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MLH1 and MSH2 Proteins as Potential Biomarkers of Risk for Colorectal Cancer

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Abstract

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By Eduard Sidelnikov

Colorectal cancer is the third most common incident cancer in the United States and the second cause of cancer deaths in men and women combined. Impairment of DNA mismatch repair (MMR) mechanisms in colonocytes is responsible for about 15% of colorectal cancers. MLH1 and MSH2 proteins play a crucial role in DNA MMR and loss of expression of either (or both) of these proteins is the main cause of DNA MMR insufficiency.

Two investigations from a colonoscopy based case-control study of incident, sporadic colorectal adenoma, and one investigation from a randomized, placebo-controlled, 2 × 2 factorial clinical trial of calcium and vitamin D_3 were conducted to characterize the expression of the mismatch repair genes *MLH1* and *MSH2* in normal colorectal crypt in humans and to assess parameters of their expression as potential modifiable biomarkers of risk for colorectal neoplasms.

The results from the case-control study showed that MLH1 and MSH2 expression in the ascending colon was statistically significantly lower in sporadic adenoma cases than in controls, but there was little evidence of case-control differences in the rectum and sigmoid colon. The clinical trial results showed that MLH1 and MSH2 expression along the full length of crypts increased in the vitamin D and calcium groups relative to the placebo group; vitamin D appeared to have the strongest effect on the expression of both proteins.

These pilot data suggest that lower MLH1 and MSH2 expression in the normal colonic mucosa, at least in the ascending colon, may be associated with increased risk of incident, sporadic colorectal adenoma as well as with modifiable risk factors for colorectal neoplasms, specifically, regular use of NSAIDs. Higher calcium and vitamin D intakes result in increased DNA MMR system activity in the normal colorectal mucosa of sporadic adenoma patients, and the strongest effects may be vitamin D related. These data support further investigation of MLH1 and MSH2 expression as potential modifiable biomarkers of risk for colorectal neoplasms.

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Chapter 1. Background and Significance

Introduction

Colorectal cancer is the third most common incident cancer and in the US in men and women combined[1]. Although mortality from colorectal cancer has decreased 39% over the last 30 years[2], it remains the second leading cause of cancer deaths. This suggests the importance of prevention in controlling the disease.

Incidence rates of colorectal cancer vary dramatically around the globe with about a 20-fold difference between the highest rates in North America, Western Europe and Australia and the lowest in India and Bangladesh[3, 4]. Overall, in countries defined by the WHO as "less developed" the average rate is 20% the rate in the industrialized countries[4].

Even within regions with high incidence rates time trends for those rates differ from region to region. While for the last 30 years incidence rates were approximately stable in the US[1], they have moderately increased in Europe and greatly in Asia[5].

Research shows that although genetic damage plays an important role in colorectal cancer development, environmental factors such as diet, lifestyle, and physical activity are also of major importance[6, 7]. The hypothesis that colorectal cancer is highly sensitive to changes in the environment is supported by the results of migrant studies and recent changes in incidence rates in Italy, Japan, China, and other countries[8]. These facts indicate that risk for colorectal cancer should be modifiable and calls for reliable and cost-effective methods of early diagnostics and screening for colorectal adenomas.

Colorectal adenoma, a benign neoplasm, remains the only reliable biomarker of risk for colorectal cancer. Adenoma patients have markedly higher risk of developing colorectal cancer, and removing adenomatous polyps reduces the risk for future colorectal cancer[9-11].

The only reliable method for diagnosing adenomatous polyps is the colonoscopy. But this method relies heavily on highly qualified personnel, is very expensive and labor intensive, and is not well received by many patients and physicians. This prompts the need for discovery of better biomarkers or profiles of biomarkers of risk for colorectal neoplasms.

Pathogenesis and Progression of Colorectal Cancer

Colorectal cancer is one of the cancers for which the stages and natural progression are rather well understood. The colonic epithelium contains about 10^7 crypts that are the main morphologic units of the colorectal mucosa (figure 1.1). Colonic epithelial cells originate from stem cells at the base of the crypt and migrate upward towards the surface epithelium layer. The 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.

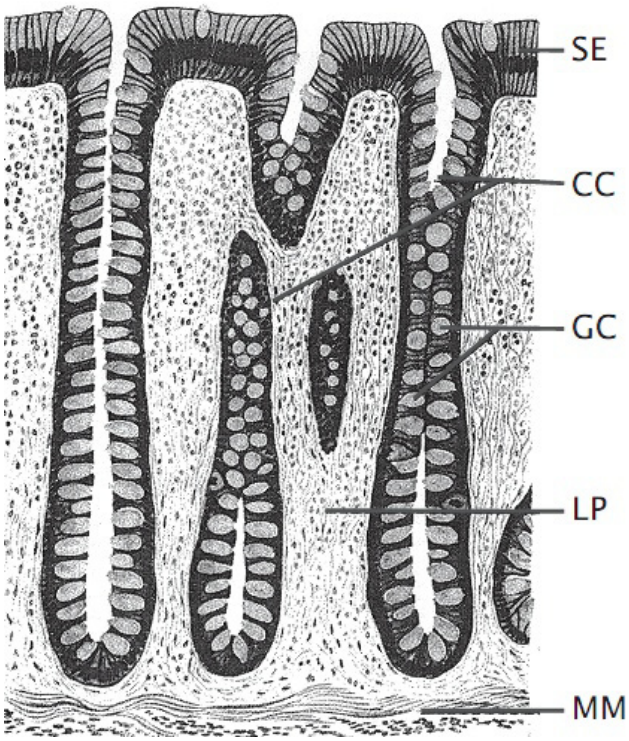

Figure 1.1. Morphology of normal colon tissue. Labels show surface epithelium (SE), colon crypts (CC), goblet cells (GC), lamina propria (LP), and muscularis mucosa (MM). The crypts open to the surface epithelium – in this cross section, some of the crypts appear partially or below the surface. From: The genetic basis of human cancer, Vogelstein B and Kinzler KW, eds., 2002.

Most colorectal cancers progress through a series of morphological stages depicted in figure 1.2. In the boxes below the schematic are the names of the genes that usually become mutated as the multi-step development of the disease progresses.

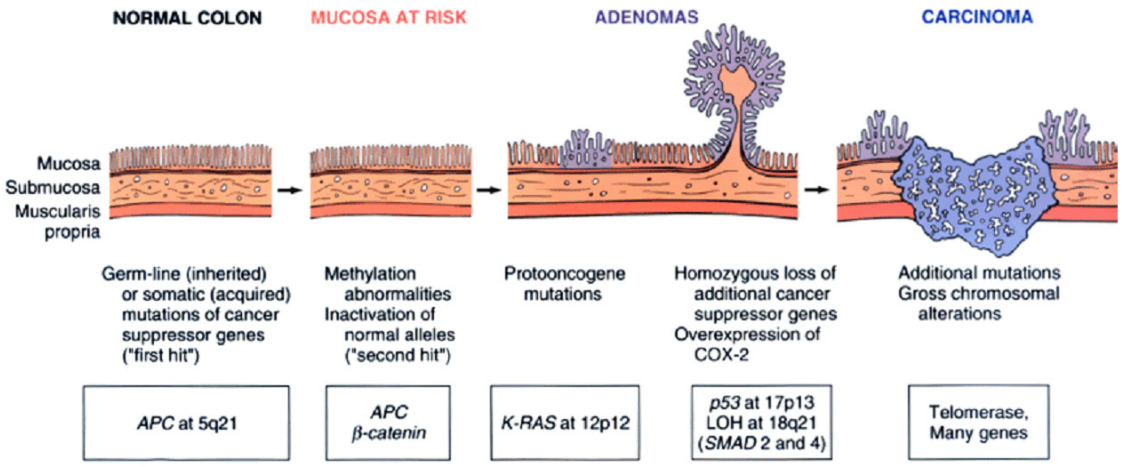

Figure 1.2. Morphologic and molecular changes in adenoma-carcinoma sequence. From: Robbins and Cotran pathologic basis of disease, Kumar V. et al., 2005.

During malignant transformation a normal colorectal epithelial cell has to accumulate multiple genetic alterations and establish successive clones characterized by relative growth advantage (increased proliferation rate, impaired apoptosis or both). It is believed that 6 to 10 clonal effects have to occur for a cancer to reach its malignant stage. In addition, a pre-cancerous clone must develop genomic instability, which allows subsequent mutations to occur with much higher probability. Genomic instability is a crucial component of carcinogenesis. Without it mutations would occur far too slowly for a cancer to develop within a person's lifetime[12].

Genomic integrity in a normal cell is constantly and very carefully maintained. The cell must pass several cell cycle and mitotic spindle checkpoints in order to complete its replication cycle. Failure to pass each of these checkpoints leads to apoptosis.

A genetic pathway of carcinogenesis is a process in which one particular type of genomic instability predominates, causing tumors to progress through characteristic histopathological stages with similar genetic alterations[12].

There are two main categories of genomic instability in colorectal cancer. The most common one is chromosomal instability (CIN), characterized by accumulation of numerical or structural chromosomal abnormalities. The second type is microsatellite instability (MSI), which is a consequence of impaired recognition and repair of mismatched bases in the daughter strand of the DNA during DNA replication. Either pathway is sufficient to drive colorectal carcinogenesis.

Approximately 70 – 85% of colorectal cancers develop via the "traditional" chromosomal instability pathway, also known as the "suppressor" or APC pathway[13]. This dissertation is focused on the other major pathway of colorectal carcinogenesis – the mismatch repair (MMR) pathway, also regarded as the microsatellite instability pathway. This pathway is responsible for about 15% of colorectal cancers. It seems, however, that patients with MMR deficient adenomas have greater risk of progressing into invasive cancer than those having CIN adenomas[12].

The main feature of the MMR pathway is interruption of normal review and repair of DNA after replication. The MMR system is composed of at least 7 proteins: hMLH1, hMLH3, hMSH2, hMSH3, hMSH6, hPMS1 and hPMS2, which associate with specific partners to form functional heterodimers[14]. hMLH1 and hMSH2 are essential components of the human mismatch repair machinery and form five functional heterodimeric proteins (hMSH2-hMSH3; hMSH2-hMSH6; hMLH1-hPMS1; hMLH1-hPMS2; hMLH1-hMLH3).

hMSH2 heterodimers are involved in recognizing DNA mismatches; the hMSH2-hMSH6 heterodimer predominantly binds to base-base mismatches and small insertion-deletion loops, and the hMSH2-hMSH3 heterodimer is primarily responsible for insertion-deletion loops (IDLs). Upon recognition, the hMSH2 heterodimer recruits the hMLH1 heterodimers to form an active complex that initiates and coordinates MMR activity. The hMLH1 component of the complex possesses ATPase activity and serves as a molecular matchmaker. It interacts with other components of the MMR system facilitating the process, and regulates termination of mismatch-provoked excision[15, 16].

Chang and colleagues reported that in the steady state substantially more hMSH2 protein was present in the cell in comparison with hMLH1 protein[17]. Increased expression of hMSH2 protein was also reported in various malignant tumors[18]; however the underlying mechanisms that caused the increased expression are still unknown. Elevated levels of hMSH2 may reflect an increased cell proliferation rate in malignant tumors or may be related to genomic instability.

DNA polymerase is prone to errors in short repeat sequences, and MMR dysfunction results in noticeable differences between tumor and germline DNA in a number of these sequences (microsatellites). Many colon cancers have mutations in only a small number of microsatellites. Five microsatellites (BAT25, BAT26, D5S346, D2S123, and D17S250) were identified as the most common sites of such mutations. Cancers that have mutations in two or more of these microsatellites are defined as high microsatellite instability (MSI-H) cancers; tumors with only one of these sites mutated are regarded as low microsatellite instability (MSI-L) cancers. Microsatellite stable (MSS) neoplasms do not have mutations in any of the five microsatellites.

MSI leads to a dramatic increase in the number of genetic errors, and several microsatellites are located in the coding regions of genes implicated in colorectal carcinogenesis, such as TGF-βRII, Bax, Caspase 5, MSH3, MSH6, β-catenin, APC, IGFII, and E2F4[12].

MSI-H cancers can develop via germline mutation of a particular MMR gene. Germline MMR gene mutations result in each cell in the body that have only one functional copy of the protein; a subsequent second somatic hit switches off the remaining normal allele of the gene rendering the cell deficient in the specific protein product and incapable of effective MMR. This is the chain of events in hereditary non-polyposis colorectal carcinoma (HNPCC or Lynch syndrome). hMLH1 and hMSH2 are the two most targeted genes in HNPCC. Epigenetic silencing of hMLH1 is a major cause of sporadic MSI-H CRCs[19, 20].

MSI-L cancers are clinicopathologically similar to MSS tumors. There is evidence of an association between MSI-L cancers and a higher prevalence of K-ras mutations and epigenetic silencing of the MGMT gene[21].

In 2006 Shibata and colleagues suggested that, at least in HNPCC, mismatch repair loss may occur early in carcinogenesis and may precede transformation by many divisions[22]. Recent research suggested that components of the MMR system, including hMSH2 and hMLH1, may participate in apoptosis signaling[22, 23]. Proteins involved in apoptotic cell mechanisms have been shown to affect levels of expression and activity of MMR proteins. Biochemical literature suggests that elevated expression of bcl-2, an anti-apoptotic protein, decreases MMR activity by down-regulating expression and activity of hMSH2 and hMSH6 – the two components that comprise the heterodimer involved in recognition of DNA base-base mismatches[24, 25].

Risk Factors for Colorectal Cancer

Among risk factors for colorectal cancer, age is one of the most important. About 90% of colorectal cancer cases occur in people of age 50 or older[1, 3, 26]. The incidence rates for colorectal cancer for both sexes and all races are very low in younger age groups, but sharply increase after age 50 years and continue to rise with age[26].

Incidence rates of colorectal cancer vary by sex. In North America, Australia, and other regions with high incidence rates of colon cancer rates in men exceed those of women[2-5, 8, 26, 27]. Rectal cancer incidence in men is about twice that in women[8].

Rates of colorectal cancer vary by race and ethnicity[3, 4, 26]. In the US, black men and women have modestly higher incidence rates than white men and women, respectively, and Asian and Native Americans have moderately lower rates, and lowest are Hispanics[26]. But while the incidence rates in Blacks are, on average, 8% higher than in Whites, the differences in mortality rates are substantially larger (44%)[2]. Blacks also tend to be diagnosed with more advanced stages of colorectal cancer[26].

Family history of colorectal cancer has been found to be positively associated with risk of this disease and not only as a result of the rare high-risk syndromes such as FAP and HNPCC, but also more generally in the population[8]. Overall about 30% of colorectal cancer cases have a history of the disease in a first degree relative which is believed to double their risk of colorectal cancer.

Dietary Factors

Animal experiments and observational epidemiologic studies suggest that diet, lifestyle factors, and physical activity are strongly associated with the occurrence of colorectal

adenomas and cancer and may partially explain much of the variability in incidence rates[3, 6, 7, 28, 29].

Although animal studies[30, 31] suggest that fruit and vegetables inhibit the development of colorectal cancer, the results of observational epidemiologic studies are not so conclusive. While studies by Lin et al[32] and Terry et al[33] support this hypothesis, others fail to detect an association despite rather large sample sizes[34, 35].

High consumption of fat and red meat have been widely studied as risk factors for colorectal cancer[36, 37]. However, it remains unclear whether these associations are confounded by other dietary factors. One of the main problems is that fat and meat consumption are often highly correlated with total energy intake, thus making it difficult to isolate the effects of these factors.

In their combined analysis of 13 case-control studies Howe et al[38] found that there was a positive association of colorectal cancer with energy intake, but after controlling for this factor, there was little evidence for such an association with fat intake alone. This conclusion was supported after an analysis of the results of cohort studies[39] and a randomized trial[40]. It appears that high energy intake, especially combined with low physical activity, is an adverse risk factor for colorectal cancer, but fat intake alone may not be.

The epidemiologic literature is inconsistent with respect to red and processed meat as risk factors for colorectal cancer. A recent meta-analysis involving 15 prospective studies that evaluated the association of red meat consumption and risk of colorectal cancer, and 14 studies on the consumption of processed meats found a RR* of 1.28 (95% C.I†.: 1.15 – 1.42)

* RR – risk ratio
† C.I. – Confidence Interval

for red meat and a RR of 1.20 (1.11 – 1.31) for processed meat. Both risk ratios compare the highest with the lowest intake category[41].

There are several plausible mechanisms that can explain such an association. Meat consumption is associated with intakes of heterocyclic amines[42] and polycyclic aromatic hydrocarbons[43]. These compounds are typically formed during cooking and are proven carcinogens in animal models. Processed meat, along with the above mentioned substances, may also contain nitrates and nitrites. Studies show that all those compounds contribute modestly but significantly to the fact that meat consumption was found to be positively associated with the risk of adenomatous polyps[44, 45]. However, a prospective study by Sanjoaquin et al[46] reported that vegetarians in the US and Europe were not at statistically significant reduced risk of colorectal cancer. Those results suggest that it is very unlikely that red meat intake alone accounts for the difference in colorectal cancer risk between industrialized and underdeveloped countries.

Several epidemiologic studies[47, 48] reported that diabetic patients had a moderately elevated risk of colorectal cancer. High blood glucose levels were found to be positively associated with colorectal cancer risk even among non-diabetic patients[49]. This suggests that intake of readily absorbed carbohydrates may be a risk factor for colorectal cancer.

Two measures are used to characterize the effect of readily absorbed carbohydrates: glycemic index and glycemic load. Glycemic index is a measure of the blood glucose response to a portion of a particular carbohydrate food, expressed as a fraction of that by an equivalent quantity of a standard carbohydrate such as glucose[50]. The glycemic load is the product of glycemic index and quantity eaten, summed for all carbohydrates in the diet[51].

European studies[52, 53] found that both glycemic index and glycemic load were positively associated with risk of colorectal cancer. An American study, on the other hand, failed to detect an association between glycemic load and the risk of adenomatous polyps[54]. This may indicate that if the association exists, it is with the later stages of the adenoma-carcinoma sequence.

The significance of fiber as a protective factor against colorectal cancer also remains controversial. The hypothesized mechanisms of action are thought to depend on physical properties of undigested cell wall polysaccharides as a bulk laxative and provider of butyrate. There are indications that butyrate suppresses mitosis, increases differentiation, and stimulates apoptosis in human cells *in vitro*[55], but those effects have been proven to be very difficult to confirm *in vivo*.[6] While some epidemiologic studies found fiber or certain types of fiber (i.e., from legume) to be inversely associated with risk of colorectal cancer[7, 32, 56-58], other prospective studies[33, 59] failed to detect such an association despite high levels of fiber intake. Moreover, several clinical trials did not detect an effect of dietary supplementation with fiber on adenoma recurrence[60]. Recently, the EPIC project[61], a large European prospective study, reported a statistically significant inverse association for the highest quintile of fiber intake vs. the lowest quintile. Furthermore, the study results suggested that the adverse effects of a high consumption of red meat may be substantially reduced in subjects with high fiber consumption. A high consumption of fiber may protect against colorectal cancer, but when consumed for a long period of time and in larger quantities than is normally consumed in most developed countries[6].

The literature on the association between consumption of fruit and vegetables and risk of colorectal cancer has been inconsistent. Earlier observational studies reported

negative associations between dietary intake of fruit and vegetables and colorectal cancer risk[62-64]. But as large prospective studies have been completed, there appears to have been a progressive loss of statistically significant evidence for this association[34, 35].

Cohort studies performed in Sweden[33] and the Nederlands[65] reported that women who were in the highest quartile of fruit and vegetable intake were at significantly lower risk of colorectal cancer, however, there was no significant association in men. On the other hand, studies by Frentzel-Beyme and Chang-Claude on did not find any effect of vegetarian diet on risk of colorectal cancer in German cohort[66]. The American study by Lin and colleagues using the data from Women's Health Initiative cohort also could not find enough evidence to support the association between dietary intake of fruit and vegetables and colorectal cancer risk[32]. Summarizing epidemiologic evidence, Potter pointed out that although overall association between dietary consumption of fruits and vegetables and colorectal cancer risk is often below 1.0, especially for women, its confidence interval almost always includes the null value[3, 67]. Fruit and vegetable intake was reported to have no significant association with rectal cancer.

Associations of fish products and n-3 unsaturated fatty acids with colorectal neoplasms were extensively studied. Epidemiologic evidence has been mixed. MacLean et al[68] recently reviewed studies of omega-3 fatty acids and colorectal cancer. They reported no significant association between n-3 fatty acids intake and disease risk; they also found no evidence to support any benefit from high fish intake. On the other hand, the EPIC study reported a highly statistically significant inverse association between fish consumption and risk of colorectal cancer[69]. Any protective effects of fish may depend on genetic polymorphisms affecting the expression of key proteins involved in peroxisome proliferator-

activated receptors (PPAR) signaling and COX-2 expression. These genes are associated with the metabolism of fatty acids, which supports the idea that it may be the particular composition of fatty acids that provide possible protective effects.

Another substance that seems to be associated with risk of colorectal cancer is folate. Folate is an amino acid that is obtainable only from diet and is essential for normal DNA synthesis and repair. A deficiency of 5,10-methylenetetrahydrofolate leads to misincorporation of uracil into DNA and excess double-strand breaks[70]. Abnormal DNA methylation causes aberrant gene expression and genomic instability, and is proposed as a major cause of genetic damage leading to cancer[71]. Folate intake and blood levels have been reported to be inversely associated with the risk of colorectal adenomas in case-control and prospective studies of different populations[72-75]. There is a concern, however, that very high levels of folate intake may adversely affect DNA methylation and favor the development of colorectal carcinomas from adenomas, particularly when the adenoma is established[76].

Calcium and Vitamin D

The analytic observational literature evidence is somewhat supportive but inconsistent regarding whether calcium reduces risk for colorectal cancer in humans. Of at least 45 analytic epidemiologic studies[72, 77-120] (22 case-control[72, 77-97] and 23 cohort studies[98-120]) that investigated the association between calcium and colorectal cancer, 34 reported inverse associations[77-90, 98-100, 102-108, 110-114, 116-120] six – positive associations[72, 91-94, 109], and four studies reported no association [95-97, 115]). The results of sixteen studies (8 case-control studies and 8 cohort studies) that reported negative associations were statistically significant. None of the studies that reported positive associations produced statistically significant results.

The results of observational epidemiologic studies and animal experiments suggested the possibility that calcium could be used as a chemopreventive agent. Seven randomized trials looked at calcium in this capacity having recurrent adenoma as their endpoint[121-128]. Six of these studies[122, 123, 125-128] found that patients receiving calcium supplements had lower risk of colorectal adenoma recurrence and one[124] found no association. Baron and colleagues reported moderate inverse association of calcium supplementation on recurrent colorectal adenomas (RR = 0.81, 95% C.I.: 0.67 – 0.99)[128]. The effect of calcium seemed to be independent of initial dietary fat and calcium intake and lasted for 5 years after the supplementation phase had ended[125].

The two most prominent hypotheses for a protective effect of calcium against colorectal cancer are the bile acid hypothesis and the direct effect on cell cycle hypothesis[129]. Bile acids are a consequence of fat intake, and are known to be mutagenic and to otherwise damage cells, provoking compensatory hyperproliferation. The bile acid hypothesis asserts that bile acids can be neutralized in the gut lumen by free calcium which is present only when calcium intake is in excess of that required for absorption and that which will be bound by phosphate. The level of calcium intake required to do this is estimated to be 1,500 – 2,000 mg daily[129]. The direct effect of calcium on cell cycle hypothesis, based on in vitro studies, states that free calcium has a direct effect on the cell cycle, decreasing proliferation and increasing differentiation. This hypothesis is supported by experimental evidence[130-134], but the exact mechanisms of the direct effect of calcium are not completely clear. These mechanisms may involve interaction with E-cadherin[135, 136], cyclic AMP[137], calmodulin[138, 139], tyrosine kinases[140], and/or ornithine decarboxylase[141]. More recently, a calcium sensing receptor was identified in the gut, and it appears that a function of calcium and the calcium

sensing receptor is promoting E-cadherin expression and suppression of β-catenin activation[142].

Vitamin D is less known as a protective factor against colorectal cancer than calcium. As for calcium, strong plausible mechanisms of action have been proposed, and the in vitro and animal experimental evidence is quite strong. Vitamin D refers to a family of related steroid hormones best known for its important role in maintaining calcium and phosphorus homeostasis, and in regulating bone metabolism[143, 144].

The expression of the nuclear vitamin D receptor in virtually all tissues of the body (including the bowel mucosa) suggests a role for vitamin D beyond mineral regulation. In particular, recent in-vitro studies show that vitamin D and vitamin D analogues can modulate cellular growth and proliferation. In human cell lines from the colon and other organs, these compounds inhibit proliferation, induce differentiation, and promote apoptosis[145-151]. D signaling can have anti-proliferative effects on the large bowel mucosa, causing, for example, inhibition of proliferation in human rectal mucosal explants and in biopsies from patients with ulcerative proctocolitis[152]. Vitamin D supplementation normalizes the colonic crypt hyperproliferation of vitamin-D deficient animals[153], and inhibits induction of mucosal ornithine decarboxylase by bile acids[154] or the bowel carcinogen DMH[155].

Randomized clinical trials have found that calcium and vitamin D supplementation reduces risk of colorectal adenoma and colorectal cancer[124, 156-158] and that calcium supplementation and vitamin D status appear to act largely together to reduce the risk[124]. Of at least 13 analytic epidemiologic studies[72, 82, 85, 86, 93, 95, 98, 102, 106, 110, 114, 159, 160] (six cohort studies[98, 102, 106, 110, 159, 160] and seven case-control studies[72, 82, 85, 86, 93, 95, 114]) that investigated the possible

association of vitamin D and colon cancer, 11 suggested an inverse association[72, 82, 85, 93, 95, 98, 102, 106, 159-161], one reported a null association[86], and none reported a positive association. All six of the cohort studies that found inverse associations reported RRs ranging from 0.3 to 0.73; four of these studies[102, 106, 114, 159] were statistically significant. Risk estimates reported by case-control studies ranged from ORs of 0.4 to 0.77; of these, three[82, 85, 93] were statistically significant.

Despite the long known intricate physiological relationship between calcium and vitamin D, there has been little study of a possible synergistic effect of the two against colon cancer. One of the goals of this dissertation project is to begin to understand their independent and synergistic effects. Several studies have reported that vitamin D supplementation has a stronger anti-neoplastic effect in animals given relatively high-calcium diets[162-164]. However, one experimental study[165] in rodents found that calcium and vitamin D3 supplementation together had a smaller protective effect than either supplement alone. Because of the measurement error associated with these two exposures, it is understandable that human observational studies may have difficulty clarifying any interactive effects. In two large cohort studies in humans[119, 120], there was clear evidence of a positive interaction between the two nutrients. Also, in the Calcium Polyp Prevention Study, there were strong indications that vitamin D enhanced the chemopreventive effect of calcium[166].

Body Mass and Physical Activity

There is a wide and consistent body of literature indicating a positive association between overweight and obesity and risk of colorectal cancer. There are a number of case-control studies[48, 167-170] that reported a positive association between obesity or body-mass index (BMI) and risk of colorectal neoplasia. This association, however, may be modified by

gender since BMI was reported to be a much stronger predictor of colorectal cancer risk in men than in women. A French study by Boutron-Ruault et al[167] investigated the associations between BMI and physical activity with colorectal adenomas and carcinomas as separate endpoints. BMI (highest vs. lowest quintile) was found to be associated with the risk of large (> 10 mm) adenomas, but no such association was observed for small adenomas or cancer. Physical activity was reported to be inversely associated with risk of cancer, but was not associated with adenomas.

An early prospective study of men by Lee et al[171] with a 26-year follow-up period observed a positive association between colon cancer and BMI: the RR was 1.08 (95% C.I.: 1.04 – 1.13) for each unit of BMI, controlling for other factors. Those in the heaviest BMI quintile had 2.4 times the risk of colorectal cancer compared to the subjects in the lightest quintile of the cohort, although the relative risks were statistically significant only among those who were less physically active. These associations have since been replicated by various prospective studies conducted around the world; these studies found similar associations for women[172-179].

There is an increasing body of evidence indicating that BMI may not be an ideal measurement of the adipose tissue content in a human body. Ross et al[180] investigated correlations of different anthropometric indicators of adiposity with MRI-verified distributions of adipose tissue within a human body. Waist circumference was identified as the strongest single predictor of total adiposity, and waist-to-hip ratio (WHR) was the best anthropometric correlate for visceral adipose tissue. Results from a later study by Janssen et al[181] led the authors to the same conclusion that fat distribution may be a more important factor in the pathogenesis of various co-morbidities of obesity than just simple total adipose

tissue. Waist circumference was found to be associated with increased risk of colorectal cancer independently of BMI[177]. This was observed in both sexes, and especially among sedentary people. Waist-to-hip ratio was positively associated with risk of colon, but not rectal, cancer in large prospective European study[178].

There is growing body of evidence that obesity and colon cancer are causally linked by chronic asymptomatic inflammation in the colonic mucosa[69, 182-185]. Obesity is often regarded as a low-grade inflammatory condition in which adverse effects are exerted on a variety of target organs probably including the colon[186, 187].

Physical activity is widely seen as a factor that decreases risk of colorectal cancer. A recent study of more than 400,000 people across Western Europe found a 20 – 25% reduction of colon cancer risk in people who engaged in two hours of moderate or one hour of vigorous physical activity per day compared to sedentary individuals[188]. No association was observed for rectal cancer. Reduced risk with higher physical activity is strongly supported by other studies[178, 189].

Other Risk Factors

Aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) have been consistently associated with a reduced risk of colorectal cancer[190-192]. The exact mechanism is not yet fully understood, but is thought to be via NSAID's inhibition of COX-2. Randomized clinical trials[193-196] 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. A cohort study by Chan and colleagues found a significant reduction in the risk of colorectal cancers that expressed COX-2, but not for those that did not[197]. The

reduction of the risk of COX-positive tumors was also found with increasing aspirin dose and duration of use[197].

Several studies indicate that cigarette smoking may damage colorectal mucosa and play a role in colorectal carcinogenesis[198]. Evidence suggests that 12% of colorectal cancer deaths may be attributable to smoking. The carcinogens found in tobacco increase tumor growth in the colon and rectum[199, 200]. It has been proposed that smoking increases risk for colorectal cancer only after a 40-year induction period. Although the association between smoking in the distant past and risk of colorectal cancer has been supported by several studies[48, 201], Le Marchand and colleagues also found a similar association for more recent smoking[48].

A positive correlation has also been found between cigarette smoking and colorectal adenomas; smokers had a greater number of colorectal polyps, and large adenomas were associated with long-time smoking[198, 199, 201]. Smoking may also expedite colorectal carcinogenesis[200].

The literature on association of alcohol consumption with colorectal cancer has not been consistent. While the majority of analytical studies point towards positive associations for both colorectal adenoma and cancer, many of those studies did not yield statistically significant results[67, 74, 174]. There are indications that the association may vary in strength depending on the specific segment of the colon. Giovanucci et al. also found that the positive association between alcohol intake and the risk of colon cancer, being present in groups with lower consumption of folate and methionine, disappeared in people who had high levels of these substances in their diet[74, 174].

Biomarkers of Risk for Colorectal Cancer

To date there is no generally accepted pre-neoplastic biomarkers of risk for colorectal cancer. Fecal occult blood test, sigmoidoscopy and colonoscopy are used for screening and diagnosing colorectal adenomas and colorectal cancer. However, colonoscopy, currently the gold standard screening and diagnostic procedure, has several important limitations: it requires colon preparation, is expensive, labor intensive, dependent on highly trained medical personnel and unpleasant for patients. These limitations make it difficult to use colonoscopy for screening, clinical and epidemiologic research and assessment of preventive interventions.

MLH1 and MSH2 have a good potential to be tissue biomarkers or parts of tissue biomarker panel for colorectal cancer. Development of tissue biomarkers of risk will greatly enhance the value of colonoscopy and will allow assessing individual risk for colorectal cancer with greater precision and refining subsequent screening strategy for a patient depending of their risk. Colorectal mucosa, the tissue where the pre-cancerous and cancerous lesions form, is the obvious place to look for tissue biomarkers. Finding reliable tissue biomarkers is also a step toward development of biomarkers that could be measured in biological fluids such as serum or urine. Biomarkers that can be easily identified in surrogate fluids will facilitate screening and indentifying people with high risk of colorectal cancer, evaluation of preventive interventions and scientific research.

Conclusion

Despite obvious advances in diagnostic and therapeutic techniques over the last several decades, colorectal cancer still remains the second leading cause of cancer deaths in the US for men and women combined[1]. The five-year survival in the United States is only

66%[2], which happens to a considerable degree because only 38% of patients are diagnosed with early stages of the disease in which the tumor is localized to the bowel wall and the prognosis is the best[202]. Since more than 90% of colorectal cancers are adenocarcinomas, which originate from adenomas[3, 8, 67], early diagnostics and removal of colorectal adenoma dramatically decreases risk of colorectal cancer[202].

Due to serious limitations, colonoscopy, currently the gold standard diagnostic procedure, is not ideal for screening and has limited use in scientific research. Alternative screening and risk detection methods that would lack those limitations need to be developed in order to facilitate screening for colorectal cancer and colorectal adenomas, enhance evaluation of preventive and therapeutic interventions, and epidemiologic and clinical research.

This dissertation contributes to developing of panels of tissue biomarkers and the long-term goal of developing profiles of pre-neoplastic biomarkers of risk for colorectal cancer that could be identified in tissue and in surrogate fluids. Measurable and reliable biomarkers of risk can be used in observational epidemiologic studies and for endpoints in dietary, lifestyle, and chemoprevention trials of modulation of risk for colorectal neoplasia. Associations of biomarkers of risk with known risk factors for colorectal cancer can provide valuable insight into colon biology and carcinogenesis as well as help to identify the most suitable risk factors for chemoprevention and risk modification.

Since the MMR pathway is an important pathway in colorectal carcinogenesis, the key proteins of the pathway are very likely candidates for inclusion in a biomarker panel.

Chapter 2. Materials and Methods

Specific Aims

The major goals of this dissertation project are:

1. From a case-control study of incident, sporadic colorectal adenoma (MAPII): a) Investigate differences in the distributions of the MLH1 protein within normal colorectal crypts in patients with incident, sporadic colorectal adenomas and adenoma-free controls; b) investigate associations of MLH1 expression in normal-appearing colorectal mucosa with risk factors of colorectal cancer such as age, sex, family history of colorectal cancer in first degree relatives, body-mass index (BMI), physical activity, aspirin and NSAID use, and total energy, calcium, vitamin D, and folate intakes; c) evaluate associations of MLH1 expression in normal-appearing rectal mucosa with adenomatous polyps overall and according to adenoma location within the large intestine, and adenoma histopathological characteristics; and from a randomized, placebo controlled 2×2 factorial clinical trial of calcium and vitamin D (CaDvMAP): d) investigate the joint and separate effects of calcium and vitamin D on the expression of MLH1 in the normal-appearing rectal mucosa of patients with recent removal of incident sporadic colorectal adenomas.

2. From a case-control study of incident, sporadic colorectal adenoma (MAPII): a) Investigate differences in the distributions of the MSH2 protein within normal colorectal crypts in patients with incident, sporadic colorectal adenomas and adenoma-free controls; b) investigate associations of MSH2 expression in normal-appearing colorectal mucosa with risk factors of colorectal cancer such as age, sex, family history of colorectal cancer in first degree relatives, body-mass index (BMI), physical activity, aspirin and

NSAID use, and total energy, calcium, vitamin D, and folate intakes; c) evaluate associations of MSH2 expression in normal-appearing rectal mucosa with adenomatous polyps overall and and according to adenoma location within the large intestine, and adenoma histopathological characteristics; and from a randomized, placebo controlled 2×2 factorial clinical trial of calcium and vitamin D (CaDvMAP): d) investigate the joint and separate effects of calcium and vitamin D on the expression of MSH2 in the normal-appearing rectal mucosa of patients with recent removal of incident sporadic colorectal adenomas.

Hypotheses

1. Increased risk of incident, sporadic colorectal adenoma is associated with lower expressions of MLH1 and MSH2 in normal appearing colorectal mucosa; lower expression of MLH1 and MSH2 in the rectal mucosa is associated with their decreased expression in more proximal sections of the colon. Expression of MLH1 and MSH2 in colorectal mucosa is inversely associated with higher age, BMI, total energy intake, and family history of colorectal cancer in a first degree relative; and positively associated with higher levels of physical activity, and higher intakes of calcium, vitamin D, and folate.

2. Six months of calcium (2,000 mg elemental calcium as calcium carbonate daily) and vitamin D (800 IU of vitamin D_3 daily) supplementation results in increased expression of MLH1 and MSH2 in normal-appearing rectal mucosa compared to participants who received placebo. The combination of calcium and vitamin D treatments works synergistically and results in even higher expression of MLH1 and MSH2 than in groups receiving each of the agents alone.

Study Designs and Data Collection

Markers of Adenomatous Polyps II (MAPII) Study

The Markers of Adenomatous Polyps II (MAP II) study is a pilot colonoscopy-based case-control study (51 cases and 154 controls) designed to investigate potential biomarkers of risk for incident, sporadic colorectal adenomas. Participants were recruited from people scheduled for elective outpatient colonoscopy at Consultants in Gastroenterology, a large gastroenterology practice in Columbia, SC. To be eligible for the study, participants must have been 30 – 74 years old, English speaking, and capable of providing informed consent. Persons of both sexes and all races were eligible to participate in the study.

Specific exclusion criteria were history of previous colorectal adenomas or inflammatory bowel disease, bowel resection, history of cancer other than non-melanoma skin cancer, medical contraindication to colorectal mucosal biopsies (medically unstable, bleeding disorders, cannot stop warfarin or aspirin), and known contraindications to a polyethylene glycol colon cleansing preparation.

Over a five-month period 351 patients were identified for recruitment; of these 232 (76%) agreed to participate in the study; and of these 205 (51 cases and 154 controls) met final eligibility criteria and were included in the study.

Prior to the colonoscopy visit, patients completed mailed questionnaires, including a modified Willett Food Frequency Questionnaire. The questionnaires were used to obtain information on medical history, family history of cancer, diet, lifestyle, and anthropometrics.

The colon site and in vivo size and shape of all polyps found were recorded, and all polyps were removed and placed in separate containers for transportation. All polyps were

examined by the study index pathologist who identified polyp type, subtype, and degree of atypia according to criteria established by the National Polyp Study.

Biopsies were performed during usual care colonoscopies after a 12-hour fast and polyethylene glycol bowel cleansing preparation. Pinch biopsies were obtained from three sites of the colon: rectum (10 cm above the anus), mid-sigmoid, and proximal ascending colon. Six biopsies per site were taken. The biopsies were harvested from normal-appearing mucosa and no biopsies were taken within 4 cm of a polypoid lesion.

Biopsies specimens were fixed by 10% normal buffered formalin for 24 hours then stored in 70% ethanol. Within a week the specimens were processed and embedded in paraffin blocks with three biopsies per colon site per block. The paraffin blocks were then cut into three micron-thick sections with each section level 30 microns apart.

Calcium, Vitamin D, and Biomarkers of Colorectal Cancer (CaDvMAP) Study

CaDvMAP is a preliminary, randomized, double-blind, colonoscopy-based, placebo-controlled, 2 x 2 factorial chemoprevention clinical trial designed to estimate the efficacy of, and the variability of response to, calcium and vitamin D on the individual components and aggregate profile of a molecular phenotype panel of biomarkers of risk for colorectal cancer.

Participants were recruited from the patient population attending the Digestive Diseases Clinic, the Emory Clinic, Emory University. To be eligible for the study, patients must have been 30-75 years of age, in general good health, and capable of informed consent. They must have had a history of at least one pathology-confirmed adenomatous colonic or rectal polyp within the past 36 months; had no contraindications to calcium or vitamin D supplementation or rectal biopsy procedures; and had no medical conditions, habits, or medication usage that would otherwise interfere with the study.

Specific exclusions were supplemental intake of calcium and/or vitamin D greater than the recommended daily allowance (RDA); supplemental daily intake of vitamin A greater than 10,000 IU/day; a major diet change within the previous six months; an inability to refrain from aspirin use for seven days; current, planned or recent participation in another clinical trial; pregnancy, trying to get pregnant, or breast-feeding; familial adenomatous polyposis; an elevated serum calcium or creatinine; supraphysiologic levels of 25-OH vitamin D at their study eligibility visit; kidney stones or sarcoidosis within the previous 20 years; a history of a bleeding disorder or current use of anticoagulant medication; use of a thiazide diuretic in an amount greater than the equivalent of 50 mg of hydrochlorothiazide daily; immunosupression; a history of osteoporosis; use of lithium, an ion exchange resin, tetracycline, or indomethacin; renal insufficiency; dementia; cardiovascular disease that moderately or severely limited activity; inflammatory bowel disease; a malignancy other than nonmelanoma skin cancer within the previous five years; hyperparathyroidism or hypoparathyroidism; uncontrolled hypothyroidism or hyperthyroidism; enema or laxative dependence; active peptic ulcer disease; gastrectomy; bowel resection; active liver or pancreatic disease; intestinal malabsorption syndromes; narcotic or alcohol dependence; on a weight loss diet; and a nondeliberate weight loss of 10% or more in previous three months.

Recruited participants (n = 92) were randomly assigned to four treatment groups (23 people per group): placebo, supplemental calcium (2,000 mg elemental calcium as calcium carbonate), vitamin D_3 (800 IU) and combination of calcium and vitamin D_3 in doses stated above. The treatment period was six months, and participants attended follow-up visits at 2 and 6 months after randomization and were contacted by telephone at monthly intervals between the second and final follow-up visits. One millimeter thick biopsy specimens were taken from the rectal mucosa 10 cm proximal to the external anal aperture.

Harvested biopsies were fixed by 10% normal buffered formalin for 24 hours and stored in 70% ethanol. Within a week the specimens processed and cut according to the same procedure that was used for MAP II case-control study (see above).

Laboratory Methods and Image Analysis

Both studies used the same laboratory methods and biomarker expression measurement procedure.

The biopsies in formalin were left undisturbed for at least six hours, transferred to 70% ethanol 24 hours after being placed in formalin, embedded in paraffin blocks within two weeks of the biopsy procedure, cut and stained within another four weeks. Five slides with four 3 micron thick section levels each taken 40 microns apart were prepared for each patient, yielding a total of 20 levels per subject per colon site (case-control study) or per visit (clinical trial). Heat-mediated antigen retrieval was used to break the protein cross-links formed by formalin to uncover the epitope. To accomplish this, slides were placed in a preheated Pretreatment (PT) Module (Lab Vision Corp., CA) with 100x Citrate Buffer pH 6.0 (DAKO S1699, DAKO Corp., Carpinteria, CA; further referred to as DAKO) and steamed for 40 minutes. Then the slides were immunohistochemically processed using the following solutions. For MLH1: an anti-MLH1 antibody (BD Pharmingen 554072) in a 1:15 dilution, a DAKO LSAB2 detection kit (DAKO K0675), and 3,3' diaminobenzidine (DAB; DAKO K3466) as the chromogen. For MSH2: an anti-MSH2 antibody (Oncogene NA27) in a 1:500 dilution, Envision+ Detection System (DAKO K4007), and 3,3' DAB (DAKO Envision+ K4007). No counterstaining was used and all stained slides were glass coverslipped with a Leica Automated Coverslipper (Leica Microsystems, Inc., IL). In each staining batch of slides, positive and negative control slides were included. Tonsil was used

as a control tissue for both biomarkers. 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.

Biomarker expression detected by immunohistochemical staining was quantified in the stained slides using densitometry implemented by image analysis methods. Image analysis procedure and biomarker expression data collection were conducted by the student using a light microscope, digital camera, drawing board, and a specially designed plug-in to ImagePro Plus (Media Cybernetics, Inc.) image analysis software.

The imaging and analysis unit was a hemicrypt, defined as one side of a colonic crypt bisected from base to colon lumen surface. Intact (at most two contiguous cells missing) hemicrypts extending from the muscularis mucosae to the colon lumen were considered eligible for quantitative image analysis ("scorable").

The two biopsies with the greatest number of "scorable" hemicrypts were selected for quantitative image analysis ("scoring"). Intact hemicrypts were "scored" in order from the first section of the first biopsy from left to right. The goal was to find at least 16 "scorable" hemicrypts per biopsy (32 per patient). If the 16th hemicrypt was reached before the level was finished, the scorer continued scoring until either the level was finished or the 20th hemicrypt was scored, whichever came first. No more than 20 hemicrypts per biopsy were scored.

If the two best biopsies for a patient had less than 32 "scorable" biopsies, an attempt was made to cut more slides. If that did not solve the issue, scoring was completed if the two best biopsies had 16 or more "scorable" hemicrypts between them. All three biopsies were

scored only if there was less than a total of 16 "scorable" hemicrypts between the two best biopsies.

To ensure adherence, a scorer was guided through the scoring protocol by the computer software. For each scored slide background correction images were obtained and controlled for by the computer program. All images were taken at 200x magnification and stored as 16-bit grayscale 1,600 × 1,200 pixel images.

"Scorable" hemicrypts were identified and manually traced by the student. A traced hemicrypt was divided by the software into segments corresponding in width to that of 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.

Statistical Methods

Populations of cases and controls from the MAPII case-control study and the treatment groups from the clinical trial were checked for comparability with respect to known important risk factors of colorectal cancer.

For MAPII case-control study data a complete array of descriptive univariate analyses were run to compare the expression of MLH1 and MSH2 in the rectum, sigmoid, and ascending colon in cases and controls; the association between the proteins' expressions and dietary and lifestyle risk factors for colorectal cancer; and associations of the proteins' expressions with various histopathological characteristics of adenomatous polyps. The distributions of MLH1 and MSH2 within colorectal crypts were evaluated by Loess nonparametric models. Statistical models that were used to answer the question of specific

aims 1 and 2 are defined below. Continuous outcome variables were checked for normality and mathematical transformations were considered if significant departure from a normal distribution were discovered. Normality of residuals and influential observations were checked using standard model diagnostic options in PROC MIXED (SAS 9.2)[203].

Definition of Statistical Models:

To evaluate associations between MLH1 and MSH2 protein expression, overall and by colon site, and risk of incident, sporadic colorectal adenoma the following linear mixed models were fit to MAPII data.

Specific Aims 1a and 2a.

To evaluate crude associations between protein expression and risk of incident, sporadic colorectal adenoma, the model defined below were fit separately for each of the three studied colon sites (rectum, sigmoid colon and ascending colon).

$$OD_{ij} = \beta_0 + \beta_1 STAT_i + \sum_k \beta_k BATCH_{ki} + b_{0i} + \varepsilon_{ij} \quad (1)$$

Where: OD_{ij} – optical density for i-th patient, j-th hemicrypt

$STAT_i$ – case-control status of the i-th patient

$BATCH_{(k-1)i}$ – indicator variable for (k – 1)-th staining batch

$$\begin{cases} 1 & \text{if a hemicrypt was stained in batch } (k-1) \\ 0 & \text{otherwise} \end{cases}$$

b_{0i} – random intercept for i-th patient; $b_{0i} \sim N(0, \sigma_b^2)$

ε_{ij} – residual error; $\varepsilon_{ij} \sim N(0, \sigma^2)$

The model defined above was also used to evaluate crude associations between overall protein expression and the risk of incident, sporadic colorectal adenoma, but the definition of the outcome variable was modified as follows: OD_{ij} – optical density for i-th patient, j-th colon site; j = 1, 2, 3. All other variables were defined as before.

Specific Aims 1b and 2b.

The multivariate linear mixed model was used to control for potential confounders. First potential confounders were considered one at a time considering potential effect modification by the appropriate interaction term (model 2). Then the variables that significantly changed estimates in the univariate analysis and for which there was no significant interaction were controlled for simultaneously. No interaction terms were considered in the model at that stage because of the limited sample size.

$$OD_{ij} = \beta_0 + \beta_1 STAT_i + \beta_2 SEX_i + \beta_3 SEX_i \times STAT_i + \sum_k \beta_k BATCH_{ki} + b_{0i} + \varepsilon_{ij} \quad (2)$$

Where SEX_i – gender of the i-th patient and all other variables are defined as in model 1.

Potential confounders were dichotomized as follows: age (<55, ≥55), family history of colorectal cancer in a first degree relative (yes, no), physical activity (low, high), BMI (<30, ≥30), smoking and alcohol consumption (never, ever), aspirin and NSAID use (yes, no), total energy intake and total intakes of fat, carbohydrates, fiber, calcium, vitamin D and folate (low, high). A person was considered a smoker if they smoked at least 100 cigarettes during their lifetime and "ever" category included former and current smokers. Subjects who took aspirin or other NSAID at least 7 times a week were considered regular aspirin or NSAID users. "Low" and "high" categories of dietary and anthropometric variables were

determined based on sex-specific distributions in the controls. All nutrient values were adjusted for total energy according to the residual regression method[204].

To control for several potential confounders at a time, the appropriate number of indicator variables representing the confounders in question were added to model 2. Predicted mean optical densities for cases and controls from models 1 and 2 were used to calculate proportional differences: $Diff = \frac{\overline{OD_{CA}} - \overline{OD_{CO}}}{\overline{OD_{CO}}} \times 100\%$, where $\overline{OD_{CA}}$ – mean optical density among cases, $\overline{OD_{CO}}$ – mean optical density among controls. Proportional differences in optical densities were used to evaluate the direction and strength of the association between protein expression in colorectal mucosa and risk of adenoma. These analyses were performed for crude and multivariate adjusted associations.

Generalized linear (logistic) mixed models were used to obtain odds ratios describing the association between MLH1 and MSH2 protein expressions in normal-appearing colorectal mucosa and risk of incident, sporadic colorectal adenoma.

$$Logit\left(P\left[OD_{isj} = 1\right]\right) = \beta_0 + \beta_1 STAT_i + b_{0i} + u_{0is} + \varepsilon_{isj} \quad (3)$$

Where: OD_{isj} – optical density for i-th patient, s-th colon site, j-th hemicrypt dichotomized at staining batch specific mean optical density in controls

$$\begin{cases} 1 & \text{if above the staining batch specific mean in controls} \\ 0 & \text{otherwise} \end{cases}$$

u_{0is} – random intercept describing variability between colon-sites within i-th patient;

ε_{isj} – residual error.

All other variables are defined the same way as for model 1.

Multivariate adjusted odds ratios were obtained by adding appropriate variables describing potential confounders. All variable definitions were identical to those for model 2.

Specific Aims 1c and 2c.

Generalized linear (logistic) mixed models were fit to evaluate the associations between MLH1 and MSH2 expression in normal appearing rectal mucosa and characteristics of incident, sporadic colorectal adenomas. The following adenoma characteristics were considered: location (right colon [cecum, ascending colon, hepatic flexure and transverse colon] or left colon [splenic flexure, descending colon, sigmoid colon and rectum]), multiplicity (single or multiple adenomas), degree of dysplasia (mild or moderate/severe), histological type (tubular or tubulovillous/villous), and shape (pedunculated or sessile). All variables were dichotomized according to the categories given in parentheses. The model is defined below:

$$Logit\left(P\left[OD_{ij} = 1\right]\right) = \beta_0 + \beta_1 CHARCT_i + b_{0i} + \varepsilon_{ij} \quad (4)$$

Where: OD_{ij} – optical density for i-th patient, j-th hemicrypt dichotomized at staining batch specific mean optical density in controls

$$\begin{cases} 1 & \text{if above the staining batch specific mean in controls} \\ 0 & \text{otherwise} \end{cases}$$

$CHARACT_i$ – an indicator variable representing a dichotomous variable for a specific adenoma characteristic.

The random intercept and the residual error are defined the same way as for model 1.

Specific Aims 1d and 2d.

Effects of calcium and vitamin D supplementation on the expression of MLH1 and MSH2 in normal-appearing rectal mucosa were evaluated by fitting the linear mixed model to the CaDvMAP data.

$$OD_{ij} = \beta_0 + \sum_3 \beta_t RxGROUP_i + \beta_4 VISIT_i + \sum_3 \beta_p (RxGRPUP \times VISIT) + \sum_k \beta_k BATCH_{ki} + b_{0i} + \varepsilon_{ij} \quad (5)$$

Where: $RxGROUP_i$ – treatment group for i-th subject. Four treatment groups (placebo, calcium, vitamin D and calcium + vitamin D) are represented by three indicator variables each defined as follows:

$\begin{cases} 1 & \text{if in the appropriate treatment group} \\ 0 & \text{otherwise} \end{cases}$, placebo group is treated as a referent group;

$VISIT_i$ – treatment visit variable $\begin{cases} 1 & \text{follow up visit} \\ 0 & \text{baseline visit} \end{cases}$

All other variables are defined the same way as for model 1.

Addressing the Issue of Multiple Hypothesis Testing

To address the potential problem of multiple hypothesis testing I calculated the probability of no association given a statistically significant finding defined by the term "false positive report probability" (FPRP). The methods of calculating FPRP are described in detail in a recent publication by Wacholder et al[205]. Although the paper describes this method in the context of genetic epidemiology, I believe that the technique is applicable to other situations requiring accounting for multiple testing. Briefly, the concept of FPRP is considered in the context of the null hypothesis (H_0), the alternative hypothesis (H_A) and a statistical test (T) aimed at evaluating the hypothesis under study. The magnitude of the

FPRP is determined by three factors: 1) prior probability π of a true association of the tested exposure with a disease ($\pi = Pr[H_A \text{ is true}]$), 2) α-level or observed p-value, and 3) statistical power $(1 - \beta)$ to detect the odds ratio of the H_A at the given α-level or p-value.

The FPRP for standard statistical significance testing is defined as Pr (H_0 is true | association is deemed statistically significant) = Pr (H_0 is true | $T > Z_\alpha$), where Z_α is the α point of the standard normal distribution. The distinction between α-level, statistical size, and FPRP is crucial; α-level is the probability of a statistically significant finding, given that the null hypothesis is true, whereas FPRP is the probability that the null hypothesis is true, given that the statistical test is statistically significant. The following table from the paper by Wacholder et al illustrates the joint probability of significance tests and truth of hypothesis.

Table 2.1. Joint probability of significance of test and truth of hypothesis

Truth of H_A	**Significance of test**		**Total**
	Significant	**Not Significant**	
True association	$(1 - \beta)\pi$ [True positive]	$\beta\pi$ [False negative]	π
No association	$(1 - \pi)\ \alpha$ [False positive]	$(1 - \alpha)\ (1 - \pi)$ [True negative]	$1 - \pi$
Total	$\pi\ (1 - \beta) + \alpha\ (1 - \pi)$	$\beta\pi + (1 - \alpha)\ (1 - \pi)$	1

Based on the table 2.1, the FPRP has the following mathematical formula:

$$FPRP = \frac{\alpha(1-\pi)}{\alpha(1-\pi)+\pi(1-\beta)} = \frac{1}{1+\left(\frac{\pi}{1-\pi}\right)\left(\frac{1-\beta}{\alpha}\right)}.$$

From the equation one can immediately notice that FPRP is high when π is much smaller than α and when the power $(1 - \beta)$ is low. The authors propose the FPRP value of 0.5 for small and moderate sample sizes to determine the results that are "noteworthy". Calculated values of FPRP for different statistical power and assumed prior probabilities are presented in table 2.2.

Table 2.2. Calculated FPRP for different prior probabilities and statistical power ($\alpha = 0.05$)

Prior probability of a true association	Power $(1 - \beta)$		
	0.7	**0.8**	**0.9**
0.25	0.18	0.16	0.14
0.1	0.39	0.36	0.33
0.01	0.88	0.86	0.85
0.001	0.99	0.98	0.98

Power Considerations*

Biopsies for 89 subjects of the MAPII study (46 cases and 43 controls) were processed and scored for MLH1 protein expression, MSH2 expression was measured in 88 patients (43 cases and 45 controls). The MAPII study design provides for biopsies taken from three colon sites: the rectum, sigmoid colon and ascending colon creating repeated measurements. CaDvMAP is a randomized placebo controlled 2×2 factorial clinical trial including 23 patients in each of the four treatment groups. Protein expression was measured on two occasions: baseline and follow-up visits that were six months apart.

The calculations evaluate statistical power that the MAPII study design with a given sample size attains for different values of minimal differences in optical density. For the MAPII study design three repeated measurement per subject (one for each colon site) were assumed for power calculation. Repeated measures ANOVA model was postulated for the power calculations.

The design of the CaDvMAP study assumes only one measurement per subject, so a one way ANOVA model was postulated for power calculation. Minimum detectable effect size was calculated for a given sample size. Effect size for a one-way ANOVA model with

* Power calculations were performed using PASS 2005 software (Hintzle J., 2005. PASS 2005. NCSS LLC. Kaysville, Utah).

four levels of a predictor variable is given by the formula: $f = \frac{\sigma_m}{\sigma}$, where σ is a within group standard deviation and σ_m is a standard deviation of the group means $\sigma_m = \sqrt{\sum_{i=1}^{4} \frac{(\mu_i - \bar{\mu})}{4}}$. No effect on protein expression over the six month period was assumed for the placebo group of the CaDvMAP study; the calcium and vitamin D groups were assumed to have the same effect on MLH1 expression, and a synergistic effect of calcium and vitamin D was assumed for the group receiving both treatments (Calcium and vitamin D).

All parameters required for the calculations were estimated from the data available. The results are summarized in tables 2.3 and 2.4.

Table 2.3. Statistical power for detecting differences between MLH1 and MSH2 proteins expressions in cases and controls, MAPII Study (Specific aims 1 and 2)

Cases	Controls	Measurements per subject	Difference to be detected (units of optical density)	Standard deviation	Auto-correlation	Alpha	Power
MLH1							
46	43	3	160	266	0.3	0.05	0.82
46	43	3	170	266	0.3	0.05	0.86
46	43	3	180	266	0.3	0.05	0.90
46	43	3	190	266	0.3	0.05	0.93
MSH2							
43	45	3	180	300	0.2	0.05	0.80
43	45	3	190	300	0.2	0.05	0.84
43	45	3	200	300	0.2	0.05	0.87
43	45	3	210	300	0.2	0.05	0.90

Table 2.4. Statistical power for detecting differences in MLH1 protein expression among four treatment groups, CaDvMAP Study (Specific aims 1 and 2)

Treatment group size	Total sample size	Minimum detectable effect size	Standard deviation within a group	Alpha	Power
MLH1					
23	92	0.40	355	0.05	0.80
23	92	0.35	355	0.05	0.90
MSH2					
23	92	0.40	410	0.05	0.80
23	92	0.35	410	0.05	0.90

Chapter 3. MutL-homolog 1 (MLH1) Expression and Risk of Incident, Sporadic Colorectal Adenoma: Search for Prospective Biomarkers of Risk for Colorectal Cancer*

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Abstract

To characterize the expression of the mismatch repair gene *MLH1* in normal colorectal crypts in humans, and assess parameters of its expression as a potential biomarker of risk for colorectal neoplasms, we conducted a pilot, colonoscopy-based case-control study (51 cases, 154 controls) of incident, sporadic colorectal adenoma. Biopsies of normal-appearing rectal, sigmoid, and ascending colon mucosa were procured, immunohistochemically processed for MLH1 protein, and analyzed using custom quantitative image analysis procedures.

MLH1 expression in the ascending colon was, on average, 49% proportionally lower in cases than controls ($p = 0.03$), but there was little evidence for case-control differences in the rectum and sigmoid colon. In cases and controls, average MLH1 expression in the ascending colon tended to be lower with increased age (by 56% [$p = 0.02$] and 25% [$p = 0.16$], respectively, for those ≥ 55 years), and with a history of colorectal cancer in a first degree relative (by 22% [$p = 0.56$] and 34% [$p = 0.16$], respectively). Among cases, but not controls, average MLH1 expression tended to be higher with current alcohol consumption,

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regular aspirin use, and higher total intakes of calcium, vitamin D, and folate. There was little indication of similar differences in the rectum.

These preliminary data suggest that lower MLH1 expression in the normal colonic mucosa, at least in the ascending colon, may be associated with increased risk of incident, sporadic colorectal adenoma as well as with modifiable risk factors for colorectal neoplasms, thus supporting further investigation of MLH1 expression as a potential "treatable" biomarker of risk for colorectal neoplasms.

Introduction

Colorectal cancer, the second leading cause of cancer mortality in the United States[206], is a multifactorial disease that appears to be the result of lifestyle factors interacting with genetic ones[3, 8]. The vast majority of so-called "sporadic" colorectal cancer develops in the adenomatous polyp, a benign intestinal tumor that is the only accepted biomarker of risk for colorectal cancer[3, 8].

The adenoma is a fairly reliable biomarker of colorectal cancer risk, and removal of this polyp reduces risk of cancer development, but screening procedures for adenoma are costly, labor intensive, require highly qualified personnel, and are not well accepted by physicians or patients. This prompts the need for discovery of pre-neoplastic biomarkers or profiles of biomarkers of risk for colorectal neoplasms 1) to identify persons most at risk, and 2) that could be treatable and thus used to monitor the efficacy of preventive interventions.

The mismatch repair (MMR) pathway is one of the two main molecular pathways of colorectal cancer development, accounting for about 15% of colorectal neoplasms[207]. The

DNA MMR system involves a complex set of proteins that identifies and repairs mismatch errors that occur during DNA replication[8, 208].

The MutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli) [Homo sapiens] (MLH1) gene is located at chromosome 3p21-23[209]. The protein product of the MLH1 gene, an important part of the MMR system, has no known enzymatic activity but probably recruits other DNA repair proteins to the mismatch repair complex[209, 210].

Because of its crucial role in the MMR pathway, the MLH1 protein is one of the potential biomarkers that we chose to investigate for possible incorporation into a biomarker profile. To the authors' knowledge, there is no literature addressing the distribution of MLH1 protein in normal-appearing colorectal mucosa and its potential as a biomarker of risk for colorectal cancer.

This paper addresses the distribution of MLH1 protein within the colorectal crypts of the normal-appearing colorectal mucosa and its association with colorectal adenoma as a first step in evaluating this potential prospective biomarker.

Participants and Methods

Study Design and Population

As reported previously[211], the Markers of Adenomatous Polyps II (MAP II) study is a pilot case-control study (51 cases and 154 controls) designed to investigate potential biomarkers of risk for incident, sporadic colorectal adenomas. Participants were recruited from people scheduled for elective outpatient colonoscopy at Consultants in Gastroenterology, a large gastroenterology practice in Columbia, SC. To be eligible for the study, participants must have been 30 – 74 years old, English speaking, and capable of

providing informed consent. Persons of both sexes and all races were eligible to participate in the study.

Specific exclusion criteria were history of previous colorectal adenomas or inflammatory bowel disease, bowel resection, history of cancer other than non-melanoma skin cancer, and medical contraindication to colorectal mucosal biopsies (medically unstable, bleeding disorders, cannot stop warfarin or aspirin) or a polyethylene glycol colon cleansing preparation.

Over a five-month period 351 patients were identified for recruitment; of these, 232 (76%) agreed to participate in the study; and of these, 205 (51 cases and 154 controls) met final eligibility criteria and were included in the study. Due to limited resources, only biopsies from all cases and a random sample of an equal number of controls were processed for MLH1 expression; from these there was adequate tissue for analysis on 46 cases and 43 controls.

Data Collection

Prior to the colonoscopy visit, patients completed mailed questionnaires, including a modified Willett Food Frequency Questionnaire. The questionnaires were used to obtain information on medical history, family history of cancer, diet, lifestyle, and anthropometrics.

The colon site and *in vivo* size and shape of all polyps found were recorded, and all polyps were removed and placed in separate containers. All polyps were examined by one study index pathologist who identified polyp type, subtype, and degree of atypia according to criteria established by the National Polyp Study[212].

After a 12-hour fast and polyethylene glycol bowel cleansing preparation, biopsies of normal-appearing mucosa were collected according to a standard protocol by gastroenterologists using standard-cup flexible endosocopy forceps during usual care colonoscopies. Six sextant pinch biopsies, approximately one millimeter thick, were obtained from the rectum (10 cm above the anus) on all participants, and from the mid-sigmoid and proximal ascending (immediately distal to the cecum) colon on 20% of participants, for a total of up to 18 biopsies. No biopsies were taken within 4.0 cm of a polypoid lesion.

Biopsies specimens were fixed by 10% normal buffered formalin for 24 hours, and then transferred to 70% ethanol. Within a week, the biopsies were processed and embedded in paraffin blocks with three biopsies per colon site per participant per block.

Immunohistochemistry

Within seven days of being embedded in paraffin blocks, 3.0 micron thick sections taken 30 microns apart were cut from each block with a microtome such that five slides with four levels each (yielding a total of 20 levels) were prepared per colon site per person. The immunohistochemistry protocol was calibrated to get the darkest biomarker labeling staining possible short of yielding non-specific background staining[213]. The slides were immunohistochemically processed using a DAKO Automated Immunostainer (DAKO Corp., Carpinteria, CA; further referred to as DAKO) and Leica H&E Autostainer (Leica Microsystems, Inc., IL). First, MLH1 antigen was unmasked via a heat-induced epitope retrieval procedure by placing the slides in a preheated Pretreatment (PT) Module (Lab Vision Corp., CA) with 100x Citrate Buffer pH 6.0 (DAKO S1699) and steamed for 40 minutes. Then the slides were immunohistochemically processed using an anti-MLH1 antibody (BD Pharmingen 554072) in a 1:15 dilution, a DAKO LSAB2 detection kit

(DAKO K0675), and 3,3' diaminobenzidine (DAB; DAKO K3466) as the chromogen. No counterstaining was used and all stained slides were glass coverslipped with a Leica Automated Coverslipper (Leica Microsystems, Inc., IL). All five slides per colon site per person were included in one staining batch of up to 48 slides that also included negative and positive control slides. A surgical specimen of normal colon was used for the control slides; the negative and the positive control slides were treated identically to study participant slides except that antibody diluent was used rather than primary antibody on the negative control slide.

Image Analysis

Since MSH1 is expressed in a density gradient along the crypt (figure 3.1) that is not quantifiable by eye (e.g., by counting cells), its expression density, detected by immunohistochemical staining, was quantified in the stained slides using image analysis densitometry methods[156, 211]. The procedure was conducted by one trained "scorer" using a light microscope (Olympus BX40, Olympus Corporation, Japan), digital camera (Polaroid DMC Digital Light Microscope Camera, Polaroid Corporation, USA), digital drawing tablet, and a custom-developed plug-in to ImagePro Plus (Media Cybernetics, Inc.) image analysis software. The scorer was blinded to case-control status and colon site.

The imaging and analysis unit was a "hemicrypt", defined as one side of a colonic crypt bisected from base to colon lumen surface. Intact (at most two contiguous cells missing) hemicrypts extending from the muscularis mucosae to the colon lumen were considered eligible for quantitative image analysis ("scorable").

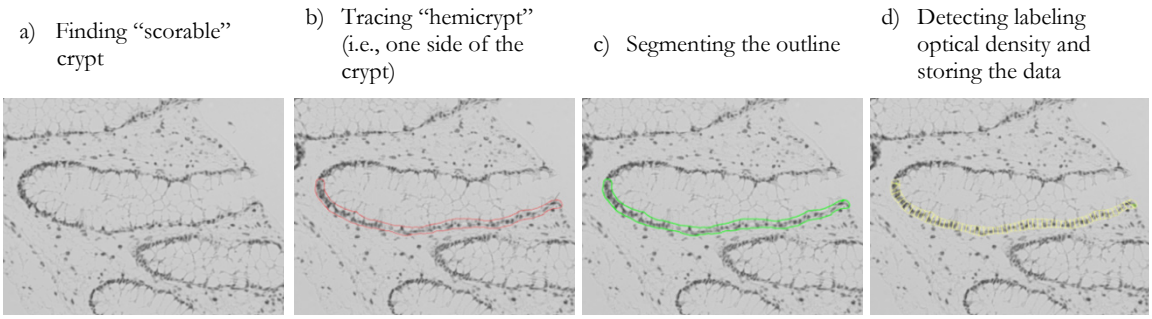

Figure 3.1. Image analysis of normal colorectal crypt immunohistochemically processed for MLH1. In the figure there is no counterstain, and all dark areas represent immunohistochemically detected MLH1. Note that MLH1 localizes in the nuclei of the crypt epithelium and is expressed in a declining density gradient from the lower to the upper portions of crypts. To quantify this: a) a full length crypt is identified by light microscopy at 200x and the image digitally captured with a digital camera, b) the nuclear zone of the selected hemicrypt is outlined manually using a digital drawing tablet, and then c) the image analysis program divides the hemicrypt into 6.59 μ width segments, conducts morphometry and d) measures the optical density of the staining in the entire hemicrypt as well as within each individual segment, and enters the data into a database.

For each patient the two of the three biopsies from each colon site with the greatest number of "scorable" hemicrypts were selected for quantitative image analysis ("scoring"). Intact hemicrypts were "scored" in order from the first hemicrypt on the first biopsy from left to right. The goal was to find at least 16 "scorable" hemicrypts per biopsy (32 per patient)[213]. If the 16th hemicrypt was reached before the level was finished, the scorer continued scoring until either the level was finished or the 20th hemicrypt was scored, whichever came first. No more than 20 hemicrypts per biopsy were scored. If the two best biopsies from a colon site on a patient had less than 32 "scorable" hemicrypts, an attempt was made to cut more slides. If that did not solve the issue, scoring was completed if the two best biopsies had 16 or more "scorable" hemicrypts between them. All three biopsies harvested from the same colon site were scored only if there was less than a total of 16 "scorable" hemicrypts between the two best biopsies.

To ensure adherence, a scorer was guided through the scoring protocol by the computer software. For each scored slide background correction images were obtained and automatically used by the computer program to yield background corrected densitometries

for all hemicrypts analyzed on that slide. All images were taken at 200x magnification (the maximum magnification at which full length colorectal crypts can be completely included in a single visual field) and stored and analyzed as 16-bit grayscale 1,600 × 1,200 pixel images.

As shown in figure 3.1, using a digital drawing tablet, hemicrypts were manually traced by the scorer from the crypt base center cell up along the crypt basement membrane to the beginning of the turn of the crypt onto the colonic mucosal surface and then back down along the crypt luminal surface of the epithelial nuclei[156, 211]. The software program divided the traced hemicrypt into segments corresponding in width to that of an average normal crypt epithelial cell (6.59 μ)[213], and then calculated overall hemicrypt- and segment-specific optical densities and entered these data into a Microsoft Access database along with various dimensional parameters of the hemicrypt[156, 211].

For quality assurance, slide sets from 10% of the participants were randomly selected by the statistical team, blinded, and re-submitted to the scorer for re-scoring[213].

Statistical Analysis

Statistical analyses were performed using SAS 9.1.3 statistical software (Copyright© 2002-2003 by SAS Institute Inc., Cary, NC, USA). The entire MAP II study population (51 cases and 154 controls) as well as a subset of participants for whom slides were immunohistochemically processed for MLH1 protein (46 cases and 43 controls) were assessed for comparability using the t-test for continuous variables, and the Fisher's exact test or χ^2-test for categorical variables as appropriate. Biopsy scoring reliability was assessed with intra-class correlation coefficients. All optical density means were calculated using linear mixed models. Potential confounders as well as staining batch were included in the models as fixed effects, and correlation among multiple optical density measurements was accounted

for by including a patient variable as a random effect. Mean proportional differences were calculated as the model-predicted mean optical density for cases minus that for controls divided by the mean for cases. Statistical significance of these measurement differences was evaluated by t-test.

The distribution of MLH1 protein within a colonic crypt was evaluated graphically with the Loess procedure as implemented in SAS 9.1.3 statistical software[203]. First, the number of cells within a hemicrypt was standardized to 50 cells (the average number of cells within a column of colonic crypt cells). Then, average colon site-specific levels of MLH1 for cases and controls predicted by the Loess model were plotted in the graphs (figure 3.2) along with smoothing lines (using a smoothing parameter of 0.5) to make graphical evaluation easier.

Potential confounders were evaluated on the basis of biological plausibility and whether the variable of interest was associated with the exposure based on existing epidemiological, medical, and basic science literature. As an additional method of selecting potential confounders, previously identified variables were added into the regression model and their ability to substantially change regression coefficients was evaluated. None of the variables changed the odds ratio (OR) by more than 10% and the adjustment was based on *a priori* considerations. Potential confounders considered in this analysis included: age, sex, physical activity, body mass index (BMI), family history of colorectal cancer in a first degree relative, smoking, alcohol consumption, aspirin and nonsteroidal anti-inflammatory drug (NSAID) 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[204]. Continuous variables were dichotomized based on their distributions in the controls. A

staining batch variable was included in the models to control for potential variability between staining batches.

The association between MLH1 expression and risk of incident sporadic colorectal adenoma was assessed by calculating odds ratios from the generalized linear mixed (logistic) models containing potential confounders and staining batch as fixed effects. The models accounted for lack of independence among hemicrypt optical density measurements within a patient by having a patient ID variable as a random effect. A 95% confidence interval was calculated for each odds ratio.

Generalized linear mixed (logistic) models were also used to model associations between the level of MLH1 expression in the rectum and adenoma location, shape, size, multiplicity, histological type, and degree of dysplasia. For this analysis the MLH1 expression variable was batch-standardized by dividing a patient-specific optical density measurement by the batch-specific mean optical density, and then dichotomized based on the colon site-specific mean of the standardized variable in the controls. The models also contained a random intercept for each patient to account for correlations due to repeated measurements (i.e., optical densities of multiple crypts per colon site per patient).

The associations of MLH1 expression within a colon site with various demographic, lifestyle, and dietary characteristics were assessed by linear mixed models stratified by colon site controlling for each of the characteristics one at a time. Each model also included a fixed effect variable to control for staining batch. Since optical density was measured on multiple hemicrypts on each patient, each model accounted for that by a random effect variable.

In sensitivity analyses, we also analyzed data without standardization for batch, as well as by using different mathematical transformations; the results from these analyses did not differ materially from those reported.

Results

The sub-population of subjects whose biopsies were stained for MLH1 and analyzed (46 cases and 43 controls) was compared to the entire MAPII 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 3.1. On average, relative to controls, cases tended to be older and more likely to be male, a current smoker, currently consume alcohol, regularly take an NSAID, and to have a higher total energy intake and lower intakes of calcium, vitamin D, and folate, although only the difference for total energy intake was statistically significant. Physical activity, BMI, aspirin use, and fat and fiber intakes did not differ substantially between cases and controls.

Among cases, 48% had multiple adenomas, 7% had an adenoma that was 1.0 cm or greater in diameter, 89% had a mild degree of atypia in their largest or most advanced adenoma, and in 41% of cases the largest or most advanced adenoma was located in the right colon (data not shown). Biopsy scoring reliability (intra-class correlation coefficient) was $r = 0.97$.

Figure 3.2 depicts the distribution of MLH1 protein within colonic crypts in the rectum, sigmoid, and ascending colon. For each of the three colon sites, the MLH1 expression curves for cases and controls closely paralleled each other. Levels of MLH1 in the rectum were slightly higher in cases than in controls, but in the sigmoid and ascending

Table 3.1. Selected characteristics of incident, sporadic colorectal adenoma cases and controls; the Markers of Adenomatous Polyps II Study

Characteristic*	N (Cases/ Controls)	Adenoma Cases	Controls	p†
Demographics				
Age (yrs.)	46/43	56.8 (7.7)	55.7 (8.4)	0.52
Male (%)	46/43	54	44	0.40
White race (%)	46/42	96	98	1.00
Family History				
1º Relative with colorectal cancer (%)	46/43	17	14	0.77
Lifestyle				
Physical activity (METs/day)	44/42	29.5 (23.5)	27.1 (20.9)	0.33‡
Body Mass Index (kg/m^2)	46/42	30.8 (7.3)	30.4 (7.0)	0.79‡
Take aspirin at least once per week (%)	46/42	39	38	1.00
Take NSAID§ at least once per week (%)	46/42	35	43	0.51
Smoking status (%)				
Never		41	52	0.30
Former	46/42	41	41	
Current		18	7	
Alcohol consumption (%)				
Never		11	14	0.74
Former	46/42	22	26	
Current		67	60	
Dietary intakes				
Total energy (kcal/day)	44/41	1,939.5 (780.0)	1,509.2 (405.5)	0.002‡
Total fat‖ (g/day)	44/42	65.7 (16.5)	65.8 (15.2)	0.99
Total dietary fiber‖ (g/day)	44/42	15.4 (5.7)	15.4 (5.9)	0.98
Total# calcium‖ (mg/day)	44/42	882.7 (487.3)	995.0 (505.4)	0.24‡
Total# vitamin D‖ (IU/day)	44/42	323.1 (289.9)	373.4 (277.8)	0.20‡
Total# folate‖ (mcg/day)	44/42	480.2 (235.0)	522.4 (266.3)	0.44

* Continuous variables presented as mean (±SD), categorical variables as proportions in percent
† Based on t-test for continuous variables, Fisher's exact test for dichotomous variables, and χ^2-test for multilevel categorical variables
‡ Variables that were not normally distributed were normalized by natural log transformation
§ NSAID – Non-steroidal anti-inflammatory drug (not including aspirin)
‖ Energy adjusted using residual method
Total = diet + supplements

colon the relationship shifted such that MLH1 levels in controls were consistently higher than those in cases. The difference in MLH1 expression levels between cases and controls was greater in the ascending than the sigmoid colon. Since case-control differences appeared

uniform throughout the lengths of the colon crypts, only analyses of overall crypt MLH1 expression data are presented in tables 3.2 – 3.5.

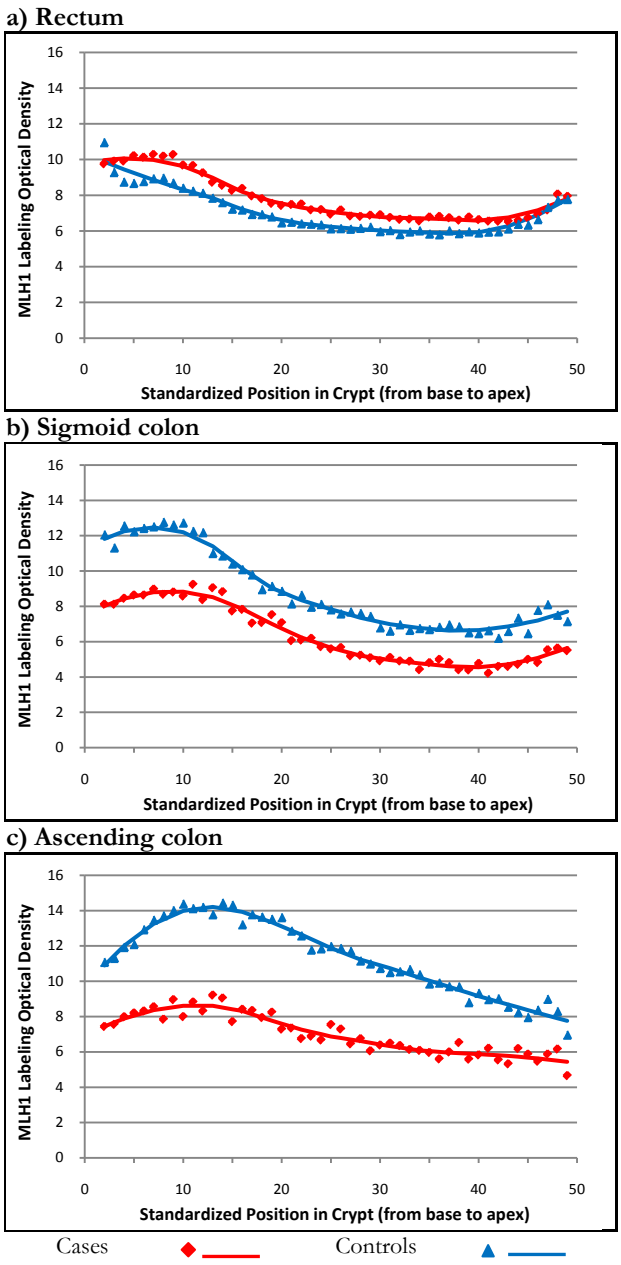

Figure 3.2. Expression of MLH1 protein at standardized positions within the crypts of normal-appearing mucosa in cases and controls for three colon sites: a) rectum, b) mid sigmoid colon, c) proximal ascending colon. The Markers of Adenomatous Polyps II Study. Data points represent average optical density for all cases or all controls at a particular standardized position in the crypt, and the curves are Loess smoothing curves (smoothing parameter 0.5).

Table 3.2 presents "crude" (controlled for staining batch only), age- and sex-adjusted, and multivariable-adjusted MLH1 expression in all cases and controls stratified by colon site, as well as the combined ORs for the associations of MLH1 expression with incident, sporadic colorectal adenoma. While, on average, expression of MLH1 protein in the rectum in adenoma cases tended to be slightly higher, in the other colon sites cases tended to have lower expression of the protein than did the controls. The proportional difference in expression between cases and controls widened from the distal to the proximal colon sites, reaching a statistically significant 49% after multivariable adjustment (table 3.2, model 3). MLH1 expression in colonic crypts

Table 3.2. Differences in full crypt MLH1 protein expression in normal-appearing mucosa between incident sporadic colorectal adenoma cases and controls, by colon site; the Markers of Adenomatous Polyps II Study

Colon Site	N	Optical Density Mean (SD)		Proportional Difference (%)*	p†
		Cases	Controls		
Model 1: controls for staining batch only					
Rectum	84	496.07 (18.66)	464.74 (20.46)	7%	0.22
Sigmoid	32	313.82 (27.44)	330.64 (29.04)	-5%	0.68
Ascending	27	380.97 (58.69)	500.30 (51.38)	-24%	0.13
Combined OR‡ (95% C.I.§)	89	0.84		(0.47 – 1.49)	
Model 2: controls for age, sex and staining batch					
Rectum	84	495.63 (18.88)	464.49 (20.79)	7%	0.22
Sigmoid	32	310.23 (28.15)	335.68 (30.13)	-8%	0.55
Ascending	27	375.92 (50.90)	481.17 (45.03)	-22%	0.10
Combined OR (95% C.I.)	89	0.87		(0.49 – 1.55)	
Model 3: controls for age, sex, history of colorectal cancer in a first degree relative, physical activity, BMI, aspirin use, total energy intake, calcium, vitamin D, and folate intakes, and staining batch					
Rectum	80	505.23 (27.48)	465.30 (28.05)	9%	0.18
Sigmoid	31	345.18 (39.71)	301.52 (34.53)	14%	0.41
Ascending	26	257.77 (84.74)	510.12 (53.19)	-49%	0.03
Combined OR (95% C.I.)	85	0.89		(0.45 – 1.76)	

* [(cases – controls)/controls]×100%
† Based on t-test for comparing the two means
‡ Combined OR – odds ratio (Cases vs. Controls) controlling for all three colon sites and the covariates indicated in the model specification. The optical density (MLH1 expression) variable was dichotomized using the mean of the colon site specific distributions in the controls.
§ C.I. – confidence interval

was non-statistically significantly inversely associated with risk of incident, sporadic colorectal adenomas (OR = 0.89, 95% confidence interval [C.I.]: 0.45 – 1.76). Although estimated differences between all cases and controls at the rectum were negligible (table 3.2), to assess whether MLH1 expression at the rectum may be associated with a subset of cases (especially those with right-sided adenomas), we investigated associations of MLH1 expression in the rectum with various adenoma characteristics (table 3.3). MLH1 expression in the rectum tended to be more strongly associated with adenomas in the right colon (OR = 1.81; 95% C.I.: 0.86 – 3.80) than in the left colon (OR = 1.19; 95% C.I.: 0.62 – 2.28). Rectal mucosal MLH1 expression also tended to be more strongly positively associated with

pedunculated (OR = 2.18; 95% C.I.: 0.67 – 7.09) and single adenomas (OR = 1.81; 95% C.I.: 0.95 – 3.44).

Table 3.3. Crude associations of batch-standardized full crypt MLH1 expression in the normal-appearing rectal mucosa with risk of incident sporadic colorectal adenomas overall and according to adenoma characteristics; the Markers of Adenomatous Polyps II Study

Adenoma characteristic	N (Cases/Controls)	MLH1 expression		95% C.I.†
		Low (OR*)	High (OR)	
All Adenomas	44/43	1.0	1.35	(0.77 – 2.38)
Location				
Right colon‡	18/43	1.0	1.81	(0.86 – 3.80)
Left colon§	26/43	1.0	1.19	(0.62 – 2.28)
Multiplicity				
Single adenoma	23/43	1.0	1.81	(0.95 – 3.44)
Multiple adenomas	21/43	1.0	1.01	(0.48 – 2.14)
Dysplasia				
Mild	39/43	1.0	1.43	(0.79 – 2.59)
Moderate/severe	5/43	1.0	1.26	(0.35 – 4.57)
Histological type				
Tubular	31/43	1.0	1.37	(0.75 – 2.51)
Tubulovillous/villous	13/43	1.0	1.51	(0.63 – 3.62)
Shape				
Pedunculated	6/43	1.0	2.18	(0.67 – 7.09)
Sessile	38/43	1.0	1.31	(0.74 – 2.34)

* OR – odds ratio
† C.I. – confidence interval
‡ Right colon includes cecum, ascending colon, hepatic flexure and transverse colon
§ Left colon includes splenic flexure, descending colon, sigmoid colon and rectum

We also assessed the potential of MLH1 expression as a modifiable biomarker of risk by evaluating associations of MLH1 expression with various risk factors for colorectal neoplasms. The associations tended to vary between the adenoma cases (table 3.4) and controls (table 3.5). In cases and controls, average MLH1 expression in the ascending colon tended to be lower with increased age (by 56% [p=0.02] and 25% [p=0.16], respectively, for those ≥55 years) and a history of colorectal cancer in a first degree relative (by 22% [p=0.56] and 34% [p=0.16], respectively), as well as among smokers (by 38% [p=0.26] and 25% [p=0.31], respectively). MLH1 expression in the ascending colon also tended to be lower in

those with higher physical activity (by 45% [p=0.42] and 15% [p=0.56], respectively; however, for those on whom ascending colon tissue was available for MLH1 evaluation, only one case and three controls were categorized as having high physical activity. Among cases, but not controls, average MLH1 expression tended to be higher with current alcohol consumption (by 58%), regular aspirin use (by 46%), and higher total intakes of calcium (by 32%), vitamin D (by 22%), and folate (by 37%), but none of these findings were statistically significant. There was little indication of similar differences in the rectum.

Table 3.4. Associations of full crypt MLH1 expression in normal-appearing colorectal mucosa with potential risk factors of colorectal cancer in incident, sporadic colorectal adenoma cases, by colon site; the Markers of Adenomatous Polyps II Study

Characteristic*	Rectum			Sigmoid			Ascending		
	N	MLH1 Expression† (SE‡)	p§	N	MLH1 Expression (SE)	p	N	MLH1 Expression (SE)	p
Age (yrs.)									
35 – 54	21	522.75 (27.77)		8	278.76 (43.38)		6	535.26 (56.12)	
≥ 55	22	470.63 (26.90)	0.20	9	349.19 (43.14)	0.30	7	234.96 (72.46)	0.02
% Difference		*-10%*			*24%*			*-56%*	
Sex									
Male	23	478.65 (27.04)		10	297.04 (43.12)		7	406.41 (82.91)	
Female	20	513.36 (26.44)	0.36	7	342.35 (40.34)	0.42	6	351.95 (87.41)	0.64
% Difference		*7%*			*15%*			*-13%*	
Family history of colorectal cancer‖									
No	37	501.02 (20.07)		14	313.09 (30.37)		10	381.85 (66.09)	
Yes	6	469.77 (49.83)	0.56	3	316.84 (76.04)	0.96	3	297.98 (127.22)	0.56
% Difference		*-6%*			*1%*			*-22%*	
Physical activity (METs/day)									
Low	25	505.02 (25.06)		13	324.25 (34.42)		12	365.40 (55.61)	
High	18	484.59 (30.49)	0.61	4	283.72 (61.54)	0.58	1	201.08 (197.00)	0.42
% Difference		*-4%*			*-12%*			*-45%*	
BMI# (kg/m²)									
< 30	21	488.87 (29.43)		9	334.19 (43.86)		8	370.13 (78.24)	
≥ 30	22	501.40 (25.84)	0.75	8	290.69 (43.22)	0.53	5	352.39 (97.79)	0.90
% Difference		*3%*			*-13%*			*-5%*	
Smoking**									
Never	19	533.08 (28.18)		9	295.99 (38.62)		7	473.75 (97.69)	
Ever	24	475.55 (25.16)	0.12	8	332.08 (44.45)	0.57	6	296.09 (97.00)	0.26
% Difference		*-11%*			*12%*			*-38%*	
Alcohol consumption††									
Former/ Never	14	529.62 (33.69)		7	326.21 (44.46)		5	249.65 (77.77)	
Current	29	483.03 (23.84)	0.28	10	305.41 (38.73)	0.74	8	393.92 (69.52)	0.17
% Difference		*-9%*			*-6%*			*58%*	

Table 3.4. Continued.

Characteristic*	Rectum			Sigmoid			Ascending		
	N	MLH1 Expression† (SE‡)	p§	N	MLH1 Expression (SE)	p	N	MLH1 Expression (SE)	p
Aspirin intake‡‡									
No	27	492.16 (23.09)		13	333.96 (32.25)		10	328.69 (65.58)	
Yes	16	504.48 (31.58)	0.75	4	236.54 (64.74)	0.21	3	478.93 (106.30)	0.26
% Difference		*3%*			*-29%*			*46%*	
NSAID§§ intake‡‡									
No	27	524.14 (24.13)		10	348.16 (44.06)		8	358.55 (75.42)	
Yes	16	456.25 (28.53)	0.07	7	262.83 (57.10)	0.32	5	372.33 (95.74)	0.92
% Difference		*-13%*			*-25%*			*4%*	
Total energy intake (kcal/day)‖‖									
Low##	9	532.93 (43.70)		7	390.31 (42.36)		7	336.05 (91.78)	
High	32	487.79 (22.17)	0.37	10	257.23 (37.65)	0.04	6	381.19 (104.69)	0.74
% Difference		*-8%*			*-34%*			*13%*	
Total* calcium intake (mg/day)**									
Low	26	504.80 (25.00)		11	296.56 (34.68)		7	312.18 (71.24)	
High	15	484.50 (33.91)	0.64	6	344.09 (51.04)	0.47	6	412.44 (78.08)	0.34
% Difference		*-4%*			*16%*			*32%*	
Total vitamin D intake (IU/day)									
Low	24	507.78 (26.10)		8	357.48 (40.93)		5	318.04 (89.30)	
High	17	482.68 (30.96)	0.55	9	258.89 (44.56)	0.15	8	388.04 (71.81)	0.56
% Difference		*-5%*			*-28%*			*22%*	
Total folate intake (mcg/day)									
Low	20	518.23 (28.16)		5	351.59 (57.40)		4	284.87 (99.11)	
High	21	477.92 (27.40)	0.31	12	294.42 (38.07)	0.46	9	389.65 (62.50)	0.39
% Difference		*-8%*			*-16%*			*37%*	

* All variables except age, sex, family history of colorectal cancer, and total energy intake adjusted for age and sex; also smoking status variable adjusted for alcohol consumption and alcohol consumption variable adjusted for smoking status.
† Mean optical density adjusted for staining batch
‡ SE – standard error
§ Based on the F-test for significance of fixed effects in a linear mixed model
‖ Family history of colorectal cancer in a first-degree relative
BMI – body mass index (kg/m2)
** Categories "Current smoker" and "Former smoker" were combined into the "Ever smoker" category due to extremely small sample size of the "Current smoker" category
†† Categories "Never consumed" and "Former consumer" were combined due to extremely small sample size of the "Never consumed" category
‡‡ Yes defined as regularly taking this medication at least once a week
§§ NSAID – nonsteroidal anti-inflammatory drugs (not including aspirin)
‖‖ Throughout the table: "Low" - below the 50th percentile of the sex-specific distribution in controls; "High" - at or above the 50th percentile of the sex-specific distribution in controls
Adjusted for physical activity
*** From diet and supplements

Table 3.5. Associations of full crypt MLH1 expression in normal-appearing colorectal mucosa with potential risk factors of colorectal cancer in controls, by colon site; the Markers of Adenomatous Polyps II Study

Characteristic*	Rectum			Sigmoid			Ascending		
	N	MLH1 Expression† (SE‡)	p§	N	MLH1 Expression (SE)	p	N	MLH1 Expression (SE)	p
Age (yrs.)									
35 – 54	21	432.34 (26.42)		9	345.20 (39.19)		8	563.59 (56.12)	
≥ 55	20	500.60 (27.28)	0.06	6	304.87 (50.95)	0.56	6	423.11 (73.52)	0.16
% Difference		*16%*			*12%*			*-25%*	
Sex									
Male	19	472.74 (28.38)		7	413.16 (42.18)		6	500.99 (89.02)	
Female	22	456.24 (26.26)	0.65	8	254.94 (39.60)	0.02	8	499.87 (76.87)	0.99
% Difference		*-3%*			*-38%*			*0%*	
Family history of colorectal cancer‖									
No	36	457.54 (21.80)		11	312.96 (36.75)		10	558.17 (63.16)	
Yes	5	510.74 (52.92)	0.34	4	378.11 (65.12)	0.42	4	367.89 (106.36)	0.16
% Difference		*12%*			*21%*			*-34%*	
Physical activity (METs/day)									
Low	25	450.66 (26.05)		10	315.14 (42.08)		10	537.37 (59.00)	
High	15	494.30 (33.71)	0.30	4	342.06 (68.38)	0.76	3	456.21 (113.06)	0.56
% Difference		*10%*			*9%*			*-15%*	
BMI# (kg/m²)									
< 30	22	466.40 (27.90)		8	371.56 (39.60)		7	528.88 (74.62)	
≥ 30	18	464.66 (29.68)	0.96	6	264.46 (44.61)	0.09	6	501.39 (71.25)	0.80
% Difference		*0%*			*-29%*			*-5%*	
Smoking**									
Never	21	474.31 (28.06)		6	250.08 (52.46)		6	591.08 (97.23)	
Ever	19	470.84 (29.14)	0.93	8	384.56 (44.11)	0.09	7	444.18 (79.87)	0.31
% Difference		*-1%*			*54%*			*-25%*	
Alcohol consumption††									
Former/ Never	16	469.52 (30.80)		6	366.07 (49.80)		5	604.07 (87.28)	
Current	24	469.39 (25.83)	1.00	8	282.16 (43.08)	0.24	8	460.08 (71.89)	0.29
% Difference		*0%*			*-23%*			*-24%*	
Aspirin intake‡‡									
No	24	478.85 (26.03)		9	314.01 (37.87)		8	536.98 (65.50)	
Yes	16	447.20 (32.58)	0.43	5	344.32 (54.23)	0.65	5	479.66 (76.55)	0.60
% Difference		*-7%*			*10%*			*-11%*	
NSAID§§ intake‡‡									
No	23	470.83 (27.32)		9	329.50 (40.43)		8	543.89 (76.35)	
Yes	17	470.86 (30.09)	1.00	5	316.50 (59.65)	0.88	5	477.62 (90.34)	0.63
% Difference		*0%*			*-4%*			*-12%*	
Total energy intake (kcal/day)‖‖									
Low§§	19	464.45 (29.85)		8	303.97 (39.87)		7	546.72 (82.97)	
High	20	472.32 (28.42)	0.84	6	319.05 (48.54)	0.82	6	493.16 (97.65)	0.69
% Difference		*2%*			*5%*			*-10%*	
Total* calcium intake (mg/day)**									
Low	20	473.18 (27.91)		8	371.85 (42.30)		7	532.49 (64.64)	
High	20	459.04 (29.21)	0.71	6	265.73 (47.96)	0.13	6	490.24 (76.26)	0.69
% Difference		*-3%*			*-29%*			*-8%*	
Total vitamin D intake (IU/day)									
Low	19	461.05 (29.68)		9	354.76 (39.13)		9	515.87 (59.18)	
High	21	471.18 (28.86)	0.80	5	285.51 (52.90)	0.32	4	501.15 (100.34)	0.90
% Difference		*2%*			*-20%*			*-3%*	

Table 3.5. Continued.

Characteristic*	Rectum			Sigmoid			Ascending		
	N	MLH1 Expression† (SE‡)	p§	N	MLH1 Expression (SE)	p	N	MLH1 Expression (SE)	p
Total folate intake (mcg/day)									
Low	19	464.52 (28.73)		9	351.62 (37.71)		9	540.55 (54.96)	
High	21	466.77 (27.82)	0.95	5	269.07 (54.11)	0.23	4	430.77 (96.94)	0.35
% Difference		0%			-23%			-20%	

* All variables except age, sex, family history of colorectal cancer, and total energy intake adjusted for age and sex; also smoking status variable adjusted for alcohol consumption and alcohol consumption variable adjusted for smoking status.
† Mean optical density adjusted for staining batch
‡ SE – standard error
§ Based on the F-test for significance of fixed effects in a linear mixed model
‖ Family history of colorectal cancer in a first-degree relative
BMI – body mass index (kg/m2)
** Categories "Current smoker" and "Former smoker" were combined into the "Ever smoker" category due to extremely small sample size of the "Current smoker" category
†† Categories "Never consumed" and "Former consumer" were combined due to extremely small sample size of the "Never consumed" category
‡‡ Yes defined as regularly taking this medication at least once a week
§§ NSAID – nonsteroidal anti-inflammatory drugs (not including aspirin)
‖‖ Throughout the table: "Low" - below the 50th percentile of the sex-specific distribution in controls; "High" - at or above the 50th percentile of the sex-specific distribution in controls
Adjusted for physical activity
*** From diet and supplements

Discussion

To our knowledge, this is the first study to report on the distribution of the MLH1 protein within normal human colorectal crypts or on associations of MLH1 expression in normal-appearing colorectal mucosa with risk for incident, sporadic colorectal neoplasms or with risk factors for colorectal cancer. Our preliminary data support the hypothesis that MLH1 expression in the normal colonic mucosa—especially in the more proximal sites of the colon—may be associated with risk of incident, sporadic colorectal adenoma. The data also suggest the possibility that MLH1 expression in the normal colon, especially in the more proximal part of it, may be associated with modifiable risk factors for colorectal neoplasms.

The expression curves for MLH1 appear to mirror the cell proliferation pattern within a colonic crypt (figure 3.2) with high expression of the protein in the lower 60% of crypts (proliferation zone), and lower expression in the upper 40%. This suggests that MLH1 expression may be correlated with the proliferative activity of colonic cells.

For chemoprevention trials or other potential outpatient applications, the most practical colon site for obtaining colorectal tissue is the rectum[213]. The procedures for obtaining rectal biopsies 10 cm above the anus are minimally invasive and do not require fasting or bowel cleansing preparations[213]. Although the estimated differences between cases and controls in the rectum in this study were negligible (slightly higher in cases, but not statistically significant), to assess whether MLH1 expression in the rectum may be associated with a subset of cases (especially those with right-sided adenomas), we investigated associations of MLH1 expression in the rectum with various adenoma characteristics (table 3.3). There was some suggestion that higher MLH1 expression in the rectum was associated with higher risk for adenomas that were right sided, pedunculated, or single. These findings were not statistically significant and may have been due to chance. On the other hand, if such findings are confirmed in a full scale study, they could suggest that there is a reciprocal relationship between MLH1 expression in the rectum and right colon; a possible explanation for this could be that MLH1 expression in the ascending colon may be more influenced by reduced expression via DNA methylation, and that MLH1 expression in the rectum may be less influenced by DNA methylation and simply reflects higher levels of proliferation (i.e., higher mismatch repair activity follows higher proliferation). It remains possible that rectal expression of MLH1 in combination with other biomarkers may increase the predictive value of such a panel of biomarkers, a subject of ongoing work. Should our findings regarding MLH1 expression in the rectum not be confirmed, assessing MLH1 expression in colonoscopic biopsies from the ascending colon may still be useful; for example, for helping assess if and when someone with a normal screening colonsocopy may need a subsequent one.

Two of the most non-controversial risk factors for colorectal cancer are increasing age and a family history of colorectal cancer in a first degree relative[3, 8, 36]. Consistent with this and our findings of lower MLH1 expression in the ascending colon in cases, we found that MLH1 expression in the ascending colon was lower in those who were older or had a first degree relative with colorectal cancer, regardless of case-control status.

Some of the most strongly supported modifiable risk factors for colorectal neoplasms are physical activity, aspirin and other nonsteroidal anti-inflammatory drug (NSAID) use, and calcium and vitamin D intakes[3, 6, 8, 36, 122, 125-127, 131, 214-216]. Folate intake has also been a subject of investigation and has been linked to DNA methylation and thus may influence MLH1 expression[6, 8, 74, 215, 217-219]. The results of our small study suggest that MLH1 expression in the ascending colon may be higher with aspirin use and higher total intakes of calcium, vitamin D, or folate, but only in persons who have developed a sporadic adenoma. MLH1 expression in the ascending colon also tended to be lower in those with higher physical activity; however, for those on whom ascending colon tissue was available for MLH1 evaluation, only one case and three controls were categorized as having high physical activity. At odds with the findings for aspirin, there was no substantial indication that use of other NSAIDs was associated with MLH1 expression in the ascending colon. Again, these results—perhaps related to the small sample size—were not statistically significant and thus may have simply been due to chance. However, if confirmed in a subsequent larger study, they suggest that aspirin, calcium, and vitamin D may be most effective as chemopreventive agents in persons already at increased risk for colorectal neoplasms.

Important strengths of this study included: (a) all participants underwent colonoscopy, which ensured accurate classification of cases and controls; (b) all self-reported

information (including dietary data) was collected before colonoscopies and thus determination of case-control status, thus minimizing possible recall bias; (c) detailed information on potential confounders, such as anthropometrics, diet, vitamin and mineral supplements, and medications, used was collected; and (d) the rigorous procedures for biopsy collection, processing, and quantitative assessment of the density of immunohistochemically-detected MLH1 expression using our custom-developed software, which minimized possible measurement error.

Since this study was a pilot study, its main limitation was the small sample size. Due to limited resources, the biopsies on only a subset of the patients were evaluated for MLH1, which further reduced the sample size. The same reasons explain why biopsies for all three colon sites were often not available. Using an automated immunostainer did not completely eliminate staining variability between staining batches, which introduced an additional source of variability into the analysis that had to be accounted for. The participants of this study were drawn from people who underwent a colonoscopy, and so the results of this study may not be directly applicable to the general population. Data collected by food frequency questionnaires and self-reported data have shortcomings that are well described in the literature, but, since these data were collected before case-control status was determined, any possible bias is likely non-differential

Basic science and epidemiologic literature[220-223] describes the central role of MLH1 in the function of the MMR machinery and the inability of this machinery to function when the *MLH1* gene is damaged or silenced. The MLH1-based protein complex participates in repairing all known kinds of DNA mismatches, and its concentration increases when cell proliferation activity increases. Changes in MLH1 expression and its distribution within a

colonic crypt may indicate changes in the cell proliferation pattern and give some information about cancer risk in a patient. Analytical epidemiologic studies[3, 8, 224, 225] have not been convincingly consistent with respect to the importance of various dietary factors as risk factors for colorectal adenoma and cancer. The literature about relationships of these factors with MLH1 expression is very limited[224, 226-231]. It was hypothesized that certain dietary components, such as folate, alcohol, and others may play a role in carcinogenesis because of their involvement in DNA methylation. Hypermethylation of CpG islands near promoter regions and subsequent transcriptional silencing of the *MLH1* gene is a common pathway for inactivating this gene.

Slattery et al[229] investigated associations between dietary (fiber, folate, alcohol, methionine, and vitamins B_6 and B_{12}) and lifestyle (BMI, physical activity, and use of aspirin and NSAIDs) factors and colon cancer. The investigators found that high folate and fiber intakes were inversely associated with risk of incident carcinomas, irrespective of *MLH1* promoter methylation; however, in those with the high-methylator phenotype, the association appeared stronger, suggesting involvement of these compounds in the DNA methylation process. On the other hand, a prospective cohort study performed in the Netherlands found no association between folate or fiber and MLH1 protein deficient colorectal cancer[230]. Our data support a possible inverse association between folate and MLH1 expression in the sigmoid and ascending colon in adenoma free controls, but suggest a positive association in incident, sporadic colorectal adenoma patients. These findings suggest that folate's role in DNA methylation may also be important in the development of colorectal adenomas.

Consistent with evidence that folate and methionine may influence methyl group availability, Giovannucci et al[74] found that methyl-deficient diets might be associated with early stages of colorectal neoplasia. The hypothesis that folate may be inversely associated with DNA hypomethylation was supported by the results of a clinical trial[217] and several observational studies[218, 219]. Our findings are also consistent with this hypothesis. On the other hand, several observational studies that specifically investigated the association between folate and MLH1 did not find significant evidence of such an association[230, 232]. So far, the evidence for a role for folate in DNA methylation is inconclusive and further investigation by more definitive studies is needed.

Several recent clinical trials[122, 125-127, 216] found that calcium reduced colorectal adenoma recurrence. Associations of calcium and vitamin D with incident adenomas have been investigated only in case-control studies[214, 233-236] and are consistent with reduced risk. As discussed above, the results of our small study suggest that MLH1 expression in the ascending colon may be higher with higher total intakes of calcium or vitamin D, but only in persons who have developed a sporadic adenoma. To our knowledge, this is the first study to investigate this association specifically.

In summary, we developed a reliable procedure for detecting and describing MLH1 expression in normal colorectal crypts, and report, to our knowledge, the first study of the distribution of the MLH1 protein within normal colorectal crypts or associations of MLH1 expression in normal-appearing colorectal mucosa with risk for incident, sporadic colorectal neoplasms or with risk factors for colorectal cancer. We found that the distribution of the MLH1 protein within normal colonic crypts parallels that of the normal proliferation zone of normal crypts. The data from this preliminary study suggest that lower MLH1 expression

in the normal colonic mucosa, at least in the ascending colon, may be associated with increased risk of incident, sporadic colorectal adenoma as well as with modifiable risk factors for colorectal neoplasms, and thus support further investigation of MLH1 expression, alone or in combination with other biomarkers, as a potential "treatable" biomarker of risk for colorectal neoplasms.

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Chapter 4. Colorectal Mucosal Expression of MSH2 as a Potential Modifiable Biomarker of Risk for Colorectal Neoplasms*

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Abstract

To characterize the expression of the mismatch repair gene *MSH2* in normal colorectal crypts in humans, and assess parameters of its expression as a potential modifiable biomarker of risk for colorectal neoplasms, we conducted a pilot, colonoscopy-based case-control study (51 cases, 154 controls) of incident, sporadic colorectal adenoma. Biopsies of normal-appearing rectal, sigmoid, and ascending colon mucosa were procured, immunohistochemically processed for MSH2 protein, and analyzed using custom quantitative image analysis procedures.

MSH2 expression in adenoma cases was lower than in controls by 49% ($p = 0.01$) and 23% ($p = 0.06$) in the ascending colon and rectum, respectively, but not in the sigmoid colon. MSH2 expression in the rectum was 39% ($p = 0.04$) higher in subjects who regularly took a nonsteroidal anti-inflammatory drug than in those who did not, and it tended to be lower in those with adenomas in the right colon and those who had an adenoma with more advanced characteristics.

These preliminary data suggest that lower MSH2 expression in the normal colonic mucosa, at least in the ascending colon and rectum, may be associated with increased risk of

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incident, sporadic colorectal adenoma as well as with modifiable risk factors for colorectal neoplasms, thus supporting further investigation of MSH2 expression as a potential modifiable biomarker of risk for colorectal neoplasms.

Introduction

Despite noticeable advances in cancer diagnostics and treatment , colorectal cancer remains the second leading cause of cancer mortality in the United States[237]. It is a multi-factorial disease that appears to be the result of interacting lifestyle and genetic factors[3, 8, 99, 130, 133, 157, 238]. The adenomatous polyp, the precursor to the vast majority of so-called "sporadic" colorectal cancer, currently is the only accepted reliable biomarker of risk for colorectal cancer[3, 8], and its removal markedly reduces risk of cancer development. However, colonoscopy and sigmoidoscopy, the procedures currently used to screen for adenoma, are costly, labor-intensive, require highly qualified personnel, and are not well accepted by many patients and physicians. Pre-neoplastic biomarkers or profiles of biomarkers of risk for colorectal neoplasms will help address these challenges. The developed biomarkers can be used to individualize colonoscopy recommendations and schedules, and to monitor the efficacy of preventive interventions.

The mismatch repair (MMR) pathway is one of the two main molecular pathways of colorectal cancer development, accounting for about 15% of colorectal cancers[207]. The DNA MMR system is a highly conservative mechanism that involves a complex set of proteins that identifies and repairs mismatch errors that occur during DNA replication[8, 15, 239]. A crucial part of this system is the *MutS-homolog 2*, colon cancer, nonpolyposis type 1 (*E. coli*) [*Homo sapiens*] (*MSH2*) gene, which is located at chromosome 2p22[209]. The product of this gene, MSH2 protein, recognizes DNA mismatches by forming two functional heterodimers:

MSH2/MSH6, which recognizes single base mismatches and short insertion-deletion loops, and MSH2/MSH3, which recognizes larger loops[18, 209, 221, 222].

Expression of MSH2 protein in the colon cell is likely to indicate the functional level of the DNA mismatch repair mechanism, which makes MSH2 expression in normal colorectal mucosa a candidate as a biomarker of risk for colorectal cancer. To the authors' knowledge, there is no literature addressing the distribution of MSH2 protein in normal-appearing colorectal mucosa, its potential as a biomarker of risk for colorectal cancer, or its associations with risk factors for colorectal neoplasms. To address this, we conducted a case-control study of incident, sporadic colorectal adenoma in which we measured the overall expression and distribution of MSH2 protein within the crypts of the normal-appearing colorectal mucosa and estimated their associations with colorectal adenoma and known risk factors for colorectal neoplasms as a first step in evaluating MSH2 as a prospective biomarker of risk for colorectal neoplasms.

Participants and Methods

Study Design and Population

The Markers of Adenomatous Polyps II (MAP II) study is a pilot case-control study (51 cases and 154 controls) designed to investigate potential biomarkers of risk for incident, sporadic colorectal adenomas. Participants were recruited from people scheduled for elective outpatient colonoscopy at Consultants in Gastroenterology, a large gastroenterology practice in Columbia, SC. Persons 30 – 74 years old, of both sexes and all races, English speaking, and capable of providing informed consent were considered to be potentially eligible for the study.

Specific exclusion criteria included history of previous colorectal adenomas or inflammatory bowel disease, bowel resection, history of cancer other than non-melanoma skin cancer, and medical contraindication to colorectal mucosal biopsies (medically unstable, bleeding disorders, cannot stop warfarin or aspirin) and polyethylene glycol colon cleansing preparations.

Over a five-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 92 participants (45 cases and 47 controls) were processed for MSH2 expression and used for the analysis reported here.

Data Collection

All patients completed mailed questionnaires, including a modified Willett Food Frequency Questionnaire, prior to the colonoscopy visit and knowledge of case-control status. The questionnaires were used to obtain information on medical history, family history of cancer, diet, lifestyle, and anthropometrics.

The colon site and *in vivo* size and shape of all polyps found were recorded. All polyps were removed and examined by one study index pathologist who identified polyp type, subtype, and degree of atypia according to criteria established by the National Polyp Study[212].

After a 12-hour fast and polyethylene glycol bowel cleansing preparation, biopsies of normal-appearing mucosa were collected according to a standard protocol by gastroenterologists using standard-cup flexible endosocopy forceps during usual care colonoscopies. Six sextant pinch biopsies, approximately 1 mm thick, were obtained from

the rectum (10 cm above the anus) on all participants, and from the mid-sigmoid and proximal ascending (immediately distal to the cecum) colon on 20% of participants, for a total of up to 18 biopsies. No biopsies were taken within 4.0 cm of a polypoid lesion.

Biopsy specimens were fixed by 10% normal buffered formalin for 24 hours, and then transferred to 70% ethanol. Within a week, the biopsies were processed and embedded in paraffin blocks with three biopsies per colon site per participant per block.

Immunohistochemistry

Within seven days of being embedded in paraffin blocks, 3.0 μm thick sections taken 30 μm apart were cut from each block with a microtome such that five slides with four levels each (yielding a total of 20 levels) were prepared per colon site per person.

The immunohistochemistry protocol was calibrated to produce the darkest biomarker labeling staining possible short of yielding non-specific background staining[213]. The slides were immunohistochemically processed using a DAKO Automated Immunostainer (DAKO Corp., Carpinteria, CA; further referred to as DAKO) and Leica H&E Autostainer (Leica Microsystems, Inc., IL). First, MSH2 antigen was unmasked via a heat-induced epitope retrieval procedure by placing the slides in a preheated Pretreatment (PT) Module (Lab Vision Corp., CA) with DAKO TBS Buffer (DAKO S1968) and steamed for 40 minutes. Then, the slides were immunohistochemically processed using an anti-MSH2 antibody (Oncogene NA27) in a 1:500 dilution, a DAKO Envision+ detection system (DAKO K4007), and 3,3' diaminobenzidine (DAB; DAKO K3466) as the chromogen. No counterstaining was used and all stained slides were glass coverslipped with a Leica Automated Coverslipper (Leica Microsystems, Inc., IL).

All five slides per colon site per person were included in one staining batch of up to 48 slides that also included negative and positive control slides. A surgical specimen of normal colon was used for the control slides; the negative and the positive control slides were treated identically to study participant slides except that antibody diluent was used rather than primary antibody on the negative control slide.

Image Analysis

Since MSH2 is expressed in a density gradient along the crypt (figure 4.1) that is not quantifiable by eye (e.g., by counting cells), its expression density, detected by immunohistochemical staining, was quantified in the stained slides using image analysis densitometry methods[156, 211]. The procedure was conducted by one trained "scorer" using a light microscope (Olympus BX40, Olympus Corporation, Japan), digital camera (Polaroid DMC Digital Light Microscope Camera, Polaroid Corporation, USA), digital drawing tablet, and a custom-developed plug-in to ImagePro Plus (Media Cybernetics, Inc.) image analysis software. The scorer was blinded to case-control status and colon site.

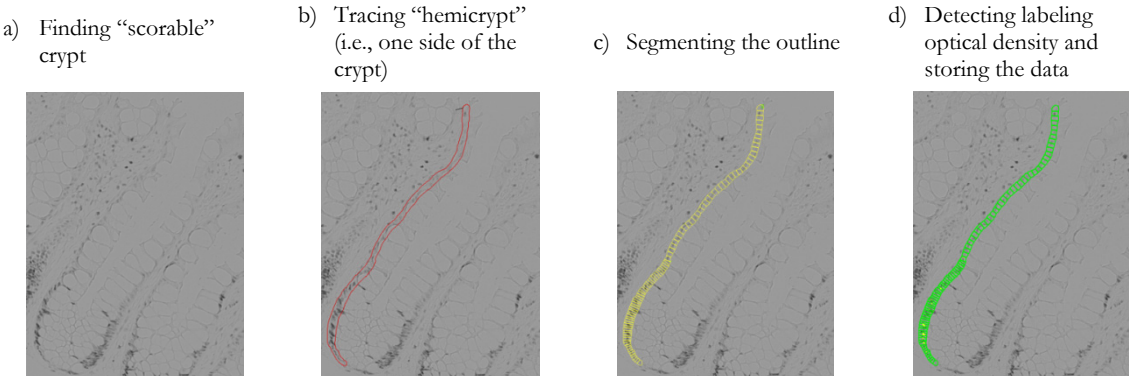

Figure 4.1. Quantitative image analysis of MSH2 labeling optical density consists of several steps: a) finding eligible crypts (see text for details); b) manually tracing one side of the crypt ("hemicrypt"); c) automated division of the outline into segments with width of an average colonocyte; d) automated background-corrected densitometry of overall and segment-specific labeling of the biomarker and entering the results into the database

The imaging and analysis unit was a "hemicrypt", defined as one side of a colonic crypt bisected from base to colon lumen surface (figure 4.1). Intact (at most two contiguous

cells missing) hemicrypts extending from the muscularis mucosae to the colon lumen were considered eligible for quantitative image analysis ("scorable").

For each patient the two of the three biopsies from each colon site with the greatest number of "scorable" hemicrypts were selected for quantitative image analysis ("scoring"). Intact hemicrypts were "scored" in order from the first hemicrypt on the first biopsy from left to right. The goal was to find at least 16 "scorable" hemicrypts per biopsy (32 per patient)[213]. If the 16th hemicrypt was reached before the level was finished, the scorer continued scoring until either the level was finished or the 20th hemicrypt was scored, whichever came first. No more than 20 hemicrypts per biopsy were scored. If the two best biopsies from a colon site on a patient had less than 32 "scorable" hemicrypts, an attempt was made to cut more slides. If that did not solve the issue, scoring was completed if the two best biopsies had 16 or more "scorable" hemicrypts between them. All three biopsies harvested from the same colon site were scored only if there was less than a total of 16 "scorable" hemicrypts between the two best biopsies.

To ensure scoring protocol adherence, a scorer was guided through the scoring protocol by the computer software. For each scored slide background correction images were obtained and automatically used by the computer program to yield background corrected densitometries for all hemicrypts analyzed on that slide. All images were taken at 200x magnification (the maximum magnification at which full length colorectal crypts can be completely included in a single visual field) and stored and analyzed as 16-bit grayscale 1,600 × 1,200 pixel images.

As shown in figure 4.1, using a digital drawing tablet, hemicrypts were manually traced by the scorer from the crypt base center cell up along the crypt basement membrane

to the beginning of the turn of the crypt onto the colonic mucosal surface and then back down along the crypt luminal surface of the epithelial nuclei[156, 211]. The software program divided the traced hemicrypt into segments corresponding in width to that of an average normal crypt epithelial cell (6.59 μm)[213], and then calculated overall hemicrypt- and segment-specific labeling optical densities and entered these data into a Microsoft Access database along with various dimensional parameters of the hemicrypt[156, 211].

For quality assurance, slide sets from 10% of the participants were randomly selected by the statistical team, blinded, and re-submitted to the scorer for re-scoring[213].

Statistical Analysis

Statistical analyses were performed using SAS 9.2 statistical software (Copyright© 2002-2008 by SAS Institute Inc., Cary, NC, USA). The entire MAP II study population (51 cases and 154 controls) as well as the subset of participants for whom slides were immunohistochemically processed for MSH2 protein (45 cases and 47 controls) were assessed for comparability using the t-test for continuous variables, the Fisher's exact test for dichotomous variables, and the Freeman-Halton extension to Fisher's exact test for categorical variables as appropriate. All labeling optical density means were calculated using linear mixed models. Potential confounders as well as staining batch were included in the models as fixed effects, and correlation among multiple optical density measurements within each patient was accounted for by including a patient variable as a random effect. Mean proportional differences were calculated as the model-predicted mean optical density for cases minus that for controls divided by the mean for cases. Statistical significance of these measurement differences was evaluated by t-test. The intra-class correlation coefficient was used to assess slide scoring reliability and found to be $r = 0.96$.

The distribution of MSH2 protein within colonic crypts was evaluated graphically with the Loess procedure as implemented in SAS version 9 statistical software[203]. First, the number of cells within a hemicrypt was standardized to 50 segments (the average number of cells within a column of colonic crypt cells). Then, average Loess model predicted segment-specific levels of MSH2 for cases and controls by colon site were plotted in the graphs (figure 4.2) along with smoothing lines to make graphical evaluation easier.

Potential confounders were evaluated on the basis of biological plausibility and whether the variable of interest was associated with the exposure based on existing epidemiological, medical, and basic science literature. Potential confounders considered in this analysis included: age, sex, physical activity, body mass index (BMI), family history of colorectal cancer in a first degree relative, smoking, alcohol consumption, aspirin and nonsteroidal anti-inflammatory drug (NSAID) 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[204]. Continuous variables were dichotomized based on their distributions in the controls.

The association between MSH2 expression and risk of incident sporadic colorectal adenoma within each colon site was assessed with linear mixed models using individual hemicrypt measurements. Potential confounders and staining batch were entered into the models as fixed effects; a random intercept was added to each model to account for multiple correlated optical density measurements within each subject. The overall association between MSH2 expression in the colorectal mucosa and risk of incident, sporadic colorectal adenoma was evaluated by calculating odds ratios (OR) from repeated measures logistic (GEE) models based on average hemicrypt MSH2 expression from all three colon sites within an

individual. Both mixed and GEE models contained the same set of potential confounders. A 95% confidence interval was calculated for each odds ratio. 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.

Repeated measures logistic models (GEE) were also used to model associations between the level of MSH2 expression in the rectum and various adenoma characteristics such as location, multiplicity, degree of dysplasia, histological type, and shape. For this analysis the batch-standardized optical density variable was dichotomized based on the colon site-specific mean in the controls.

The associations of MSH2 expression in the rectum with various demographic, lifestyle, and dietary characteristics were assessed by mixed models. Potential confounders were entered into the model one at a time as fixed effects. 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. A random intercept was included in the model to account for lack of independence among hemicrypt measurements within each patient. The number of biopsies from the more proximal portions of the colon was too small for reliable similar analyses.

In sensitivity analyses, we also analyzed data without standardization for batch, as well as by using different mathematical transformations; the results from these analyses did not differ materially from those reported.

Results

The sub-population of subjects whose biopsies were stained for MSH2 and analyzed (45 cases and 47 controls) was compared to 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 4.1. On average, cases tended to be older, more likely to be male, more likely to be a current smoker and currently consume alcohol, less likely to regularly take an NSAID, and tended to have higher total energy intakes and lower intakes of calcium, vitamin D, and folate than controls, but only the difference for total energy intake was statistically significant. Physical activity, BMI, aspirin use, and fat and fiber intakes did not differ substantially between cases and controls.

Among cases, 30% had multiple adenomas, 8% had an adenoma that was ≥ 1.0 cm in diameter, and 14% had moderate or severe dysplasia in their largest or most advanced adenoma. In 73% of cases the largest or most advanced adenoma was located proximal to the rectum, and of these, 59% were located in the right colon (data not shown).

The distribution of MSH2 protein within colonic crypts in the rectum, sigmoid, and ascending colon by colon site is presented in figure 4.2 a – c, respectively. In all three colon sites MSH2 expression was higher at the base of the crypt and progressively decreased towards the hemicrypt's opening into the colon lumen. In the rectum, MSH2 expression was highest in the lower 20% of the crypt and then rapidly decreased and leveled off the rest

Table 4.1. Selected characteristics of incident, sporadic colorectal adenoma cases and controls; the Markers of Adenomatous Polyps II Study

Characteristic*	N (Cases/ Controls)	Adenoma Cases	Controls	p†
Demographics				
Age (yrs.)	45/47	56.2 (7.3)	55.1 (8.4)	0.49
Male (%)	45/47	51	43	0.53
White race (%)	45/46	96	98	0.62
Family History				
1° Relative with colorectal cancer (%)	45/47	16	17	1.00
Lifestyle				
Physical activity (METs/day)	45/46	29.0 (23.6)	27.2 (21.2)	0.69
Body Mass Index (kg/m²)	45/46	30.8 (7.4)	30.6 (7.4)	0.87
Take aspirin at least once per week (%)	45/46	40	37	0.83
Take NSAID‡ at least once per week (%)	45/46	36	48	0.29
Smoking status (%)				
Never		42	50	0.25
Former	45/46	40	43	
Current		18	7	
Alcohol consumption (%)				
Never		11	13	0.74
Former	45/46	24	30	
Current		65	57	
Dietary intakes				
Total energy (kcal/day)	45/45	1,910.8 (794.7)	1,523.6 (411.9)	0.005
Total fat§ (g/day)	45/46	65.6 (16.3)	64.5 (15.1)	0.74
Dietary fiber§ (g/day)	45/46	15.3 (5.7)	15.4 (5.9)	0.81
Total‖ calcium§ (mg/day)	45/46	893.0 (486.7)	959.5 (498.3)	0.42
Total‖ vitamin D§ (IU/day)	45/46	327.4 (288.1)	358.9 (286.9)	0.58
Total‖ folate§ (mcg/day)	45/46	476.3 (233.8)	515.0 (279.3)	0.69

* 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, Fisher's exact test for dichotomous variables, and modified Fisher's exact test for multilevel categorical variables
‡ NSAID – Non-steroidal anti-inflammatory drug (not including aspirin)
§ Energy adjusted using residual method
‖ Total = diet + supplements

of the way up the crypt. In the sigmoid and ascending colon, MSH2 expression was highest in the lower 60% of the crypt (the proliferation zone) and lower in the upper 40% of the crypt (the differentiation zone)[130, 133, 213]. MSH2 expression in the rectum and sigmoid colon appeared virtually identical in cases and controls; in the ascending colon MSH2 expression in

cases was noticeably lower than in controls. For each of the three colon sites investigated in the study, the MSH2 expression curves for cases and controls closely paralleled each other, indicating that the differences in MSH2 expression between cases and controls were uniform along the full lengths of the crypts and were not confined to a functional zone of the crypt or to distribution differences; therefore, only the results from analyses of total crypt expression are reported below and in the tables. Table 4.2 presents "crude", age- and sex-adjusted, and multivariable-adjusted MSH2 expression in all cases and controls stratified by colon site, as well as the combined OR for the association of MSH2 expression with incident, sporadic colorectal adenoma. On average, after adjusting for potential confounders, expression of MSH2 protein in the rectum and ascending colon was 23% (p = 0.06) and 49% (p = 0.01), respectively, lower in adenoma

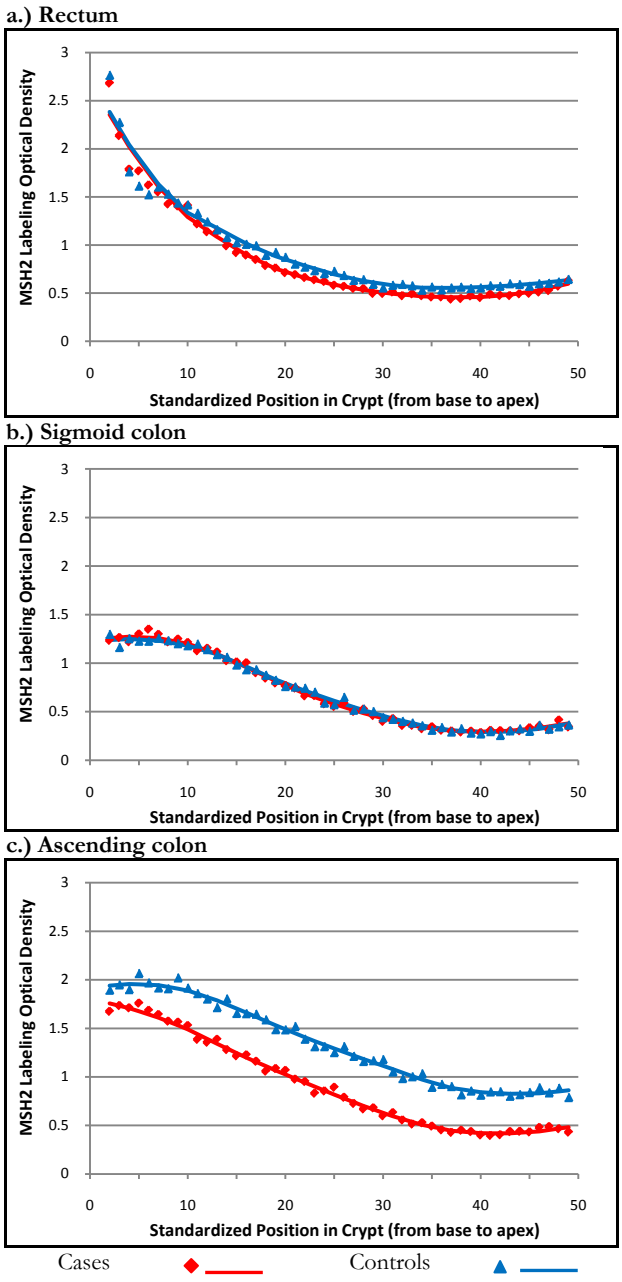

Figure 4.2. Expression of MSH2 protein at standardized positions within crypts of normal-appearing mucosa in incident, sporadic colorectal adenoma cases and controls for three colon sites: a) rectum, b) mid sigmoid colon, c) proximal ascending colon. The Markers of Adenomatous Polyps II Study.

cases than in controls. In the sigmoid mucosa, on the other hand, MSH2 expression was, on average, 25% higher in cases than in controls, but the difference was not statistically significant (p = 0.42). Risk of incident, sporadic colorectal adenomas was inversely associated with MSH2 expression in colonic crypts from all three colon sites combined, but the association was not statistically significant (combined OR = 0.77, 95% confidence interval [C.I.]: 0.38 – 1.58).

Table 4.2. Differences in MSH2 protein expression* in normal-appearing mucosa between incident sporadic colorectal adenoma cases and controls, by colon site; the Markers of Adenomatous Polyps II Study

Colon Site	N (Cases/ Controls)	MSH2 Labeling Optical Density Mean (SE)				Proportional Difference (%)†	p‡
		Cases		Controls			
Model 1: controls for staining batch only							
Rectum	37/41	346.96	(36.93)	410.50	(34.89)	-15%	0.21
Sigmoid	14/16	364.68	(57.52)	313.97	(50.90)	16%	0.52
Ascending	14/16	434.86	(87.90)	712.89	(81.00)	-39%	0.03
Combined OR§ (95% C.I.‖)	43/47	0.78 (0.39 - 1.53)					
Model 2: controls for age, sex, and staining batch							
Rectum	37/40	346.53	(37.47)	415.88	(36.33)	-17%	0.19
Sigmoid	14/16	379.19	(58.13)	302.45	(51.48)	25%	0.34
Ascending	14/16	432.09	(93.60)	714.45	(85.15)	-40%	0.03
Combined OR (95% C.I.)	43/46	0.76 (0.38 - 1.50)					
Model 3: controls for age, sex, FHCRC#, aspirin/NSAIDs, physical activity, calcium, and staining batch**							
Rectum	37/39	330.75	(46.00)	428.8	(47.50)	-23%	0.06
Sigmoid	14/15	406.35	(96.48)	325.6	(67.80)	25%	0.42
Ascending	14/15	377.80	(128.40)	738.02	(98.01)	-49%	0.01
Combined OR (95% C.I.)	43/45	0.77 (0.38 - 1.58)					

* Expression detected immunohistochemically and labeling quantified by image analysis densitometry methods; results shown as labeling optical density (OD)

† [(cases – controls)/controls]×100%

‡ Based on t-test for comparing the two means

§ Combined OR – odds ratio (Cases vs. Controls) controlling for all three colon sites and the covariates indicated in the specified model. The labeling optical density (MSH2 expression) variable was dichotomized using the mean of the colon site specific distributions in the controls.

‖ C.I. – confidence interval

Family history of colorectal cancer in a first degree relative

** Take aspirin or other nonsteroidal anti-inflammatory drug at least once a week

To assess whether MSH2 expression in the rectum may be associated with a subset of cases (especially those with right-sided adenomas) we investigated associations of MSH2

protein in the rectum with various adenoma characteristics (table 4.3). The overall inverse association of MSH2 expression with adenomas tended to be stronger for adenomas in the right colon and for those with more advanced characteristics (villous component or moderate/severe dysplasia), but the sample size was small and the findings were not statistically significant.

Table 4.3. Crude associations of batch-standardized MSH2 expression in the normal-appearing rectal mucosa with risk of incident sporadic colorectal adenomas overall and according to adenoma characteristics; the Markers of Adenomatous Polyps II Study

Adenoma characteristic*	N (Cases/Controls)	MSH2 expression		95% C.I.‡
		Low (OR†)	High (OR)	
All Adenomas	37/41	1.00	0.87	(0.44 - 1.71)
Location				
Right colon§	26/41	1.00	0.81	(0.39 - 1.67)
Left colon‖	11/41	1.00	1.02	(0.34 - 3.06)
Multiplicity				
Single adenoma	26/41	1.00	0.75	(0.37 - 1.54)
Multiple adenomas	11/41	1.00	1.20	(0.40 - 3.62)
Dysplasia				
Mild	32/41	1.00	0.97	(0.48 - 1.94)
Moderate/severe	5/41	1.00	0.39	(0.08 - 1.83)
Histological type				
Tubular	24/41	1.00	0.95	(0.45 - 1.99)
Tubulovillous/villous	13/41	1.00	0.74	(0.27 - 2.05)

* Size of adenoma not included because only 3 cases had an adenoma ≥ 1 cm in diameter
† OR – odds ratio
‡ C.I. – confidence interval
§ Right colon includes cecum, ascending colon, hepatic flexure, and transverse colon
‖ Left colon includes splenic flexure, descending colon, sigmoid colon, and rectum

We also assessed the potential of MSH2 expression in the rectum as a modifiable biomarker of risk by evaluating associations of MSH2 expression with various risk factors for colorectal cancer (table 4.4). The results from the similar analyses for the two proximal colon sites are not presented because the very limited sample size for these colon sites prohibited reliable estimation. The only statistically significant finding was that MSH2

expression in the rectal mucosa was 39% (p = 0.04) higher in subjects who took aspirin or another non-steroidal anti-inflammatory drug (NSAID) at least once a week.

Table 4.4. Associations of MSH2 expression* in normal-appearing rectal mucosa according to potential risk factors for colorectal neoplasms; the Markers of Adenomatous Polyps II Study

Characteristic†		N	MSH2 Labeling Optical Density‡ Mean (SE§)		p‖
Age (yrs.)					
	35 – 54	41	367.31	(35.16)	0.56
	≥ 55	36	398.52	(39.01)	
	% Difference		*8%*		
Sex					
	Male	37	356.60	(38.41)	0.42
	Female	40	401.97	(37.84)	
	% Difference		*13%*		
Family history of colorectal cancer#					
	No	66	366.03	(29.15)	0.34
	Yes	11	443.07	(72.20)	
	% Difference		*21%*		
Physical activity**					
	Low	43	367.33	(37.22)	0.64
	High	33	395.30	(41.11)	
	% Difference		*8%*		
BMI†† (kg/m²)					
	< 30	39	398.60	(38.54)	0.47
	≥ 30	38	357.59	(38.77)	
	% Difference		*-10%*		
Smoking					
	Former/Never	67	402.18	(28.04)	0.29
	Current	9	291.98	(99.29)	
	% Difference		*-27%*		
Alcohol consumption‡‡					
	Former/ Never	30	374.69	(43.35)	0.79
	Current	46	390.25	(35.31)	
	% Difference		*4%*		
Take aspirin/NSAID§§					
	No	24	303.96	(46.29)	0.04
	Yes	52	423.28	(31.23)	
	% Difference		*39%*		
Total energy intake**					
	Low	27	325.39	(51.75)	0.28
	High	48	394.16	(33.29)	
	% Difference		*21%*		

Table 4.4. Continued

Characteristic†	N	MSH2 Labeling Optical Density‡ Mean (SE§)		p¤
Total‖‖ calcium intake**				
Low	37	414.55	(38.36)	0.26
High	39	350.36	(37.70)	
% Difference		*-15%*		

* Expression detected immunohistochemically and labeling quantified by image analysis densitometry methods; results shown as labeling optical density (OD)
† 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 calcium intake was also adjusted for total energy intake; total energy intake was also adjusted for physical activity.
‡ Mean labeling optical density
§ SE – standard error
‖ Based on t-test for significance of variables from a mixed model
Family history of colorectal cancer in a first-degree relative
** "Low" - below 50th percentile of sex-specific distribution in controls; "High" - at or above 50th percentile of sex-specific distribution in controls
†† BMI – body mass index
‡‡ Categories "Never consumed" and "Former consumer" combined due to small sample size of "Never consumed" category
§§ Aspirin/NSAID – takes aspirin or other nonsteroidal anti-inflammatory drug at least once a week
‖‖ From diet and supplements

Discussion

To our knowledge, this is the first study to report on the distribution of MSH2 protein within normal colorectal crypts in humans or on associations of MSH2 expression in normal-appearing colorectal mucosa with risk for incident, sporadic colorectal neoplasms or with risk factors for colorectal cancer. Our preliminary data support the hypothesis that MSH2 expression in the normal colonic mucosa, especially in the ascending colon, is inversely associated with risk of incident, sporadic colorectal adenoma. The data also suggest that MSH2 expression in the normal colon may be associated with modifiable risk factors for colorectal neoplasms. These findings are similar to those we previously reported for the other key protein in the MMR pathway, MLH1[239]. Our findings may be explained by possible inactivation of the *MSH2* gene via DNA methylation, which results in a loss of expression of the protein in the mucosa. Seifert, *et al* demonstrated that most inactivating mutations in the

MSH2 gene lead to a lack of expression or the expression of a truncated protein not detectable by antibodies used in many studies[18].

We also found that, as would be expected from the known function of MSH2, the colon crypt expression distribution curves for MSH2 appear to follow the cell proliferation distribution within colonic crypts (figure 4.2) with higher expression of the protein in the lower 60% of crypts (proliferation zone), and lower expression in the upper 40% of the crypt (differentiation zone)[130, 133, 213].

The rectum is the most practical colon site for chemoprevention trials and potential clinical outpatient applications because the procedures for obtaining rectal biopsies 10 cm above the anus are minimally invasive and do not require fasting or bowel cleansing preparations[132, 133]. The data from our pilot study suggest that MSH2 expression in the rectum may be associated with risk for incident colorectal adenomas, and that this association parallels that in the ascending colon (table 4.2). We also found that lower MSH2 expression in the rectum tended to be more strongly associated with adenomas in the right colon than in the left colon (table 4.3). These findings are consistent with previous research indicating that MMR deficient colorectal neoplasms tend to be located in the right colon[3, 8]. Our preliminary results, if confirmed by a full-scale study, may indicate that MSH2 expression in the rectum may be a good indicator of its expression and thus risk in the proximal colon, which is much less accessible for screening procedures. We also observed that higher MSH2 expression in the rectum may be associated with lower risk for more advanced adenomas; however, these associations were based on a small sample size and were not statistically significant.

Our analyses to assess associations of MSH2 expression with various risk factors for colorectal neoplasms were severely limited by our small sample size. However, we did find that MSH2 expression in the rectum was statistically significantly substantially higher among persons who regularly took an NSAID, one of the clearest modifiers of risk for colorectal neoplasms. A similar, but not statistically significant association between higher doses of aspirin and MSH2 expression was observed *in vitro*[240]. The exact mechanisms underlying the chemopreventative effects of NSAIDs against colorectal neoplasms are not yet fully understood. In addition to COX-2 inhibition, NSAIDs may also act through non-COX-2-dependent mechanisms[241, 242]. NSAIDs are effective against MMR-deficient colorectal cancers despite the fact that COX-2 expression is often reduced in MMR-deficient colorectal cancer cells[242], so non-COX-2-dependent mechanisms may play a crucial role in this type of cancer.

Since this study was a pilot study, its main limitation was the small sample size. Due to limited resources and the complexity of the procedure, only a fraction of the patients' biopsies were processed for MSH2 expression, further reducing the sample size. For the same reasons biopsies for all three colon sites were often not available. Using a commercial automated immunostainer did not completely eliminate staining variability between staining batches, which introduced an additional source of variability into the analysis that had to be accounted for. The participants in this study were drawn from people who underwent a colonoscopy, and so the results of this study may not be directly applicable to the general population. Data collected by food frequency questionnaires and self-reported data have shortcomings that are well described in the literature, but, since these data were collected before case-control status was determined, any possible bias is expected to be non-differential.

On the other hand, this study had several important strengths: (a) all participants underwent colonoscopy, which ensured accurate identification of cases and controls; (b) all self-reported data (including dietary information) were collected before the case-control status of each participant was determined, thus minimizing possible recall bias; (c) detailed information on potential confounders such as anthropometrics, diet, vitamin and mineral supplements, and medications used was collected; and (d) the rigorous procedures for biopsy collection, processing, and quantitative assessment of the labeling optical density of MLH1 expression detected by immunohistochemistry (IHC) using our specially developed software, which minimized possible measurement error.

In summary, we developed a reliable procedure for detecting and describing MSH2 expression in normal colorectal crypts, and report, to our knowledge, the first study to describe the distribution of the MSH2 protein within normal colorectal crypts, or on associations of MSH2 expression in normal-appearing colorectal mucosa with risk for incident, sporadic colorectal neoplasms or with important risk factors for colorectal cancer. We found that the distribution of the MSH2 protein within normal colonic crypts parallels that of the normal proliferation zone of normal crypts. The data from this preliminary study suggest that lower MSH2 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, and thus support further investigation of MSH2 expression, alone or in combination with other biomarkers, as a potential modifiable biomarker of risk for colorectal neoplasms.

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Chapter 5. Effects of Calcium and Vitamin D on MLH1 and MSH2 Expression in Rectal Mucosa of Sporadic Colorectal Adenoma Patients*

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Abstract

To further clarify and develop calcium and vitamin D as chemopreventive agents against colorectal cancer in humans, understand the mechanisms by which these agents reduce risk for the disease, and develop modifiable biomarkers of risk for colorectal cancer, we conducted a pilot, randomized, double-blind, placebo-controlled, 2×2 factorial clinical trial to test the effects of calcium and vitamin D_3, alone and in combination on key DNA mismatch repair proteins in the normal colorectal mucosa. Ninety-two men and women with at least one pathology-confirmed colorectal adenoma were treated with 2.0 g/d calcium or 800 IU/d vitamin D_3, alone or in combination, versus placebo over six months. Colorectal crypt overall expression and distribution of MSH2 and MLH1 proteins in biopsies of normal-appearing rectal mucosa were detected by automated immunohistochemistry and quantified by image analysis. After six months of treatment MSH2 expression along the full length of the crypt increased by 61% (p=0.11) and 30% (p=0.36) in the vitamin D and calcium groups, respectively, relative to the placebo group. The estimated calcium and vitamin D treatment effects were more pronounced in the upper 40% of the crypt

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(differentiation zone) where MSH2 expression increased by 169% (p=0.04) and 107% (p=0.13) in the vitamin D and calcium groups, respectively. These findings suggest that higher calcium and vitamin D intakes result in increased DNA MMR system activity in the normal colorectal mucosa of sporadic adenoma patients, and that the strongest effects may be vitamin D related and in the differentiation zone of the colorectal crypt.

Introduction

Colorectal cancer is a common, lethal disease in Western nations, and its incidence and mortality is increasing dramatically in the rest of the world. Currently, in the United States, colorectal cancer is the second leading cause of cancer deaths[1]. The vast majority of so-called "sporadic" colorectal cancer develops in the adenomatous polyp, a benign intestinal tumor that is the only accepted biomarker of risk for colorectal cancer[3, 8].

Higher intakes of calcium and higher levels of circulating 25-OH-vitamin D have been associated with reduced risk for colorectal cancer[100, 107, 243]. Also, calcium intake is inversely associated with risk of incident colorectal adenomas, and calcium supplementation reduces adenoma recurrence[107, 216, 243, 244]. However, the individual and combined anti-neoplastic effects of calcium and vitamin D in humans remain unclear. Proposed, likely complementary, anti-neoplastic mechanisms of calcium include protection of the colorectal mucosa against bile and fatty acids[141, 245], direct effects on the cell cycle[246], and modulation of E-cadherin and β-catenin expression via the calcium sensing receptor (CaSR)[246-248]. Vitamin D, beyond its role in calcium metabolism and homeostasis, promotes bile acid degradation and xenobiotic metabolism; regulates cell proliferation, differentiation, and apoptosis; and influences DNA repair, angiogenesis, inflammation, and immune response[248-250].

The DNA mismatch repair (MMR) pathway is responsible for ~15% of colorectal cancers, which involves silencing of one of two (or both) essential genes: *MLH1* and *MSH2*[12]. Silencing of either of these genes interrupts the normal review and repair of DNA errors after replication, which eventually leads to microsatellite instability (MSI) and cancer development. Levels of expression of MLH1 and MSH2 protein in colonic cells are likely to indicate the functional level of the MMR mechanism.

To the authors' knowledge there are no published studies that specifically investigated effects of calcium or vitamin D on DNA mismatch repair proteins. This paper reports findings from a pilot clinical trial that addresses the individual and combined effects of calcium and vitamin D_3 supplementation on the expression of MLH1 and MSH2 proteins in the normal-appearing colorectal mucosa of colorectal adenoma patients.

Participants and Methods

This study was approved by the Emory University IRB. Written informed consent was obtained from each study participant.

Participant Population

Participants were recruited from the patient population attending the Digestive Diseases Clinic, the Emory Clinic, Emory University. To be eligible for the study, patients must have been 30-75 years of age, in general good health, and capable of informed consent. They must have had a history of at least one pathology-confirmed adenomatous colonic or rectal polyp within the past 36 months; had no contraindications to calcium or vitamin D supplementation or rectal biopsy procedures; and had no medical conditions, habits, or medication usage that would otherwise interfere with the study as described below.

Specific exclusions were supplemental calcium and/or vitamin D intake greater than the recommended daily allowance (RDA); supplemental vitamin A intake greater than 10,000 IU/day; major diet change within the previous six months; inability to refrain from aspirin use for seven days; current, planned or recent participation in another clinical trial; pregnancy, trying to get pregnant, or breast-feeding; familial adenomatous polyposis; elevated serum calcium or creatinine; supraphysiologic levels of 25-OH-vitamin D at their study eligibility visit; kidney stones or sarcoidosis within the previous 20 years; history of a bleeding disorder or current use of anticoagulant medication; use of a thiazide diuretic in an amount greater than the equivalent of 50 mg of hydrochlorothiazide daily; immunosupression; a history of osteoporosis; use of lithium, an ion exchange resin, tetracycline, or indomethacin; renal insufficiency; dementia; cardiovascular disease that moderately or severely limited activity; inflammatory bowel disease; a malignancy other than nonmelanoma skin cancer within the previous five years; hyperparathyroidism or hypoparathyroidism; uncontrolled hypothyroidism or hyperthyroidism; enema or laxative dependence; active peptic ulcer disease; gastrectomy; bowel resection; active liver or pancreatic disease; intestinal malabsorption syndromes; narcotic or alcohol dependence; on a weight loss diet; and a non-deliberate weight loss of 10% or more in previous three months.

Clinical Trial Protocol

All age-eligible patients who had been diagnosed as having at least one pathology confirmed adenomatous colonic or rectal polyp within the past 36 months were identified as potential study participants. All patients passing initial chart screening for eligibility were sent an introductory letter, followed by a telephone interview. During the telephone interview, a few preliminary screening questions were asked and, if a person was willing, still appeared eligible, and could be available for the next eight months, an in-person eligibility visit was

scheduled. Potential participants were asked to bring all medications and vitamins and minerals being taken to this appointment.

During the eligibility visit, potential participants were interviewed, signed a consent form, completed questionnaires (included questions on socio-demographics, medical history and medication use, nutritional supplement use, lifestyle, family history, and others), and provided a blood sample. Diet was assessed with a semi-quantitative Willett food frequency questionnaire[251]. Medical and pathology records were reviewed. Those still eligible and willing to participate then entered a 30-day placebo run-in trial. Only participants without significant perceived side effects and who had taken at least 80% of their tablets were eligible for randomized assignment. Adherence for the run-in trial was assessed by questionnaire, interview, and pill count.

Eligible participants then had their vital signs taken, underwent a baseline rectal biopsy and, if still willing to participate, were randomly assigned (stratified by sex and nonsteroidal anti-inflammatory drug [NSAID] use) to one of four treatment groups. Of patients who passed initial chart eligibility, 42% were contacted and 20% were eligible and consented to participate.

Participants (n=92) were randomly assigned to the following four treatment groups: a placebo control group, a 2.0 g elemental calcium supplementation group (as calcium carbonate in equal doses twice daily), an 800 IU vitamin D_3 supplementation group (400 IU twice daily), and a calcium plus vitamin D_3 supplementation group taking 2.0 g elemental calcium plus 800 IU of vitamin D_3 daily. Each group consisted of 23 participants.

Study tablets were custom manufactured by Tishcon Corporation, NY, USA. The corresponding supplement and placebo pills were identical in size, appearance, and taste. The placebo was free of calcium, magnesium, vitamin D, and chelating agents.

Calcium carbonate was chosen because it delivers more elemental calcium for a given tablet than other forms, therefore, fewer tablets are required, enhancing adherence; it was the form used in the Calcium and Polyp Prevention adenoma recurrence[121] and the Calcium and Colorectal Epithelial Cell Proliferation[133] trials, and in the majority of the larger studies using long term calcium supplementation for other reasons, therefore, its safety record had been well established; and it was the least expensive and most widely available calcium supplement form.

Vitamin D_3 was the chosen form of vitamin D for several reasons, the most important of which was to avoid the toxicity risks associated with 1,25$(OH)_2$-vitamin D or 25(OH)-vitamin D. Multivitamins and calcium/vitamin D supplements typically provide 400 IU per day of vitamin D_3, but numerous intervention studies show that this dose will not suppress PTH in the overwhelming majority of North American adults[252, 253]. So, we chose a more effective dose of 800 IU per day, which raises serum 25-(OH) vitamin D levels toward the desired range, and leaves a substantial margin of safety, even after taking into consideration dietary intake.

The treatment period was six months to replicate the treatment period of the Calcium and Colorectal Epithelial Cell Proliferation trial[133] and to ensure approximately 2 – 3 months of 25-OH-vitamin D steady state levels. Participants attended follow-up visits at 2 and 6 months after randomization and were contacted by telephone at monthly intervals between the second and final follow-up visits. At follow-up visits, pill-taking adherence was

assessed by questionnaire, interview, and pill count. Participants were instructed to remain on their usual diet and not take any nutritional supplements not in use on entry into the study. At each of the follow-up visits participants were interviewed, filled out questionnaires, and had their vital signs taken. At the first and last visits all participants had their blood drawn and underwent a rectal biopsy procedure. All participants were asked to abstain from aspirin use for seven days prior to each biopsy visit. All visits for a given participant were scheduled at the same time of day to control for possible circadian variability in the outcome measures.

Factors hypothesized to be related to risk for colorectal neoplasms or to the expression of MMR proteins in normal colon mucosa (e.g., diet, medications, etc.) were assessed at baseline, several were reassessed at the first follow-up visit, and all were reassessed at the final follow-up visit. Participants did not have to be fasting for their visits and did not take a bowel cleansing preparation or enema.

Six sextant approximately one millimeter-thick biopsy specimens were taken from normal-appearing rectal mucosa 10 cm proximal to the external anal aperture through a rigid sigmoidocsope with a jumbo cup flexible endoscopic forceps mounted on a semi-flexible rod. No biopsies were taken within 4.0 cm of a polypoid lesion. The biopsies were then immediately placed in phosphate buffered saline and examined and reoriented under a dissecting microscope to ensure that they were not twisted or curled on the bibulous paper. The biopsies were then immediately placed in 10% normal buffered formalin.

Immunohistochemistry Protocol

The biopsies in formalin were left undisturbed for at least six hours, transferred to 70% ethanol 24 hours after being placed in formalin, embedded in paraffin blocks (two

blocks of three biopsies each) within two weeks of the biopsy procedure, cut and stained within another four weeks, and analyzed within another four weeks. From one block, five slides with four section levels each taken 40 microns apart were prepared for each antigen, yielding a total of 20 levels per antigen.

Heat-mediated antigen retrieval was used to break the protein cross-links formed by formalin to uncover the epitope. To accomplish this, slides were placed in a preheated Pretreatment (PT) Module (Lab Vision Corp., CA) with 100x Citrate Buffer pH 6.0 (DAKO S1699, DAKO Corp., Carpinteria, CA; further referred to as DAKO) and steamed for 40 minutes. After antigen retrieval, slides were placed in a DAKO Automated stainer (DAKO) and rinsed with warm PT Module Buffer. The Autostainer was programmed for each immunohistochemistry (IHC) run and the following reagents were used: antibody (MLH1 antibody manufactured by BD Pharmingen, catalog no. 554072, dilution 1:15; or MSH2 antibody manufactured by Calbiochem, catalog no. NA27, dilution 1:500) diluted with Antibody Diluent (DAKO S0809 for MLH1 and S3022 for MSH2, DAKO), LSAB2 Detection System (DAKO K0675, DAKO) for MLH1 and Envision+ Detection System (DAKO K4007, DAKO) for MHS2, diaminobenzidine (DAB) (DAKO K3466 for MLH1 and K3438 for MSH2, DAKO), and TBS buffer (DAKO S1968, DAKO). The slides were not counterstained. After staining, the slides were coverslipped automatically 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. A surgical specimen of normal colon tissue was used as a control tissue for both MMR biomarkers. The control tissue was processed in the same manner as the patient's tissue, and 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.

Protocol for Quantifying Staining Density of Immunohistochemically Detected Biomarkers in Normal Colon Crypts ("Scoring")

The imaging and analysis unit was a "hemicrypt", defined as one side of a colonic crypt bisected from base to colon lumen surface. Intact (at most two contiguous cells missing) hemicrypts extending from the muscularis mucosae to the colon lumen were considered eligible for quantitative image analysis ("scorable"; figure 5.1). Before analysis, negative and positive control slides were checked for staining adequacy, and the patient's slides were scanned to assess the adequacy of the biopsy specimen (i.e., whether "scorable" crypts were present).

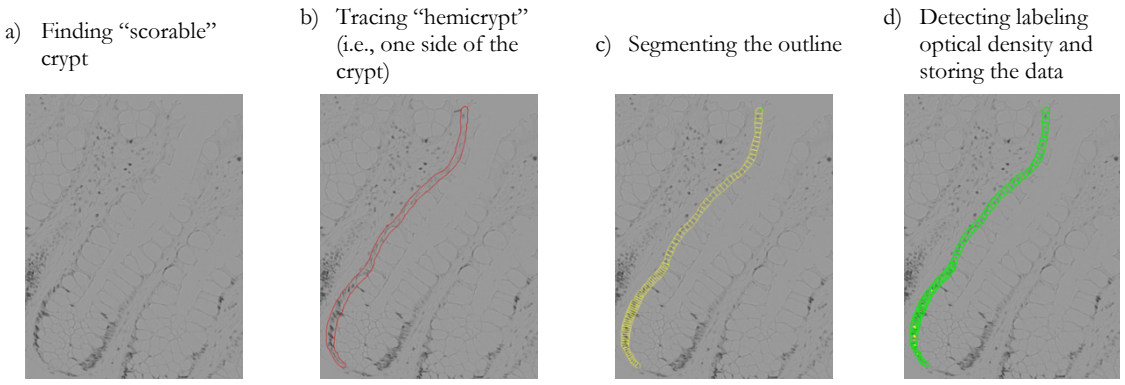

Figure 5.1. Quantitative image analysis of MLH1 and MSH2 labeling optical density consists of several steps: a) finding eligible crypts (see text for details); b) manually tracing one side of the crypt ("hemicrypt"); c) automated division of the outline into segments of width of an average colonocyte; d) automated background-corrected densitometry of overall and segment-specific labeling of the biomarker and entering the results into the database

The major equipment and software for the image analysis procedures were: personal computer, light microscope (Olympus BX40, Olympus Corporation, Japan) with appropriate filters and attached digital light microscope camera (Polaroid DMC Digital Light Microscope Camera, Polaroid Corporation, USA), digital drawing board, ImagePro Plus image analysis software (Media Cybernetics, Inc., MD), our in-house developed plug-in software for colorectal crypt analysis, and Microsoft Access 2003 relational database software (Microsoft Corporation, WA).

The following preparations were performed before starting the scoring program: 1) ensuring standardized settings on the microscope, digital camera, and imaging software; and 2) cleaning and visually scanning the slides. Then, participant ID number, scorer ID, visit number, and antigen, followed by the number of the first biopsy to be scored, whether it had "scorable" crypts, whether it was labeled, and if so, the section level number on the biopsy on which scoring was begun was recorded. Slides were oriented in a standardized fashion and the section levels on the slides were viewed in sequence using light microscopy. All images were taken at 200x magnification and stored as 16-bit grayscale 1,600 × 1,200 pixel images.

For each patient the two biopsies from each colon site with the greatest number of "scorable" hemicrypts were selected for quantitative image analysis ("scoring"). Intact hemicrypts were "scored" in order from the first section of the first biopsy from left to right. The goal was to score at least 16 "scorable" hemicrypts per biopsy (32 per patient). If the 16^{th} hemicrypt was reached before the level was finished, the scorer continued scoring until either the level was finished or the 20^{th} hemicrypt was scored, whichever came first. No more than 20 hemicrypts per biopsy were scored.

If the two best biopsies taken from the same colon site of a patient had less than 32 "scorable" biopsies, an attempt was made to cut more slides. If that did not solve the issue, scoring was completed if the two best biopsies had 16 or more "scorable" hemicrypts between them. All three biopsies harvested from the same colon site were scored only if there was less than a total of 16 "scorable" hemicrypts between the two best biopsies.

To ensure adherence, a scorer was guided through the scoring protocol by the computer software. For each scored slide background correction images were obtained and

controlled for by the computer program. Hemicrypts were manually traced by the scorer (figure 5.1). A traced hemicrypt was divided by the software into segments corresponding in width to that of 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.

One slide reader analyzed all of the MLH1 and MSH2 stained slides throughout the study. A reliability control sample previously analyzed by the reader was re-analyzed during the course of the trial to determine intra-reader reliability.

Protocol for Measuring Serum 25-OH-vitamin D and 1,25-(OH)2-vitamin D Levels

Laboratory assays for serum 25-OH-vitamin D and 1,25-$(OH)_2$-vitamin D were done by Dr. Bruce W. Hollis at the Medical University of South Carolina using a RIA method as previously described[254, 255]. Serum samples for baseline and follow-up visits for all subjects were assayed together, ordered randomly, and labeled to mask treatment group, follow-up visit, and quality control replicates. The average intra-assay coefficient of variation for serum 25-OH-vitamin D was 2.3%, and for 1,25-$(OH)_2$-vitamin D, 6.2%.

Statistical Analysis

Statistical analyses were performed using SAS 9.2 statistical software (Copyright© 2002-2008 by SAS Institute Inc., Cary, NC, USA). Treatment groups were assessed for comparability of characteristics at baseline and at final follow-up by the Fisher's exact test for categorical variables and analysis of variance (ANOVA) for continuous variables. Slide scoring reliability was analyzed using intra-class correlation coefficients.

Labeling optical densities for MLH1 and MSH2 were adjusted for staining batch by dividing each individual's measurement by their corresponding batch-specific mean. Batch-specific means were calculated among all subjects for the baseline visit and among the placebo group for the follow-up visit. We decided *a priori* to investigate overall (total) crypt expression, expression in the upper 40% (differentiation zone) and lower 60% (proliferation zone) of the crypts, and the ratio of expression in the upper 40% to the full length of the crypts as a measure of within-crypt distribution (distribution index or DI) of the MMR markers[130, 133, 213].

Treatment effects were evaluated by assessing differences in mean labeling optical densities from baseline to the 6-month follow-up visit between patients in each active treatment group relative to the placebo group using linear mixed models to account for correlated data. Primary analyses were based on assigned treatment at the time of randomization, regardless of adherence status (intent-to-treat analysis). Two continuous outcomes – MLH1 and MSH2 labeling optical density measurements – were analyzed separately. To provide perspective on the magnitude of the absolute treatment effects ([follow-up – baseline in the active treatment group] – [follow-up – baseline in the placebo group]) of each outcome variable, we also calculated relative effects, defined as: [treatment group follow-up mean/treatment group baseline mean] / [placebo follow-up mean/placebo baseline mean]. The interpretation of the relative effect is somewhat analogous to that of an odds ratio (e.g., a relative effect of 2.0 would mean that the relative proportional change in the treatment group was twice as great as that in the placebo group). No adjustment was made for other covariates in the primary intent-to-treat analyses.

Possible effects of calcium and/or vitamin D on the distribution of MLH1 and MSH2 in rectal crypts were also assessed graphically with the Loess procedure as implemented in SAS version 9 statistical software[203]. First, the number of cells within a hemicrypt was standardized to 50 segments (the average number of cells within a column of colonic crypt cells). Then, average Loess model predicted segment-specific levels of MSH2 for cases and controls by colon site were plotted in the graphs (figure 5.2) along with smoothing lines to make graphical evaluation easier.

In sensitivity analyses, we also analyzed data without standardization for batch, by including batch as a covariate, and using different transformations; the results from these analyses did not differ materially from those reported.

Statistical analyses were done using SAS System software (v.9.2; SAS Institute, Inc., NC). A cutoff level of $P \leq 0.05$ (2-sided) was used for assessing statistical significance.

Results

Characteristics of Study Participants

Treatment groups did not differ significantly on characteristics measured at baseline (table 5.1) or at the end of follow-up (data not shown). On average, participants were 61 years old, and 70% were male, 71% were white, and 19% had a history of colorectal cancer in a first degree relative. Most of the participants were college graduates, overweight, and non-smokers. Adequate biopsy specimens for image analysis for MSH2 and MLH1 were obtained from 87 and 78 participants at baseline and from 82 and 72 participants after 6-months follow-up, respectively.

Adherence to visit attendance averaged 92% and did not differ significantly among the four treatment groups. On average, at least 80% of pills were taken by 93% of

Table 5.1. Selected baseline characteristics of the study participants* (n=92)

Characteristics	Treatment Group				p†
	Placebo	Calcium	Vitamin D	Calcium + vitamin D	
	(n=23)	(n=23)	(n=23)	(n=23)	
Demographics					
Age, years	58.5 (8.2)	61.9 (8.2)	60.2 (8.1)	62.1 (7.5)	0.39
Men (%)	70	70	70	70	1.00
White (%)	74	83	65	61	0.13
College graduate (%)	65	64	57	45	0.53
Medical history					
History of colorectal cancer in 1° relative (%)	17	30	17	13	0.60
Take NSAID‡ regularly§ (%)	22	13	9	17	0.77
Take aspirin regularly§ (%)	22	52	30	57	0.05
Habits					
Current smoker (%)	9	4	0	0	0.61
Take multivitamin (%)	30	30	26	39	0.86
Physical activity (METs/day)	14.5 (11.6)	17.9 (17.9)	20.7 (12.0)	20.9 (14.7)	0.43
Mean dietary intakes					
Total energy intake, kcal/d	1,596 (528)	1,788 (691)	1,848 (821)	1,845 (752)	0.59
Total calcium‖, mg/d	618 (308)	746 (335)	843 (526)	824 (714)	0.41
Total vitamin‖ D, IU/d	277 (230)	336 (202)	360 (317)	415 (316)	0.40
Total fat, gm/d	67 (32)	72 (35)	70 (32)	74 (28)	0.59
Dietary fiber, gm/d	15 (7)	17 (9)	18 (9)	17 (11)	0.97
Alcohol, gm/d	9 (14)	11 (15)	14 (18)	10 (20)	0.84
Anthropometrics					
Body mass index (BMI), kg/m^2	30.6 (7.2)	29.4 (5.5)	28.9 (5.6)	31.6 (6.0)	0.44
Waist-to-hip ratio	0.9 (0.1)	0.9 (0.1)	0.9 (0.1)	1.0 (0.1)	0.17
Adenoma characteristics					
Multiple adenomas# (%)	17	22	39	26	0.45
Large adenoma ≥ 1 cm** (%)	19	32	17	9	0.32
Villous/tubulovillous adenoma†† (%)	4	9	9	4	1.00
Mild dysplasia‡‡ (%)	100	96	100	100	1.00
Baseline vitamin D serum levels					
25-OH-vitamin D (ng/ml)	20.44 (7.5)	25.67 (7.6)	21.04 (8.3)	20.93 (9.6)	0.12
1,25-$(OH)_2$-vitamin D (pg/ml)	39.2 (12.2)	45.4 (35.3)	44.5 (22.6)	37.9 (12.5)	0.60

* Data are given as means (SD) unless otherwise specified.
† By Fisher's exact test for categorical variables, and by ANOVA for continuous variables.
‡ Nonsteroidal anti-inflammatory drug.
§ At least once a week.
‖ Diet plus supplements.
At least two adenomas.

participants at the first follow-up visit and 84% at the final follow-up visit. There were no treatment or biopsy complications. Seven people (8%) were lost to follow-up due to perceived drug intolerance (n=2), unwillingness to continue participation (n=3), physician's advice (n=1), and death (n=1). Dropouts included one person from the vitamin D supplementation group, and two persons from each of the other three groups. Intra-class correlation coefficients for biopsy "scoring" reliability were 0.95 and 0.98 for MLH1 and MSH2, respectively.

At baseline, there were no significant differences in serum levels of 25-OH-vitamin D or 1,25-$(OH)_2$-vitamin D among the four study groups. As previously reported[156], at the end of follow up, 25-OH-vitamin D serum levels had statistically significantly increased in the vitamin D and calcium plus vitamin D groups, and decreased minimally in the placebo and calcium groups; the decrease, however, was not statistically significant. As expected, serum levels of 1,25-$(OH)_2$-vitamin D did not change within any treatment group (data not shown).

Effects of Calcium and/or Vitamin D3 Supplementation on MSH2 Expression

At baseline the four treatment groups did not differ significantly in their expression of MSH2 or MLH1 in the rectal mucosa. The graphical assessment of MSH2 distribution showed that after the treatment period the MSH2 protein retained its normal within-rectal crypt distribution in all four treatment groups with most MSH2 expression concentrated in the lower 60% of the crypt (the proliferation zone; figure 5.2). After 6-months of treatment, MSH2 expression along the full lengths of crypts increased by 30% (p=0.36) and 61% (p=0.11) in the calcium and vitamin D groups, respectively, relative to the placebo group, but did not change appreciably in the calcium plus vitamin D treatment group (table 5.2, A).

Table 5.2. MLH1 and MSH2 expression in colorectal crypts at baseline and 6-months follow-up shown as staining batch standardized* optical density of staining of the immunohistochemically-detected biomarkers

	Baseline				6-Month Follow-up				Absolute Treatment Effect§				Relative Effect‖
	N	Mean*	SE†	p‡	N	Mean	SE	p	N	Mean	SE	p	
A. Entire crypts													
MSH2													
Placebo	20	1.01	0.14		20	1.01	0.14		17				1.00
Calcium	23	0.86	0.13	0.41	21	1.11	0.14	0.60	21	0.26	0.28	0.36	1.30
Vitamin D	22	0.75	0.13	0.17	20	1.20	0.14	0.34	19	0.46	0.28	0.11	1.61
Calcium + vitamin D	22	1.13	0.13	0.57	21	1.10	0.14	0.66	20	-0.03	0.28	0.93	0.98
MLH1													
Placebo	17	1.04	0.07		18	1.05	0.07		13				1.00
Calcium	18	0.98	0.07	0.59	19	1.11	0.07	0.58	17	0.11	0.15	0.44	1.11
Vitamin D	21	0.93	0.07	0.27	18	1.11	0.07	0.59	18	0.17	0.14	0.24	1.18
Calcium + vitamin D	22	1.05	0.07	0.93	17	1.11	0.07	0.56	16	0.05	0.14	0.71	1.05
B. Upper 40% of crypts													
MSH2													
Placebo	20	0.10	0.02		20	0.06	0.02		17				1.00
Calcium	23	0.07	0.02	0.27	21	0.09	0.02	0.31	21	0.06	0.04	0.13	2.07
Vitamin D	22	0.06	0.02	0.18	20	0.11	0.02	0.12	19	0.08	0.04	0.04	2.69
Calcium + vitamin D	22	0.07	0.02	0.30	21	0.09	0.02	0.41	20	0.05	0.04	0.18	1.90
MLH1													
Placebo	17	0.33	0.03		18	0.31	0.03		13				1.00
Calcium	18	0.31	0.03	0.64	19	0.33	0.03	0.57	17	0.04	0.06	0.47	1.14
Vitamin D	21	0.30	0.03	0.44	18	0.35	0.03	0.26	18	0.07	0.05	0.18	1.26
Calcium + vitamin D	22	0.35	0.03	0.58	17	0.36	0.03	0.20	16	0.03	0.06	0.58	1.10

Table 5.2. Continued.

	Baseline				6-Month Follow-up				Absolute Treatment Effect§				Relative Effect‖
	N	Mean*	SE†	p‡	N	Mean	SE	p	N	Mean	SE	p	
C. Lower 60% of crypts													
MSH2													
Placebo	20	0.91	0.13		20	0.94	0.13		17				1.00
Calcium	23	0.79	0.12	0.48	21	1.02	0.13	0.68	21	0.20	0.26	0.44	1.25
Vitamin D	22	0.68	0.12	0.20	20	1.09	0.13	0.43	19	0.38	0.26	0.16	1.54
Calcium + vitamin D	22	1.05	0.12	0.44	21	1.01	0.13	0.73	20	-0.08	0.26	0.77	0.92
MLH1													
Placebo	17	0.72	0.05		18	0.75	0.05		13				1.00
Calcium	18	0.68	0.05	0.58	19	0.78	0.05	0.62	17	0.07	0.10	0.45	1.11
Vitamin D	21	0.63	0.05	0.22	18	0.76	0.05	0.86	18	0.10	0.10	0.32	1.15
Calcium + vitamin D	22	0.70	0.04	0.85	17	0.76	0.05	0.89	16	0.02	0.10	0.81	1.03

* Standardization for staining batch done by dividing each individual's labeling optical density measurement by the mean measurement of their staining batch. Batch-specific means were calculated among all subjects for the baseline visit and among the placebo group for the follow-up visit.

† SE – standard error

‡ Evaluates the difference between each treatment group and the placebo group.

§ Absolute Treatment Effect = (treatment group follow-up – treatment group baseline) – (placebo group follow-up – placebo group baseline).

‖ Relative effect = [(treatment group follow-up/treatment group baseline) / (placebo follow-up/placebo baseline)]; interpretation as for odds ratio (e.g., a relative effect of 1.6 indicates a proportional increase of 60% in the treatment group relative to that in the placebo group)

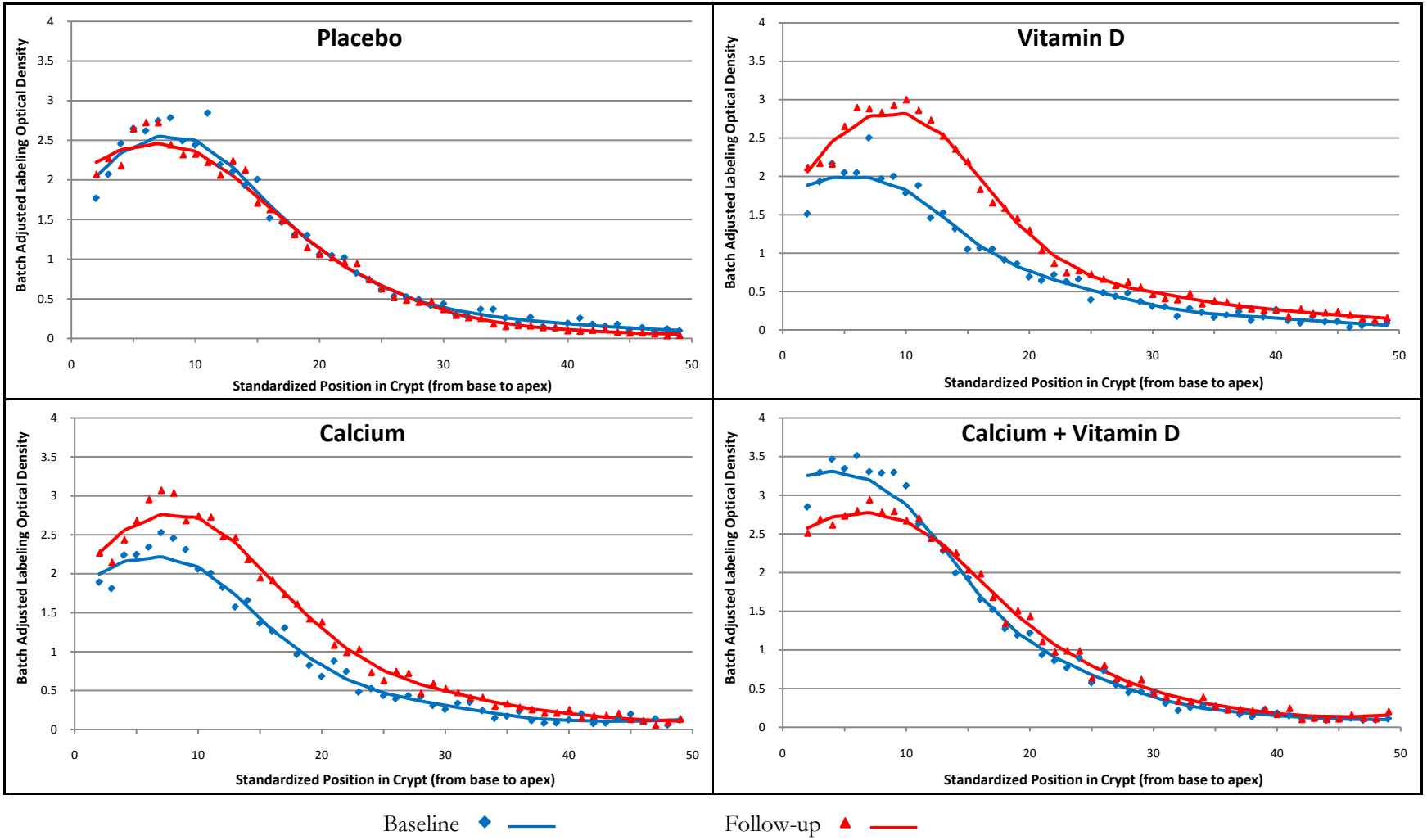

Figure 5.2. Expression of MSH2 protein at standardized positions within the crypts of normal-appearing rectal mucosa in four treatment groups. The Calcium, Vitamin D vs. Markers of Adenomatous Polyps Trial. Data points represent average optical density for all cases or all controls at a particular standardized position in the crypt, and the curves are Loess smoothing curves (smoothing parameter 0.5).

Most of the *absolute* change in the calcium, vitamin D, and calcium plus vitamin D groups occurred in the lower 60% of crypts (table 5.2, B, C; figure 5.2); the relative treatment effect in this crypt zone was very similar to that for the entire crypt in each treatment group (table 5.2, A, C). On the other hand, the greatest *relative* change occurred in the upper 40% of the crypt (the differentiation zone) where MSH2 expression increased by 107% (p=0.13), 169% (p=0.04), and 90% (p=0.18) in the calcium, vitamin D, and calcium plus vitamin D groups, respectively, relative to the placebo group (table 5.2, B). Because of the overall low expression of MSH2 in the differentiation zone the absolute differences here were much lower than in the lower portion of the crypt. The proportion of MSH2 in the upper 40% of the crypt (DI) did not change appreciably in any of the treatment groups (data not shown).

Effects of Calcium and/or Vitamin D Supplementation on MLH1 Expression

Graphical assessment of MLH1 expression within the crypt indicated that the most baseline to follow-up change occurred in the vitamin D group in which MLH1 expression appeared to increase uniformly along the entire length of the crypt. In the other three groups the expression curves for the baseline and the follow-up visits were virtually identical (figure 5.3).

Changes in MLH1 expression in the calcium and/or vitamin D supplementation groups relative to the placebo group were similar but less pronounced than those for MSH2 (table 5.2). At the end of the treatment period MLH1 expression in the entire crypt increased by 11% (p=0.44), 18% (p=0.24), and 5% (p=0.71) in calcium, vitamin D, and calcium plus vitamin D groups, respectively, relative to the placebo group (table 5.2, A). The increase in MLH1 expression occurred uniformly along the crypt length and was of approximately the same magnitude in the proliferation and differentiation zones of the crypt

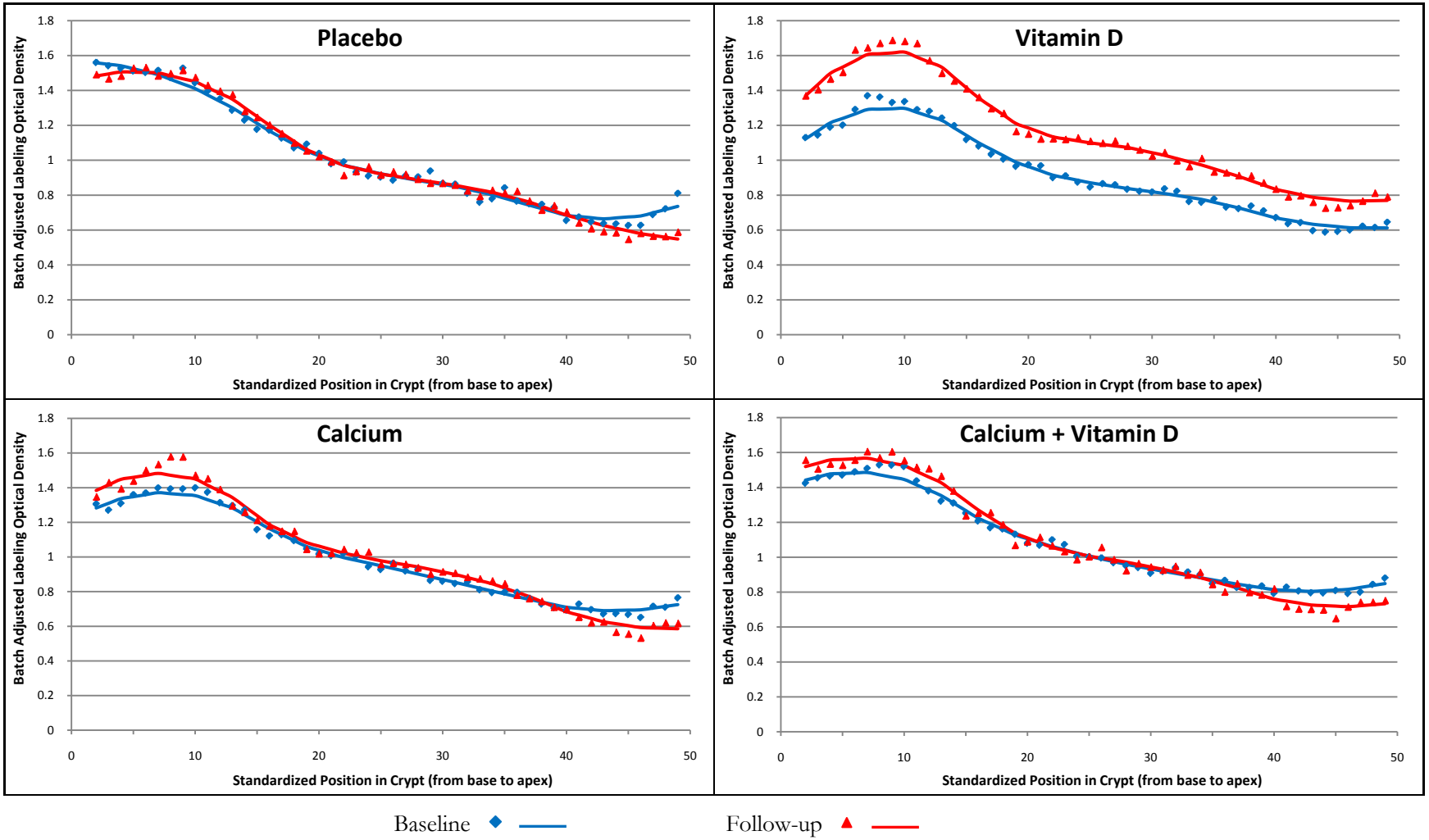

Figure 5.3. Expression of MLH1 protein at standardized positions within the crypts of normal-appearing rectal mucosa in four treatment groups. The Calcium, Vitamin D and Markers of Adenomatous Polyps Trial. Data points represent average optical density for all cases or all controls at a particular standardized position in the crypt, and the curves are Loess smoothing curves (smoothing parameter 0.5).

(table 5.2, B and C); this resulted in no change in the DI in any of the treatment groups (data not shown).

Discussion

This clinical trial had two missions: a) to add to the body of knowledge of the mechanisms by which calcium and vitamin D decrease risk of colorectal cancer, and b) to develop modifiable biomarkers of risk for colorectal cancer. MLH1 and MSH2 were chosen as prospective biomarkers of risk for colorectal cancer because of their crucial role in the human DNA MMR mechanism. Loss or insufficient function of either of these proteins is the main cause of MMR mechanism impairment and is responsible for about 15% of colorectal cancers[12, 16]. To our knowledge the study reported here is the first study to investigate individual and combined effects of calcium and/or vitamin D supplementation on the expression of MLH1 and MSH2 in the normal appearing rectal mucosa in sporadic adenoma patients. Although not statistically significant, our findings suggest that calcium and/or vitamin D supplementation may increase expression of MLH1 and MSH2 in rectal crypts, and that expression of both proteins increases uniformly along the entire crypt. Calcium and vitamin D appear to have greater effects on expression of MSH2 than of MLH1. Our data suggest that individual effects of calcium and vitamin D supplementation may be stronger than the combined effect of calcium plus vitamin D, and that vitamin D may have a stronger effect than calcium on MSH2 and MLH1 expression.

There are no known mechanisms of direct effects of calcium or vitamin D on MLH1 and MSH2 expression. Since in sporadic colorectal carcinomas in which the *MLH1* and/or *MSH2* gene is silenced, the silencing is primarily through epigenetic phenomena[256], the effects of calcium and vitamin D may be through epigenetic modification of the *MLH1* and

MSH2 genes. The results of this study suggest that this may be a fruitful avenue for basic science mechanistic investigations.

Although we hypothesized that the combined effect of calcium plus vitamin D on the MMR proteins would be greater than from either agent alone, we found that it was the smallest among all active treatment groups. At least one experiment in rodents found that calcium and vitamin D individually suppressed cancer development, but their combination was ineffective[165]. The Women's Health Initiative randomized clinical trial also found no overall treatment effect from the combination of calcium plus vitamin D on colorectal cancer incidence; however, this trial used lower daily doses of calcium (1000 mg) and vitamin D (400 IU) and had substantial treatment drop in and drop out[257]. On the other hand, many animal studies that investigated the combination of calcium and vitamin D reported that the anti-neoplastic effect of vitamin D was stronger in animals given relative high-calcium diets[162, 164], and at least two large cohort studies[119, 120] found clear indications of a positive interaction between the two nutrients. In a randomized clinical trial of recurrent colorectal adenoma, there was strong evidence that vitamin D may enhance the chemopreventive effect of calcium; the investigators found that calcium supplementation reduced colorectal adenoma recurrence only in people with blood levels of 25-OH-vitamin D of more than 29.1 ng/ml[124]. In our trial all treatment groups had mean baseline levels of 25-OH-vitamin D below 29.1 ng/ml and only the vitamin D supplementation group exceeded that level at the end of follow up, which may be another explanation of why we did not see any appreciable effect in the calcium plus vitamin D group.

Previous human studies of calcium and/or vitamin D and MLH1 and MSH2 have been limited to investigations of associations of calcium and/or vitamin D with colorectal

carcinomas with microsatellite instability (MSI). MSI develops due to impaired function of MLH1 and/or MSH2 and total absence of one of the proteins leads to high degree microsatellite instability (MSI-H)[15, 209, 258]. Two American case-control studies reported inverse associations between increased calcium intake and colorectal carcinomas with MSI[224, 259]. We did not investigate MSI neoplasms, but our findings suggest that calcium may decrease risk of MSI by directly or indirectly increasing the abundance of MLH1 and MSH2 proteins. On the other hand, a Dutch study reported that increased calcium intake was associated with increased risk of MSI colorectal carcinomas, but their results were not statistically significant[226].

The increase in MLH1 and MSH2 expression in the calcium and vitamin D groups that we observed in our study suggests that calcium and vitamin D increased the activity of the DNA MMR mechanism. Such an increase in activity may be due to an increased capacity of a previously impaired MMR mechanism or it may be a response of the MMR mechanism to an increase in the number of DNA mismatches caused by increased cell proliferation. The latter is unlikely because 1) in this same study[260] and in our previous trial[133] calcium supplementation did not affect the overall colorectal cell proliferation rate, and 2) in both studies there was a downward shift of the proliferative zone[133], whereas there was no evidence for a crypt zone shift for either MSH2 or MLH1 expression in the current study.

In our study, we observed stronger effects of calcium and vitamin D on MSH2 expression along the length of colorectal crypts than on MLH1 expression. A biological mechanism for this finding is unclear. One possible explanation is that since in the steady state there is substantially more MSH2 than MLH1 protein in the cell[17], an increase in MMR function would also require a greater increase in MSH2 concentration.

Our study has several strength and limitations. It is the only randomized, double-blind, placebo-controlled trial to have assessed the independent and combined effects of supplemental calcium and vitamin D on DNA mismatch repair markers in the normal rectal epithelium; there was high protocol adherence by study participants; immunostaining was automated; and, via the use of novel quantitative image analysis procedures, biopsy analysis reliability was high. On the other hand, MLH1 and MSH2 are not proven biomarkers of risk for colorectal cancer, but substantial basic science and epidemiologic literature support their role in colorectal carcinogenesis[3, 8, 12, 16]. This study cannot prove that calcium and/or vitamin D increase the capacity of DNA MMR system, but its results suggest that calcium and vitamin D could have at least an indirect effect on MLH1 and MSH2 expression and thus the entire MMR mechanism.

Overall, the results of this pilot clinical trial suggest that a) calcium and vitamin D individually may increase expression of MLH1 and MSH2 proteins in normal appearing rectal mucosa; b) the effect of vitamin D on both MLH1 and MSH2 expression may be stronger than that of calcium; c) combined treatment with calcium and vitamin D may have an appreciable effect on MSH2 and MLH1 expression only in the differentiation zone of the crypt, but this effect may be weaker than the separate effects of calcium or vitamin D; and d) MLH1 and MSH2 proteins may be potential modifiable biomarkers of risk for colorectal cancer, but further investigation in a full-scale study is required to obtain definitive results. Our trial adds to the body of knowledge supporting calcium and vitamin D as potential chemopreventive agents against colorectal neoplasms.

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Chapter 6. Strengths and Limitations of the Project

Strengths

1. Accurate ascertainment of cases and controls in MAPII case-control study using colonoscopy – the gold standard procedure for diagnosing colorectal adenomas, and pathological verification of colorectal biopsies;

2. Detailed information on potential confounders such as anthropometrics, diet, vitamin and mineral supplements, and medications used was collected;

3. CaDvMAP is the only randomized, double-blind, placebo-controlled trial to have assessed the effect of calcium and/or vitamin D supplementation on the MMR markers in the normal colorectal epithelium. The study achieved high protocol adherence by study participants;

4. The multi-disciplinary approach and integration of laboratory, clinical, epidemiologic, and biostatistical methods;

5. The rigorous procedures for biopsy collection, processing, and quantitative assessment of the optical density of protein expression detected by immunohistochemistry (IHC) using our specially developed software, which minimizes possible measurement error;

6. The novel approach integrating the most current knowledge of the complex molecular basis of colon crypt structure and function and of colon carcinogenesis.

Limitations

1. As pilot studies, MAPII and CaDvMAP have limited sample sizes;

2. Due to limited resources and the complexity of the procedure, only a fraction of the biopsies obtained from MAPII participants were processed into slides and stained for scoring, which further reduced the sample size;

3. This research primarily focused on adenomas rather than carcinomas; however, the adenoma is the precursor of the vast majority of sporadic colon cancers;

4. The participants of both studies were drawn from people who underwent a colonoscopy, and so the results of the study may not be directly applicable to the general population;

5. Data collected by food frequency questionnaires and self-reported data have shortcomings that are well described in the literature, but, since these data were collected before case-control status was determined, any possible bias is expected to be non-differential with respect to case-control status.

Chapter 7. Conclusions

International ecologic and migrant studies point out the importance of modifiable risk factors such as diet and physical activity in the etiology of colorectal cancer. This underscores the importance of early identification of high-risk populations and suggests the importance of preventive interventions. Currently, the colorectal adenoma is the only reliable biomarker of risk for colorectal cancer, and colonoscopy is the only reliable full colon length screening method. However, because of its several limitations, asymptomatic people who are likely to be in the high risk group, sometimes opt out of colonoscopy for financial reasons and because of the general physical and emotional discomfort associated with the procedure. The nature of colonoscopy limits its application in epidemiologic and clinical research.

Alternative methods of screening and risk detection are needed to facilitate screening and research. Biomarkers of risk have been used for other chronic diseases (e.g., cardiovascular diseases) and their utility is proven. This dissertation project is a step in the development of a panel of tissue biomarkers of colorectal cancer. We examined the two key DNA MMR proteins, MLH1 and MSH2, as potential biomarkers of risk for colorectal cancer.

The first study was an analysis of a pilot study to investigate the abundance and distribution of MLH1 and MSH2 proteins in the crypts of the normal colorectal mucosa of incident, sporadic colorectal adenoma cases and adenoma free controls, and the potential for the proteins to serve as modifiable tissue biomarkers of risk for colorectal cancer. It was, to our knowledge, the first study to investigate the distributions of MLH1 and MSH2 within the normal colorectal crypt or associations of the expression of these proteins in normal-

appearing colorectal mucosa with risk for incident, sporadic colorectal neoplasms or with risk factors for colorectal cancer.

We found that MLH1 and MSH2 distributions correspond with these of proliferation markers within the colorectal crypt; i.e., higher expression in the lower 60% of the crypt (the proliferation zone) and lower expression in the upper 40% (the differentiation zone). Levels of expression of both proteins in the rectum were about the same in cases and controls; however, in the ascending colon MLH1 and MSH2 expression was markedly lower in adenoma cases than in controls. This finding suggests possible MMR system deficiency in the proximal colon of sporadic adenoma patients and is consistent with the fact that MMR-deficient colorectal neoplasms tend to be located in the proximal colon.

We found that, while rectal expression of MSH2 and MLH1 tended to be associated with various characteristics of incident colorectal adenomas, the associations were of opposite directions (positive for MLH1 and inverse for MSH2). The respective associations were stronger for adenomas with a villous component or that were located in the proximal colon, but the sample size was small and the associations were not statistically significant. These results may be indicative of early damage to the MMR mechanism due to MSH2 deficiency.

Our results suggest that lower expression of MLH1 and MSH2 in the normal colonic mucosa, at least in the ascending colon, may be associated with increased risk of incident, sporadic colorectal adenoma as well as with modifiable risk factors for colorectal neoplasms, such as regular use of NSAIDs, thus supporting further investigation of MLH1 and MSH2 expression as a potential "treatable" biomarkers of risk for colorectal neoplasms.

The second study was a pilot randomized, placebo-controlled, 2×2 factorial clinical trial to investigate the separate and combined effects of calcium and vitamin D on potential biomarkers of risk for colorectal cancer in people with history of colorectal adenomas. In my dissertation project I investigated the effects of calcium and vitamin D on rectal expression of MLH1 and MSH2.

We found that after six months of treatment, calcium and vitamin D tended to increase MLH1 and MSH2 expression within the rectal crypts. The combined effect of calcium and vitamin D was substantially weaker than the separate effect of calcium or vitamin D; the greatest effect for both MLH1 and MSH2 was observed in the vitamin D group. In the calcium and vitamin D group MSH2 expression increased to a greater degree compared to MLH1 expression. While the increase in MLH1 expression was quite uniform along the entire length of the crypt, MSH2 expression in the upper 40% of the crypt (the differentiation zone) was much more noticeable than in the lower 60% (the differentiation zone). None of the treatment regimens, however, changed the proportion of either proteins' expression in the upper 40% of the crypt (φh).

The results of the trial suggest that calcium and vitamin D may increase the capacity of the DNA MMR system in the colonocyte, and that the strongest treatment effect may be vitamin D related and occur in the upper region of the colorectal crypt.

Although the results of these studies are interesting and may have important implications, they require further investigation. Both studies were pilot studies and, as such, had limited sample sizes, so most of the observed associations were not statistically significant and could have been due to chance. However, the observed associations suggest that MLH1 and MSH2 have high potential to become modifiable tissue biomarkers of risk

for colorectal cancer. Larger, more powerful observational studies and clinical trials are needed to test our findings further and establish whether MLH1 and MSH2 can be used as tissue biomarkers of risk for colorectal cancer and whether one should use them alone or as a part of a broader panel of biomarkers.

Tissue biomarkers will increase the diagnostic value of colonoscopy by adding new information about the personal risk of developing colorectal cancer for people who still have no detectable lesions. This information may then be used for developing further screening strategies that are based on an individual's risk. The development of tissue biomarkers will also lead to the next logical step: identification of biomarkers of risk in surrogate fluids.

Finding biomarkers of risk for colorectal cancer that could be easily identified in surrogate fluids such as blood or urine will facilitate screening and identifying people with high risk for developing colorectal cancer, evaluating preventive interventions, and scientific research. This dissertation project adds to the body of knowledge leading to identification of the panels of the most probable biomarkers of risk for colorectal cancer to be used in subsequent research eventually leading to clinical application.

Chapter 8. Implications and Future Research Directions

Implications for Cancer Research and Public health

This dissertation project is the first step in the development of MLH1 and MSH2 as tissue biomarkers of risk for colorectal cancer. If developed, the biomarkers will have several applications in research, public health and clinical practice:

1. Increase the screening value of colonoscopy and sigmoidoscopy by providing additional information about a patient's risk of colorerectal adenoma or cancer even when there were no detectable polyps during the procedure.

2. Enable a physician to modify a future screening and diagnostic schedule based on an individual's risk profile. This will decrease the number of unnecessary procedures and divert resources to the people who need them most. Biomarkers of risk for colorectal cancer that can be easily detected in tissue or surrogate fluids will help medical professionals evaluate the effectiveness and monitor the progress of prescribed therapies.

3. Provide surrogate endpoints to use in cancer research and evaluation of public health interventions. Modifiable biomarkers of risk for colorectal cancer will help to identify dietary and lifestyle changes that decrease risk for colorectal cancer development and will provide a means for monitoring the effects of these changes on an individual's risk of the disease.

Future Research Directions

The results of this dissertation project suggest that lower expression of MLH1 and MSH2 in normal colorectal mucosa may be associated with increased risk for sporadic

colorectal adenoma; however these results are based on data from pilot studies and cannot be considered definitive and need validation by larger studies.

Our group has developed an innovative image analysis procedure to measure protein expression in human tissue; however, currently, the procedure only allows relative concentration measurements based on differences in biomarker labeling optical density. Modifications of image analysis software are currently being developed by our research team to improve the image analysis algorithms and minimize human interference and resulting human error.

Slide staining procedures also can be improved to allow for measuring protein concentration in colonocytes. Our current immunohistochemistry procedure is being modified by including a scale of pre-determined dilutions of measured protein (e.g., MLH1 or MSH2) in every slide. After the slide is immunohistochemically processed, this scale can be used to compare biomarker labeling optical density measured within colonocytes to that of the scale thus measuring the concentration of a protein. Currently, a new protocol for slide staining using quantum dots is being developed. The quantum dot staining method allows using the same slide to stain for different proteins, minimizing the cost of the procedure. Also, it will be possible to measure concentrations of quantum dots bound to a certain antigen within a cell and use this information to measure concentrations of that antigen.

In a significant fraction of MSI-positive sporadic colon tumors that have MMR deficiency, mutations have not been identified in MMR genes. Epigenetic silencing of hMLH1 via promoter hypermethylation strongly down-regulates MMR in this case[15]. There is evidence suggesting that lifestyle and dietary factors may play a role in the development of

epigenetic alterations in normal and neoplastic tissue[261-263]. Investigation of the association between MLH1 and MSH2 expression in normal colorectal mucosa and DNA methylation would provide additional information that may help establish MLH1 and MSH2 proteins expression as biomarkers of risk of colorectal cancer.

Biochemical research suggests that Bcl2, an anti-apoptotic protein, potently suppresses MMR by decreasing MutSα (MHS2-MSH6) heterodimer activity[25]. So, measuring the relative abundance of Bcl2 and MSH2 in the colorectal mucosa may be of interest as another potential biomarker of risk for colorectal cancer. A ratio variable $\frac{Bcl2}{MSH2}$ may be created to measure the relative concentration of these proteins in the colorectal mucosa. An increase of this ratio would mean decreased MMR capacity and vice versa. Pilot data from the MAPII and CADvMAP studies can be used to initially test this hypothesis, and then Bcl2 and MSH2 data from larger studies using improved expression measurement techniques described above may be used to further develop the ratio measurement as a potential biomarker of risk of colorectal cancer.

It is unlikely that one protein or even several proteins that take part in one pathway of carcinogenesis will provide enough information to be an effective biomarker of risk for colorectal cancer. More likely, a panel of biomarkers will be developed that will describe the status of key systems within colonocytes whose malfunction can lead to the development of colorectal cancer. The proteins that were found to be promising biomarkers of risk in pilot studies should be tested in a full scale clinical trial to prove their effectiveness and to justify their inclusion in the functional panel of biomarkers of risk for colorectal cancer. The results of the trial would be necessary for implementing guidelines for using the panel of biomarkers in research, public health interventions, and clinical practice.

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Appendices

Appendix A. Supplementary Tables for MAPII Case-Control Study

Table A.1. Selected characteristics of controls whose biopsies were processed for MMR markers and the entire study population; the Markers of Adenomatous Polyps II Study

Characteristic*	N (Selected/ Not Selected)	Selected for IHC	Not Selected for IHC	p†
Demographics				
Age (yrs.)	47/154	55.1 (8.4)	55.3 (7.9)	0.89
Male (%)	47/154	45	49	0.74
White race (%)	46/148	98	95	1.00
Family History				
1° Relative with colorectal cancer (%)	47/154	17	19	1.00
Lifestyle				
Physical activity (METs/day)	46/149	28.8 (22.0)	29.0 (22.0)	0.95
Body Mass Index (kg/m^2)	46/148	31.1 (7.6)	29.8 (7.1)	0.32
Take aspirin at least once per week (%)	46/148	37	40	0.86
Take NSAID‡ at least once per week (%)	46/148	48	36	0.17
Smoking status (%)				
Never		50	47	0.52
Former	46/149	43	40	
Current		7	13	
Alcohol consumption (%)				
Never		13	19	0.46
Former	46/149	30	22	
Current		57	59	
Dietary intakes				
Total energy (kcal/day)	45/148	1632.3 (693.9)	1631.2 (624.0)	0.99
Total fat§ (g/day)	46/149	65.1 (15.4)	65.1 (15.4)	0.99
Carbohydrate intake§ (g/day)	46/149	198.6 (29.1)	199.1 (34.5)	0.94
Dietary fiber§ (g/day)	46/149	15.4 (5.8)	15.2 (4.9)	0.77
Total‖ calcium§ (mg/day)	46/149	968.4 (491.3)	901.1 (464.4)	0.40
Total‖ vitamin D§ (IU/day)	46/149	288.5 (361.4)	256.7 (319.2)	0.57
Total‖ folate§ (mcg/day)	46/150	366.0 (285.0)	347.0 (292.3)	0.70

* 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, Fisher's exact test for dichotomous variables, and modified Fisher's exact test for multilevel categorical variables
‡ NSAID – Non-steroidal anti-inflammatory drug (not including aspirin)
§ Energy adjusted using residual method
‖ Total = diet + supplements

Table A.2. Differences in full crypt MLH1 protein expression in normal-appearing mucosa between incident sporadic colorectal adenoma cases and controls, by colon site; the Markers of Adenomatous Polyps II Study

Colon Site	N (Cases/ Controls)	MSH2 Labeling Optical Density Mean (SE)				Proportional Difference (%)*	p†
		Cases		Controls			
Model 1: controls for staining batch only							
Rectum	37/41	346.96	36.93	410.5	34.89	-15%	0.21
Sigmoid	14/16	364.68	57.52	313.97	50.90	16%	0.52
Ascending	14/16	434.86	87.9	712.89	81.00	-39%	0.03
Combined OR‡ (95% C.I.§)	43/47	0.78 (0.39 - 1.53)					
Model 2: controls for age, sex, and staining batch							
Rectum	37/40	346.53	37.47	415.88	36.33	-17%	0.19
Sigmoid	14/16	379.19	58.13	302.45	51.48	25%	0.34
Ascending	14/16	432.09	93.6	714.45	85.15	-40%	0.03
Combined OR (95% C.I.)	43/46	0.76 (0.38 - 1.50)					
Model 3: Aspirin/NSAID‖ use, FHCRC#, physical activity, calcium, and staining batch							
Rectum	37/39	329.88	45.52	429.77	46.95	-23%	0.05
Sigmoid	14/15	398.20	94.26	340.44	67.31	17%	0.55
Ascending	14/15	415.56	121.32	741.46	96.28	-44%	0.02
Combined OR (95% C.I.)	43/45	0.75 (0.37 - 1.53)					
Model 4: Age, sex, aspirin/NSAID use, FHCRC, physical activity, calcium, and staining batch							
Rectum	37/39	330.75	46.00	428.8	47.50	-23%	0.06
Sigmoid	14/15	406.35	96.48	325.6	67.80	25%	0.42
Ascending	14/15	377.80	128.40	738.02	98.01	-49%	0.01
Combined OR (95% C.I.)	43/45	0.77 (0.38 - 1.58)					
Model 5: Aspirin/NSAID use, FHCRC, physical activity, calcium, age, alcohol consumption, and staining batch							
Rectum	37/39	353.55	54.09	453.10	54.57	-22%	0.06
Sigmoid	14/15	425.90	113.91	382.51	86.96	11%	0.69
Ascending	14/15	424.73	156.79	709.29	129.94	-40%	0.10
Combined OR (95% C.I.)	43/45	0.75 (0.37 - 1.50)					
Model 6: controls for age, sex, staining batch, FHCRC, physical activity, aspirin/NSAID use, alcohol, and total energy intake							
Rectum	37/38	337.28	57.71	440.31	56.87	-23%	0.08
Sigmoid	14/15	431.51	106.62	351.99	87.21	23%	0.48
Ascending	14/15	346.37	154.84	700.19	119.2	-51%	0.04
Combined OR (95% C.I.)	43/44	0.75 (0.35 - 1.63)					

* [(cases – controls)/controls]×100%

† Based on t-test for comparing the two means

‡ Combined OR – odds ratio (Cases vs. Controls) controlling for all three colon sites and the covariates indicated in the model specification. The optical density (MLH1 expression) variable was dichotomized using the mean of the colon site specific distributions in the controls.

§ C.I. – confidence interval

‖ NSAID - Non-steroidal anti-inflammatory drugs

\# FHCRC – family history of colorectal cancer in a first-degree relative

Table A.3. Associations of full crypt MSH2 expression in normal-appearing colorectal mucosa with potential risk factors of colorectal cancer in incident, sporadic colorectal adenoma cases, by colon site; the Markers of Adenomatous Polyps II Study

Characteristic*	Rectum			Sigmoid			Ascending		
	N	MSH2 Expression† (SE‡)	p§	N	MSH2 Expression (SE)	p	N	MSH2 Expression (SE)	p
Age (yrs.)									
35 – 54	19	357.62 (52.63)	0.78	6	336.17 (99.53)	0.72	6	500.84 (150.21)	0.59
≥ 55	18	336.11 (53.57)		8	386.37 (82.79)		8	392.52 (119.97)	
% Difference		*-6%*			*15%*			*-22%*	
Sex									
Male	19	333.97 (54.42)	0.77	7	255.36 (71.78)	0.03	7	522.50 (131.17)	0.35
Female	18	357.20 (55.54)		7	488.92 (76.28)		7	347.23 (118.90)	
% Difference		*7%*			*91%*			*-34%*	
Family history of colorectal cancer‖									
No	32	333.65 (41.91)	0.51	13	368.13 (59.81)	0.56	13	426.36 (95.03)	0.62
Yes	5	412.67 (106.96)		1	229.15 (227.75)		1	610.91 (352.20)	
% Difference		*24%*			*-38%*			*43%*	
Physical activity (METs/day)									
Low	18	288.41 (57.14)	0.18	9	324.82 (75.07)	0.48	9	540.27 (153.36)	0.12
High	19	398.24 (51.52)		5	412.80 (95.85)		5	200.64 (109.24)	
% Difference		*38%*			*27%*			*-63%*	
BMI# (kg/m^2)									
< 30	19	379.86 (53.79)	0.39	6	463.12 (108.52)	0.30	6	474.10 (150.65)	0.74
≥ 30	18	312.70 (53.95)		8	303.17 (82.53)		8	401.37 (134.35)	
% Difference		*-18%*			*-35%*			*-15%*	
Smoking**									
Never	16	391.75 (56.05)	0.30	8	451.82 (76.58)	0.08	8	353.11 (129.65)	0.43
Ever	21	313.24 (48.66)		6	224.26 (87.42)		6	526.01 (150.63)	
% Difference		*-20%*			*-50%*			*49%*	
Alcohol consumption††									
Former/ Never	13	330.65 (65.67)	0.77	7	368.35 (83.72)	0.93	7	272.29 (153.98)	0.22
Current	24	354.97 (46.86)		7	357.93 (85.56)		7	553.48 (127.11)	
% Difference		*7%*			*-3%*			*103%*	
Aspirin intake‡‡									
No	21	355.73 (48.48)	0.84	12	379.14 (71.21)	0.52	12	359.60 (89.23)	0.06
Yes	16	340.16 (59.69)		2	255.81 (165.34)		2	891.34 (244.24)	
% Difference		*-4%*			*-33%*			*148%*	
NSAID§§ intake‡‡									
No	24	323.05 (45.78)	0.39	8	388.32 (83.87)	0.65	8	449.20 (104.07)	0.65
Yes	13	389.63 (61.26)		6	327.62 (95.78)		6	369.88 (133.65)	
% Difference		*21%*			*-16%*			*-18%*	
Total energy intake (kcal/day)‖‖									
Low##	7	230.58 (88.83)	0.15	6	425.15 (93.48)	0.36	6	444.60 (141.77)	0.88
High##	30	376.86 (41.43)		8	309.62 (79.56)		8	414.89 (126.52)	
% Difference		*63%*			*-27%*			*-7%*	

Table A.3. Continued

Characteristic*	Rectum			Sigmoid			Ascending		
	N	MSH2 Expression† (SE‡)	p§	N	MSH2 Expression (SE)	p	N	MSH2 Expression (SE)	p
Total* fat intake (mg/day)**									
Low	18	332.72 (53.21)	0.74	2	140.40 (158.73)	0.15	2	512.60 (264.77)	0.74
High	19	357.52 (50.61)		12	406.38 (63.58)		12	418.38 (94.47)	
% Difference		*7%*			*189%*			*-18%*	
Total* calcium intake (mg/day)**									
Low	19	375.45 (52.36)	0.42	8	239.03 (69.80)	0.03	8	444.74 (123.43)	0.93
High	18	311.21 (55.41)		6	500.69 (82.60)		6	428.89 (136.83)	
% Difference		*-17%*			*109%*			*-4%*	
Total* vitamin D intake (IU/day)**									
Low	20	388.07 (55.30)	0.23	7	338.95 (95.34)	0.73	7	375.53 (130.65)	0.56
High	17	296.73 (49.87)		7	385.20 (87.03)		7	497.85 (141.31)	
% Difference		*-24%*			*14%*			*33%*	
Total* folate intake (mcg/day)**									
Low	15	396.98 (56.90)	0.26	5	422.91 (108.79)	0.52	5	350.75 (151.97)	0.48
High	22	310.25 (48.84)		9	330.26 (79.69)		9	501.44 (128.79)	
% Difference		*-22%*			*-22%*			*43%*	

* All variables except age, sex, family history of colorectal cancer, and total energy intake adjusted for age and sex; also smoking status variable adjusted for alcohol consumption and alcohol consumption variable adjusted for smoking status.
† Mean optical density adjusted for staining batch
‡ SE – standard error
§ Based on the F-test for significance of fixed effects in a linear mixed model
‖ Family history of colorectal cancer in a first-degree relative
BMI – body mass index (kg/m2)
** Categories "Current smoker" and "Former smoker" were combined into the "Ever smoker" category due to extremely small sample size of the "Current smoker" category
†† Categories "Never consumed" and "Former consumer" were combined due to extremely small sample size of the "Never consumed" category
Yes defined as regularly taking this medication at least once a week
§§ NSAID – nonsteroidal anti-inflammatory drugs (not including aspirin)
‖‖ Throughout the table: "Low" - below the 50th percentile of the sex-specific distribution in controls; "High" - at or above the 50th percentile of the sex-specific distribution in controls
Adjusted for physical activity
*** From diet and supplements

Appendix B. Supplementary Table for CADvMAP Clinical Trial

Table B.1. MLH1 and MSH2 Expression in Colorectal Crypts at Baseline and 6-months Follow-Up Shown as Staining Batch Standardized* Optical Density of Staining of the Immunohistochemically-detected Biomarkers

	Baseline				6-Months Follow-up				Absolute Treatment Effect§				Relative
	N	Mean*	SE†	p‡	N	Mean	SE	p	N	Mean	SE	p	Effect‖
A. Ratio of upper 40% to the entire crypt													
MSH2													
Placebo	20	0.10	0.01		20	0.06	0.01		17				1.00
Calcium	23	0.08	0.01	0.41	21	0.07	0.01	0.43	21	0.03	0.03	0.25	1.57
Vitamin D	22	0.09	0.01	0.58	20	0.08	0.01	0.33	19	0.03	0.03	0.28	1.56
Calcium + vitamin D	22	0.07	0.01	0.19	21	0.07	0.01	0.57	20	0.04	0.03	0.19	1.68
MLH1													
Placebo	17	0.31	0.01		18	0.29	0.01		13				1.00
Calcium	18	0.31	0.01	0.90	19	0.29	0.01	0.87	17	0.00	0.02	0.98	1.00
Vitamin D	21	0.32	0.01	0.55	18	0.31	0.01	0.12	18	0.02	0.02	0.43	1.05
Calcium + vitamin D	22	0.32	0.01	0.28	17	0.32	0.01	0.07	16	0.01	0.02	0.51	1.05

Table B.1. Continued.

	Baseline				6-Months Follow-up				Absolute Treatment Effect[§]				Relative
	N	Mean[*]	SE[†]	p[‡]	N	Mean	SE	p	N	Mean	SE	p	Effect[‖]
B. Ratio of upper 20% to lower 20% of crypts													
MSH2													
Placebo	20	0.09	0.02		20	0.03	0.02		17				1.00
Calcium	23	0.07	0.02	0.54	21	0.05	0.02	0.68	21	0.03	0.05	0.47	1.79
Vitamin D	22	0.05	0.02	0.17	20	0.06	0.02	0.39	19	0.07	0.05	0.12	3.63
Calcium + vitamin D	22	0.04	0.02	0.13	21	0.07	0.02	0.26	20	0.09	0.05	0.06	4.55
MLH1													
Placebo	17	0.47	0.04		18	0.42	0.04		13				1.00
Calcium	18	0.51	0.04	0.56	19	0.41	0.04	0.84	17	-0.04	0.07	0.55	0.91
Vitamin D	21	0.53	0.04	0.35	18	0.50	0.04	0.17	18	0.03	0.07	0.71	1.07
Calcium + vitamin D	22	0.54	0.04	0.21	17	0.49	0.04	0.23	16	0.00	0.07	1.00	1.02

[*] Standardization for staining batch done by dividing each individual's labeling optical density measurement by the mean measurement of their staining batch. Batch-specific means were calculated among all subjects for the baseline visit and among the placebo group for the follow-up visit.

[†] SE – standard error

[‡] Evaluates the difference between each treatment group and the placebo group.

[§] Absolute Treatment Effect = (treatment group follow-up – treatment group baseline) – (placebo group follow-up – placebo group baseline).

[‖] Relative effect = [(treatment group follow-up/treatment group baseline) / (placebo follow-up/placebo baseline)]; interpretation as for odds ratio (e.g., a relative effect of 1.6 indicates a proportional increase of 60% in the treatment group relative to that in the placebo group)