree relative, smoking, alcohol consumption, aspirin and nonsteroidal anti-inflammatory drug (NSAID) use, current hormone replacement therapy (HRT) use, and total intakes of energy, fat, fiber, folate, calcium, and vitamin D. All nutrient values were adjusted for total energy according to the residual regression method (140). Continuous variables were dichotomized based on their means in the controls.

The association between MIB1 expression and risk of incident sporadic colorectal adenoma was assessed with log-linear models using means of hemicrypt measurements for each patient. The overall association between MIB1 expression in the colorectal mucosa and risk of incident, sporadic colorectal adenoma was evaluated by calculating odds ratios (OR) from logistic models. Both linear and logistic models contained the same set of potential confounders. A 95% confidence interval (95% CI) was calculated for each OR. To build the most parsimonious model that adequately controlled for confounding, first, all a priori identified potential confounding variables were ranked based on published literature on their hypothesized relative contributions to risk for colorectal neoplasms and then again on the strengths of their associations with the biomarkers investigated in this study. Next, a summary rank was calculated and covariates were added to the age- and sex-adjusted model one at a time according to their rank from highest to

lowest. The model that adequately controlled for confounding and had the smallest number of parameters was selected as the final multivariable-adjusted model.

The associations of MIB1 expression in the rectum with various demographic, lifestyle, and dietary characteristics were assessed by log-linear models. Potential confounders were entered into the model one at a time. The model also included a fixed effect to control for case-control status and an appropriate interaction term to check for potential modification of the effect of each characteristic by case-control status.

3. Resault

The sub-population of subjects whose biopsies were stained for MIB1 (30 cases and 50 controls) was compared with the entire MAP II study population (51 cases and 154 controls) and found completely comparable with respect to all considered characteristics (data not shown). Selected characteristics of cases and controls of the population considered in this analysis are shown in Table 1. On average, cases tended to be older, more likely to be male, more likely to be a current smoker and currently drink alcohol, more likely to regularly take a NSAID, less likely to regularly take aspirin, and tended to have higher intakes of total energy, fat, fiber, and calcium, and lower intakes of vitamin D, vitamin E, and folate than controls, but only the difference for total energy intake was significant at 95% level. Physical activity and BMI did not differ substantially between cases and controls.

Table 2 presents 1) "crude", age- and sex-adjusted, and multivariable-adjusted[1] mean MIB1 expression in cases and controls, and 2) odds ratios for associations of MIB1 measurements with risk for adenoma. After adjusting for potential confounders[2], expression of MIB1 in the whole crypt, in the bottom 60% of the crypt, and in the top 40% of the crypt was, respectively, 18% (p = 0.04), 18% (p = 0.03), and 28% (p = 0.16) lower in adenoma cases than in controls. The distributional index was, on average, 12% lower in cases than in controls, but the difference was not statistically significant (p = 0.47).

Risk of incident, sporadic colorectal adenomas was inversely associated with MIB1 labeling index in the whole crypt (OR_{LI} = 0.39; 95% CI: (0.15, 1.05)), in the bottom 60% of the crypt (OR_{LIb60} = 0.50 (0.19, 1.31)), and in the top 40% of the crypt (OR_{LIt40} = 0.62; 95% CI: (0.25, 1.54)), as well as with the MIB1 distributional index ($OR_{\Phi h}$ = 0.88; 95% CI: (0.35, 2.17)). The

[1] Selection of covariate is based on Table 4 and Table 5 in appendix.

[2] Age, sex, BMI, total energy intake, fat, calcium, and hormone therapy.

inverse association for LI_{b60} was stronger after controlling for age and sex (OR_{LIb60} = 0.44 (0.16, 1.19)). After additionally adjusting for BMI and total energy intake, the association for LI as well as LI_{t40} became stronger and the LI was statistically significantly associated with colorectal adenoma (OR_{LI} = 0.29; 95% CI: (0.09, 0.95)). The multivariable-adjusted[1] association of the Φ_h with adenoma was more strongly inverse than was the crude association but it also was not statistically significant ($OR_{\Phi h}$ = 0.72; 95% CI: (0.24, 2.12)).

We also assessed the potential of MIB1 expression in the rectum as a modifiable biomarker of risk by evaluating associations of MIB1 expression with various risk factors for colorectal cancer (Table 3). The only statistically significant finding was that MIB1 expression in the rectal mucosa was 36% (p = 0.05) higher in subjects whose total (dietary plus supplemental) calcium intakes were above the mean levels in the controls[2].

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[1] Age, sex, BMI, total energy intake, fat, calcium, and hormone therapy.

[2] Mean of calcium consumption in the control group.

4. Discussion

To our knowledge, this is the first reported case-control study to investigate associations of colorectal epithelial cell proliferation in the normal-appearing colorectal mucosa with risk of incident, sporadic colorectal adenoma and various risk factors for colorectal neoplasms, and thus the potential validity of colorectal epithelial cell proliferation as a potential modifiable biomarker of risk for colorectal neoplasms. Opposite to our hypotheses, our preliminary data suggest that MIB1 expression level in the normal rectal mucosa and an expansion of the proliferation zone into the upper portion of the crypt may be inversely associated with risk of incident, sporadic colorectal adenoma (Table 2). These findings were unexpected and opposite to the commonly held belief that hyperproliferation and/or an expansion of the proliferative zone to the upper portion of crypts in the normal-appearing colorectal mucosa are associated with increased risk of colorectal neoplasms. Our data also suggest that MIB1 expression in the rectum may be associated with modifiable risk factors for colorectal neoplasms (Table 2) in directions opposite to those hypothesized.

A possible explanation for our findings may be that unhealthy lifestyles and diets may not only damage cells but decrease their ability to rapidly replace them, perhaps via a compensatory decrease in apoptosis, which, in turn, would allow continuation of potentially deleterious damaged clones of cells. The question raised, then, is whether hyperproliferation or the diminished ability to renew damaged cells is most relevant to risk of incident, sporadic colorectal adenoma. Other than chance, another possible explanation is that MIB1 or other cell cycle S-phase-based proliferation markers are poor indicators of perhaps more relevant average, long-term proliferation as would be, for example, telomerase expression (as indicated by hTERT).

Proliferative abnormalities (hyperproliferation and an upward expansion of the proliferation zone) in the normal colorectal mucosa have been proposed as a possible marker of enhanced susceptibility to colorectal cancer. In earlier, small clinical studies using [^{3}H]thymidine ([^{3}H]dThd) (126, 134) and bromodeoxyuridine (BrdUrd) (135-137) as a marker of cell proliferation, hyperproliferation and an upwards expansion of the proliferative zone seemed to be a common feature in cases with colorectal adenoma or cancer. Terpstra's study (13 colon carcinoma, 11 large adenoma, 21 one or more small adenomas, 16 controls) using [3H]dThd as the indicator of cell proliferation observed higher cell proliferation for people with colorectal either carcinoma or adenoma (134). A smaller study (6 colorectal carcinoma, 8 adenomatous polyps, and 10 controls) using BrdUrd to assess cell proliferation found proliferation index in patients with colonic polyps and in those with colon cancer was significantly higher than in controls(135). Another case-control study (21 colorectal cancer, 19 adenomatous polyps, and 19 controls) using [3H]dThd as an indicator of cell proliferation found a significant upwards expansion of the proliferative zone of intestinal glands in patients with either polyps or cancer of the large bowel (126). A clinical study (75 patients) of cell proliferation using BrdUrd also found a general shifting of proliferative zone to the upper part of the crypts (137). Another study (200 adenoma; 150 adenocarcinoma; 50 adenoma plus adenocarcinoma, and 400 controls) of proliferation detected by BrdUrd found that hyperproliferation and the proliferative compartment shift coexist but are independent in the flat rectal mucosa of patients with colorectal neoplasia (136). However, all the previous studies but the last one had very small sample sizes and all used clinical population convenience samples., which were not true case-control studies with unbiased selection of cases and controls or ascertainment of potential confounding or effect modifying variables. The results of our study are not consistent with the findings of these previous studies. A somewhat similar situation was also found in Gwin's study of breast carcinomas in which some cases with a low recurrence score exhibited unexpected, unexplainably high MIB1 expression (141). The surprising proliferative activity observed in our study may relate to the accumulative

damage to the epithelia which diminished the ability to renew cells. A study on cell proliferation in different lesions in colorectal adenomas reported that MIB1 was expressed in 96% of the high-grade dysplasia cells but only in 3.5% of the carcinoma-in-situ cells ($p < 0.05$). So, it is possible that an unhealthy diet and lifestyle may continuously, slowly damage colorectal epithelial cells, and the cumulative damage may diminish the ability of colon crypts to rapidly renew the colorectal epithelial lining. This would be reflected in lower cell proliferation measurements and increase the probability that damaged cells could be retained for the more immediate need to maintain a barrier against the gut lumen environment, at the expense of allowing potentially deleterious damaged clones to be propagated and increasing future risk for colorectal neoplasms.

Our study had several strengths and limitations. Our study is the first reported case-control study to evaluate the potential validity of cell proliferation as a potential biomarker of risk for colorectal neoplasms. The study has several other strengths: 1) integration of laboratory, clinical, and epidemiologic methods; 2) detailed information on risk factors for colorectal neoplasms was collected before colonoscopy and adenoma diagnosis, thus minimizing possible recall bias; 3) the cases and controls were based on colonoscopy-detected, pathology-confirmed adenomatous polyps, thus limiting the chances of misclassification; 4) our study was tissue based, moving beyond traditional cell culture studies and animal models, taking advantage of being able to analyze the more relevant cell proliferation in human colon crypts. Analysis of the normal colorectal epithelium provides greater insight into colon carcinogenesis in humans, which is the result of gene-gene interactions, as well as gene-environment interactions.

However, our study had a small sample size and our results should be interpreted with caution. Larger sample sizes will be needed to validate our study results. A limitation of this study is that since it was retrospective, it cannot be determined which came first, the adenomas or the proliferation profiles. Also, since this study was colonoscopy-based, cases and controls may

have been more similar than if the study had been conducted in the general population. However, such a limitation would most likely have attenuated our results. Another limitation of this study was the exclusion of high risk genetic conditions (e.g., FAP, HNPCC); however, sporadic colon cancer makes up the majority of colon cancers. Proliferation measurements were made only on rectal mucosa, and thus it is unknown whether the case-control differences we found would have been seen at other levels of the colon; however, other studies suggest that risk group differences may be found throughout the colon (134). Usually for correlated data, repeated-measures models which contain a random intercept to account for multiple correlated cells counted within each patient are used. In our study, in order to increase the normality of the proliferation parameters, we used the mean of the proliferation parameters for each biopsy sample to represent the parameters of the patients. Since our study used tightly integrated laboratory, clinical, and epidemiologic methods, and the scoring quality was well controlled, our statistical results would likely be very similar to the results if using mixed models. Finally, as is generally true of most colonoscopy-based case-control studies of colorectal adenoma, a family history bias was noted in our study. However, colorectal epithelial cell proliferation was not associated with a family history of colorectal cancer in a first degree relative, nor did inclusion of family history into our models materially affect our results.

In summary, despite the commonly held belief that increased colorectal epithelial cell proliferation is a biomarker of increased risk for colorectal neoplasms, in this preliminary case-control study, we found proliferation to be inversely associated with risk for colorectal adenoma as well as with risk factors known or suspected to be associated with increased risk for colorectal neoplasms. Previous studies directed at assessing the potential validity of proliferation as a biomarker of risk for colorectal neoplasms were small and used convenience samples rather than unbiased selection of cases and controls. There is also biological plausibility for our findings. Additional, larger, properly-conducted case-control and

prospective studies are needed to resolve whether or not colorectal epithelial cell proliferation is a valid biomarker of risk for colorectal neoplasms.

Chapter III. Summary, and Possible Future Directions

In summary, to our knowledge, this is the first study to evaluate the potential validity of cell proliferation as a biomarker of risk for colorectal neoplasms using a case-control study design and MIB as an indicator of proliferation, in particular. Our preliminary data suggest that lower MIB1 expression in the normal colonic mucosa may be associated with increased risk of incident, sporadic colorectal adenoma as well as with modifiable risk factors for colorectal neoplasms in opposite to hypothesized directions. These finding are unexpected and opposite to our common belief that hyperproliferation of the colorectal mucosa with a shift of the proliferative zone to the upper portion of the crypt is associated with risk of colorectal adenoma. From our study, we argue that diminished ability to renew damaged cells may be more relevant to risk of incident, sporadic colorectal adenoma than is hyperproliferation.

Colorectal cancer pathogenesis involves combinations of cell proliferation, differentiation, and apoptosis. Therefore, further studies should investigate the other markers, such as apoptosis, which is involved in colorectal carcinogenesis. It is possible that MIB1 or other cell cycle S-phase-based proliferation markers are poor indicators of perhaps more relevant average, long-term proliferation than would be, for example, telomerase expression (as indicated by hTERT). In addition, a study with a larger sample size is recommended to allow further clarification of differences based on adenoma characteristics, such as degree of dysplasia, adenoma size, or adenoma location. Ultimately, a prospective study would be the best type of study to assess whether proliferation is associated with future colorectal neoplasms.

Table 1. Selected characteristics of incident, sporadic colorectal adenoma cases and controls; the Markers of Adenomatous Polyps II Study (sub-population).

Characteristics*	N (cases/ controls)	Cases	Controls	P†
Demographics				
Age (yrs.)	30/50	56.1 (6.7)	55.5 (7.8)	0.70
Male (%)	30/50	63.3	52.0	0.32
Caucasian (%)	30/50	96.7	98.0	0.86
Body mass index (kg/m^2)	29/49	29.6 (6.1)	30.3 (7.4)	0.87
1^{st} relative with colorectal cancer (%)	28/46	17.9	21.7	0.69
Lifestyle				
Physical activity (METs/day)	30/49	48.4 (10.9)	47.0 (12.4)	0.33
Alcohol consumption (%)	30/49			
Never		13.3	14.3	0.68
Former		16.7	14.5	
Current		70.0	61.2	
Smoking (%)				
Never	30/49	46.7	51.0	0.70
Former		36.7	38.8	
Current		16.7	10.2	
Take NSAID‡ at least once per week (%)	29/49	31.0	24.5	0.53

Take aspirin at least once per week (%)	29/49	37.9	42.9	0.67
Take hormone therapy (women) (%)	11/20	45.5	45.0	1.00
Dietary intakes				
Total energy (kcal/d)	30/49	2,028.2 (612.9)	1,471.1 (586.7)	<.01
Total fat[§] (g/d)	30/49	67.5 (15.8)	65.2 (13.0)	0.48
Dietary fiber[§] (g/d) *	30/49	16.4 (5.7)	14.7 (4.1)	0.09
Total[‖] vitamin D[§] (IU/d)	30/49	373.2 (304.1)	377.0 (269.1)	0.88
Total[‖] vitamin E[§] (mg/d)	30/49	16.0 (12.3)	17.9 (14.1)	0.56
Total[‖] folate equivalents[§] (mcg/d)	30/49	760.0 (458.4)	838.4 (472.3)	0.40
Total[‖] calcium[§] (mg/d)	30/49	914.7 (414.8)	886.2 (414.6)	0.60

[*] Continuous variables presented as mean (SD), categorical variables as proportions in percent.

[†] Based on t-test for continuous normally distributed variables, Wilcoxon's rank-sum test for continuous non-normally distributed variables, χ^2-test for categorical variables.

[‡] NSAID – Non-steroidal anti-inflammatory drug (not including aspirin).

[§] Energy adjusted using residual method.

[‖] Total = diet + supplements.

Table 2. Mib1 protein expression in normal-appearing mucosa of incident sporadic colorectal adenoma cases and controls; the Markers of Adenomatous Polyps II Study.

		Mib1 expression mean (SE)		Diff (%) *	P_{diff} †	OR ‡ (95% CI)
		Cases	Controls			
Model 1	Crude					
	LI	16.03 (1.07)	19.23 (1.05)	-17	0.03	**0.39** (0.15, 1.05)
	LI_{b60}	25.87 (1.06)	31.00 (1.05)	-17	0.02	**0.50** (0.19, 1.31)
	LI_{t40}	1.38 (1.16)	1.92 (1.12)	-28	0.08	**0.62** (0.25, 1.54)
	Φ_h	3.93 (1.13)	4.59 (1.1)	-14	0.33	**0.88** (0.35, 2.17)
Model 2	Adjusted for age and sex					
	LI	15.97 (1.07)	19.27 (1.05)	-17	0.03	**0.39** (0.15, 1.05)
	LI_{b60}	25.76 (1.07)	31.08 (1.05)	-17	0.02	**0.44** (0.16, 1.19)
	LI_{t40}	1.38 (1.16)	1.92 (1.12)	-28	0.08	**0.63** (0.25, 1.60)
	Φ_h	3.94 (1.13)	4.59 (1.1)	-14	0.35	**0.87** (0.35, 2.19)
Model 3	Adjusted for age, sex, BMI and total energy intake					
	LI	15.67 (1.07)	19.15 (1.05)	-18	0.03	**0.29** (0.09, 0.95)
	LI_{b60}	25.47 (1.07)	30.94 (1.05)	-18	0.02	**0.48** (0.15, 1.48)
	LI_{t40}	1.39 (1.18)	1.91 (1.13)	-27	0.14	**0.49** (0.17, 1.46)
	Φ_h	4.06 (1.14)	4.6 (1.1)	-12	0.48	**0.76** (0.26, 2.22)
Model 4	Adjusted for age, sex, BMI, total energy intake, fat §, calcium §, and hormone therapy					

LI	15.76 (1.08)	19.29 (1.06)	-18	0.04	**0.28** (0.08, 0.95)
LI_{b60}	25.42 (1.07)	31.12 (1.05)	-18	0.03	**0.48** (0.15, 1.56)
LI_{t40}	1.42 (1.18)	1.97 (1.14)	-28	0.16	**0.49** (0.16, 1.48)
Φ_h	4.12 (1.14)	4.7 (1.11)	-12	0.47	**0.72** (0.24, 2.12)

Abbreviations: OR, odds ratio; 95% CI, ninety-five percent confidence interval; LI, labeling index (#labeled cells in crypt/total #cells in crypt); LI_{b60}, labeling index in the bottome 60% of the crypt; LI_{t40}, labeling index in the top 40% of the crypt; Φ_h, distributional index (#labeled cells in the top 40% of th crypt/#labeled cells in crypt); BMI, body mass index,

* Difference between means (cases - controls) divided by mean in controls $\times 100\%$.

† Difference *P* value for comparison of means (analysis of covariance).

‡ The level of MIB1 expression was dichotomized using the mean in controls.

§ Included diet + supplements; energy adjusted using the residual method.

Table 3. Associations of MIB1 expression in normal-appearing rectal mucosa according to potential risk factors for colorectal neoplasms; the Markers of Adenomatous Polyps II Study

Characteristics*		MIB1 expression† mean (SE‡)			
		LI	**LI_{b60}**	**LI_{t40}**	**Φ_h**
Demographics					
Age (yrs.)					
	> 55	18.63 (1.06)	30.09 (1.06)	1.85(1.14)	4.58 (1.12)
	<=55	17.36 (1.06)	28.02 (1.05)	1.58(1.13)	4.14 (1.11)
	Diff (%)	7	7	17	11
	P_{diff} §	0.38	0.36	0.39	0.52
Sex					
	Male	18.19 (1.05)	29.32 (1.05)	1.75(1.13)	4.38 (1.11)
	Female	17.46 (1.06)	28.26 (1.06)	1.6(1.15)	4.23 (1.12)
	Diff (%)	4	4	9	4
	P_{diff}	0.61	0.63	0.64	0.82
Body mass index (kg/m^2)					
	≥ 30	17.74 (1.06)	28.90 (1.06)	1.59(1.14)	4.11 (1.12)
	< 30	17.85 (1.06)	28.54 (1.06)	1.82(1.14)	4.67 (1.12)
	Diff (%)	-1	1	-13	-12
	P_{diff}	0.94	0.87	0.47	0.41

Family history of colorectal cancer‖				
Yes	18.33 (1.1)	29.62 (1.09)	1.6(1.24)	3.98 (1.19)
No	17.80 (1.05)	28.56 (1.05)	1.81(1.11)	4.67 (1.09)
Diff (%)	3	4	-12	-15
P_{diff}	0.78	0.72	0.60	0.42
Lifestyle				
Physical activity¶				
≥ 22 METs/day	17.06 (1.06)	27.82 (1.06)	1.44 (1.14)	3.88 (1.12)
< 22 METs/day	18.61 (1.06)	29.62 (1.05)	1.99 (1.13)	4.90 (1.11)
Diff (%)	-8	-6	-27	-21
P_{diff}	0.30	0.43	0.09	0.14
Take aspirin/NSAID††				
Yes	18.43 (1.06)	29.69 (1.05)	1.74 (1.13)	4.34 (1.11)
No	16.97 (1.07)	27.50 (1.06)	1.64 (1.16)	4.42 (1.13)
Diff (%)	9	8	6	-2
P_{diff}	0.33	0.35	0.78	0.91
Smoking				
Current	18.07 (1.12)	29.43 (1.12)	1.34 (1.31)	3.37 (1.25)
Former/never	17.74 (1.04)	28.59 (1.04)	1.76 (1.10)	4.56 (1.08)
Diff (%)	2	3	-24	-26
P_{diff}	0.88	0.81	0.35	0.20
Alcohol consumption				

Current	18.38 (1.05)	29.53 (1.05)	1.82 (1.12)	4.56 (1.10)
Former/never	17.10 (1.07)	27.75 (1.07)	1.56 (1.17)	4.14 (1.14)
Diff (%)	7	6	17	10
P_{diff}	0.39	0.44	0.45	0.56
Hormone replacement therapy				
Current	17.28 (1.10)	28.17 (1.09)	1.72 (1.24)	4.67 (1.19)
Former/never	17.72 (1.09)	28.35 (1.08)	1.81 (1.22)	4.67 (1.17)
Diff (%)	-3	-1	-5	0
P_{diff}	0.84	0.96	0.87	1.00
Dietary intakes				
Total energy intake**				
High	17.61 (1.06)	28.41 (1.06)	1.60 (1.14)	4.18 (1.11)
Low	18.05 (1.07)	28.74 (1.07)	1.87 (1.17)	4.71 (1.14)
Diff (%)	-2	-1	-14	-11
P_{diff}	0.70	0.89	0.46	0.49
Total fat**				
High	17.30 (1.06)	28.08 (1.06)	1.62 (1.14)	4.32 (1.11)
Low	18.22 (1.06)	29.15 (1.06)	1.80 (1.14)	4.52 (1.12)
Diff (%)	-5	-4	-10	-5
P_{diff}	0.52	0.63	0.58	0.77
Dietary fiber**				
High	17.65 (1.05)	28.24 (1.05)	1.71 (1.13)	4.46 (1.10)

Low	18.03 (1.07)	29.58 (1.06)	1.61 (1.16)	4.07 (1.13)
Diff (%)	-2	-5	7	10
P_{diff}	0.80	0.57	0.74	0.56
Total‡‡ vitamin D**				
High	17.58 (1.05)	28.03 (1.05)	1.78 (1.13)	4.64 (1.10)
Low	18.11 (1.07)	29.67 (1.06)	1.63 (1.16)	4.13 (1.13)
Diff (%)	-3	-6	9	12
P_{diff}	0.73	0.49	0.65	0.47
Total‡‡ vitamin E**				
High	17.32 (1.06)	27.51 (1.05)	1.72 (1.14)	4.57 (1.11)
Low	18.42 (1.06)	30.17 (1.06)	1.73 (1.15)	4.30 (1.12)
Diff (%)	-6	-9	0	6
P_{diff}	0.48	0.26	1.00	0.71
Total‡‡ folate equivalents**				
High	17.73 (1.06)	28.14 (1.05)	1.77 (1.14)	4.57 (1.11)
Low	17.91 (1.06)	29.35 (1.06)	1.65 (1.15)	4.25 (1.12)
Diff (%)	-1	-4	7	7
P_{diff}	0.90	0.59	0.71	0.64
Total‡‡ calcium**				
High	17.68 (1.06)	27.86 (1.05)	1.96 (1.13)	5.08 (1.11)
Low	17.85 (1.06)	29.59 (1.06)	1.46 (1.14)	3.75 (1.12)

Diff (%)	-1	-6	35	36
P_{diff}	0.91	0.44	0.11	0.05

† Expression detected immunohistochemically and labeling quantified by counting method; results shown as labeling index of the full crypts (LI), labeling index of the bottom 60% of the crypts (LI_{b60}), labeling index of the top 40% of the crypts (LI_{t40}), and distributional index (Φ_h).

* All variables except age and sex adjusted for age and sex; smoking status also adjusted for alcohol consumption and alcohol consumption also adjusted for smoking status; total energy intake also adjusted for physical activity; total fat, fiber, vitamin D, vitamin E, folate, and calcium intakes also adjusted for total energy intake.

‡ SE - standard error.

§ Difference *P* value for comparison of means (analysis of covariance).

‖ Family history of colorectal cancer in a first-degree relative.

¶ Physical activity and total energy intake mutually adjusted for each other

** "Low" - below 50th percentile of sex-specific distribution in controls; "High" - at or above 50th percentile of sex-specific distribution in controls.

†† Aspirin/NSAID - takes aspirin or other nonsteroidal anti-inflammatory drug at least once a week.

‡‡ Total = diet + supplements.

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Appendix

Table 4. Crude and adjusted associations of MIB1 expression in normal-appearing rectum with the risk of incident, sporadic colorectal adenomas; the Markers of Adenomatous Polyps II Study (confounder assessment).

Covariates	LI		LI_{b60}		LI_{t40}		Φ_h	
	OR	Diff%	OR	Diff%	OR	Diff%	OR	Diff%
Crude 1 (no confounder)	*0.39*	*Ref.*	*0.50*	*Ref.*	*0.62*	*Ref.*	*0.88*	*Ref.*
Age, sex	0.39	-1	0.44	**-13.3**	0.63	2.3	0.87	-0.3
Age, sex, famcrc[*]	0.39	-0.8	0.40	-8	0.49	**-21.5**	0.73	**-16.7**
Age, sex, famcrc, BMI[†]	0.34	**-11.9**	0.38	-6.5	0.44	**-11.1**	0.66	-9.1
Crude 2 (including age, sex, famcrc and BMI)	*0.34*	*Ref.*	*0.38*	*Ref.*	*0.44*	*Ref.*	*0.66*	*Ref.*
Age, sex, famcrc, BMI, mets_ex[‡]	0.35	2.9	0.39	3.5	0.45	3.4	0.69	3.8
Age, sex, famcrc, BMI, drink[§]	0.31	-8.5	0.35	-6.9	0.38	**-12.5**	0.68	2.9
Age, sex, famcrc, BMI, smoke	0.35	2.3	0.38	2.4	0.45	1.6	0.70	5.9
Age, sex, famcrc, BMI, aspirin/NSAID[‖]	0.31	-9.4	0.35	-6.1	0.42	-5	0.63	-3.9

Age, sex, famcrc, BMI, hrt[¶]	0.32	-6.5	0.37	-2.1	0.39	**-11.4**	0.58	**-11.5**
Age, sex, famcrc, BMI, calor[**]	0.30	**-13.2**	0.38	2.4	0.38	**15.5**	0.80	**21.4**
Crude 3 (including age, sex, famcrc, BMI and calor)	*0.30*	*Ref.*	*0.38*	*Ref.*	*0.38*		*0.80*	
Age, sex, famcrc, BMI, calor, fat	0.31	4.1	0.41	7.6	0.56	**11**	0.84	4.5
Age, sex, famcrc, BMI, calor, fiber	0.31	4.4	0.41	5.7	0.50	-1.2	0.80	-0.4
Age, sex, famcrc, BMI, calor, vitamin D	0.29	-3.7	0.38	-0.5	0.50	-1.2	0.80	0.4
Age, sex, famcrc, BMI, calor, vitamin E	0.26	**-11.1**	0.36	-6.3	0.49	-4.1	0.80	-0.7
Age, sex, famcrc, BMI, calor, folate	0.27	-9.5	0.36	-6	0.49	-3.7	0.77	-3.7
Age, sex, famcrc, BMI, calor, calcium	0.25	**-16.7**	0.35	-4.4	0.51	4.5	0.82	6

Abbreviations: OR, odds ratio; 95% CI, ninety-five percent confidence interval; Diff%, proportional difference = [100% × (adjusted OR - crude OR)/crude OR].

† famcrc: family history of colorectal cancer in a first-degree relative.

* BMI: body mass index.

‡ mets_ex: physical activity.

§ drink: alcohol consumption.

‖ NSAID: non-steroidal anti-inflammatory drug (not including aspirin).

[¶] hrt: hormone replacement therapy

[**] calor: total energy intake

Table 5. Parsimonious model selection for parameters of MIB expression in normal-appearing rectum; the Markers of Adenomatous Polyps II Study.

Models	OR	95% CI	Diff%
LI			
Full model (including all variables)	0.30	(0.07,1.27)	-
BMI calor[*] VE Ca folate asns[†] fiber drink[‡] age sex	0.30	(0.08,1.07)	ref
BMI calor VE Ca folate asns fiber drink	0.31	(0.09,1.09)	5.4
BMI calor VE Ca folate asns fiber	0.31	(0.09,1.12)	5.4
BMI calor VE Ca folate asns	0.30	(0.08,1.03)	-0.7
BMI calor VE Ca folate	0.29	(0.09,0.94)	-4.4
BMI calor VE Ca	0.28	(0.08,0.94)	-6.0
BMI calor Ca	0.29	(0.09,0.97)	-1.3
LI_{b60}			
Full model (including all variables)	0.43	(0.10, 1.87)	-
age sex famcrc[§] BMI calor fat	0.41	(0.12,1.38)	ref
age sex famcrc BMI	0.38	(0.13,1.10)	-9.2
age sex famcrc	0.46	(0.14,1.15)	-2.9
age sex	0.40	(0.16,1.19)	5.6
LI_{t40}			
Full model (including all variables)	0.56	(0.15, 2.10)	-

BMI drink|smoke hrt calor fat mets_ex‖ calcium age sex famcrc	0.466	(0.14,1.61)	ref
BMI drink|smoke hrt calor fat mets_ex calcium	0.46	(0.14,1.51)	-0.2
BMI hrt¶ calor e_tfat mets_ex	0.50	(0.16,1.58)	8.2
BMI hrt calor e_tfat	0.48	(0.16,1.43)	2.4
Φ_h			
Full model (including all variables)	1.04	(0.30, 3.72)	-
famcrc BMI hrt calor mets_ex smoke calcium age sex	0.77	(0.25,2.36)	ref
famcrc BMI hrt calor mets_ex smoke calcium	0.74	(0.25,2.23)	-3.9
famcrc BMI hrt calor mets_ex calcium	0.75	(0.25,2.25)	-3.1
famcrc BMI hrt calor mets_ex	0.75	(0.25,2.22)	-3.6
famcrc BMI calor mets_ex	0.82	(0.28,2.42)	6.5
BMI calor mets_ex	0.81	(0.28,2.36)	4.7
BMI calor	0.76	(0.26,2.17)	-1.9

Abbreviations: OR, odds ratio; 95% CI, ninety-five percent confidence interval; Diff%, proportional difference = [100% × (adjusted OR – ref. OR)/crude OR].

* calor: total energy intake.

† asns: non-steroidal anti-inflammatory drug (including non-steroidal anti-inflammatory drug and aspirin)

‡ drink: alcohol consumption.

§ famcrc: family history of colorectal cancer in a first-degree relative.

‖ mets_ex: physical activity.

¶ hrt: hormone replacement therapy