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Effects of Calcium and Vitamin D3 on Transforming Growth Factor alpha and Transforming Growth Factor Beta1 in Rectal Mucosa of Sporadic Colorectal Adenoma Patients in a Double-blinded Randomized Controlled Trial

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An abstract of
A thesis submitted to the Faculty of the
Rollins School of Public Health of Emory University
in partial fulfillment of the requirements for the degree of
Master of Public Health
in department of epidemiology
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By Hongjie Chen

Colorectal cancer is the fourth most common incident cancer and the second most common cause of cancer deaths in the United States. Ecologic studies indicated that colorectal cancer is heavily environmental influenced and thus preventable. An inverse relationship between calcium and colorectal cancer incidence has been widely accepted. The autocrine or paracrine growth factors TGFα and TGFβ_1 likely contribute to or at least affect colon carcinogenesis.

In this study, we constructed a randomized, placebo-controlled, double-blinded clinical trial to investigate the effects of supplemental 1,200 mg elemental calcium and/or 1000 IU vitamin D daily over one year on the autocrine/paracrine growth factors TGFα and TGFβ_1 in the normal appearing rectal mucosa of sporadic colorectal adenoma patients. We hypothesize that the supplemental calcium and vitamin D may individually or jointly decrease the expression of TGFα and the ratio of TGFα to TGFβ_1 in the human colorectal mucosa.

Slight differences were observed in several groups of discussion in both biomarkers' expression, while none of the treatment effect was statistically significant. The study result thus suggests that daily supplements of 1,200 mg elemental calcium and/or 1000 IU vitamin D may not have effect on the growth factors TGFα and TGFβ_1 in the normal appearing rectal mucosa of sporadic colorectal adenoma patients. Further investigations in larger clinical trial are needed to testify this conclusion.

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In this study, we constructed a randomized, placebo-controlled, double-blinded clinical trial to investigate the effects of supplemental 1,200 mg elemental calcium and/or 1000 IU vitamin D daily over one year on the expression of autocrine/paracrine growth factors TGFα and TGFβ_1 in the normal appearing rectal mucosa of sporadic colorectal adenoma patients. We hypothesize that the supplemental calcium and vitamin D may individually or jointly increase the expression of TGFβ_1, decrease the expression of TGFα and the ratio of TGFα to TGFβ_1 in the human colorectal mucosa.

Our results suggest that calcium 1,200 mg and/or vitamin D 1,000 IU supplementation over 12 months may not substantially affect TGF-α or TGF-β_1 expression, individually or in balance with one another, in the crypts of the normal-appearing colorectal mucosa of sporadic colorectal adenoma patients.. Further investigations in larger population are needed to testify this association.

Background

Colorectal Cancer

Colorectal cancer is the fourth most common incident cancer and the third leading common cause of cancer deaths in the United States, with more than 144,000 new cases and 56,000 deaths annually.[1] Colon cancer is essentially the only cancer that occurs with almost equal frequency in men and women. Its incidence does not differ substantially by social economic status within any region. Five-year relative survival following a diagnosis of colon cancer is approximately only 50 percent in the United States.[2] Similar survival rates are reported around the developed world.[3] Colon cancer incidence varies approximately 20-fold around the world.[4] Western countries have a significantly higher incidence rate; while the rate for developing countries is far lower.[5] Migrants from low incidence to high incidence countries develop the incidence rates of their adopted countries within one to two generation.[6] Taken together, the ecologic studies indicate that colorectal is heavily environmentally influenced and thus preventable.

Molecular basis of Colorectal Cancer

The molecular basis of colon carcinogenesis involves in variety types of genetic alterations (in *APC, K-ras, p53*). There are two main pathways in colon cancer development.[7] The first pathway, which is called 'APC pathway', accounts for Familial Adenomatosis Polyposis (FAP) and 80% of sporadic cancers. In the APC pathway, both alleles of the *APC* tumor suppressor gene are inactivated through somatic mutation or epigenetic phenomena. The second pathway, called the 'Mismatch Repair Pathway', accounts for Hereditary Non-polyposis Colon Cancer (HNPCC) and other 20% of sporadic colon cancer. Patients in this pathway have inactivation of one of the

mismatch repair genes, predominantly *MSH2* and *MLH1*. HNPCC patients are born with a mutation in one allele, while sporadic patients acquire the inactivation through epigenetic silencing or mutation.[8]

Inflammation and inflammation regulation is likely to play important roles in colon carcinogenesis and the activity of genes for elements of the system. There is the correlation between the activity of the inflammatory factor *COX-2* and *APC* gene. In animal experiments, NSAIDs reduced colon tumorigenesis. The autocrine or paracrine growth factors TGFα and $TGF\beta_1$ also likely to contribute to or at least affect colon carcinogenesis.[9] Those findings are essential to understand in the development of colorectal cancer, and may be useful for developing treatable biomarkers of risk.

Environmental factors for Colorectal Cancer Development

It has been widely accepted that the incidence of colorectal cancer is related to diet. Studies suggested that vegetable and fruit consumption was inversely associated with colon cancer incidence. Vegetables and fruits contain a myriad of potentially anti-carcinogenic compounds, including fiber, antioxidants and antioxidant enzyme-associated micronutrients, and folic acid. Diets high in animal fats were also believed to be associated with high serum cholesterol, which was related to higher risk for development of colorectal adenomas and carcinomas.[10]

Another consistent association observed for colorectal cancer is an inverse association with physical activity. Both prospective and case-control studies stated that higher level of physical activity to be associated with reduced risk of colorectal adenoma.[11] This inverse association is likely causal since the study results were widely consistent among studies in diverse populations, for variety of study designs, and considering both leisure and occupational activity type.

Alcohol's effect on colon cancer incidence was once controversial, while a pooled meta-analysis found that compared with low alcohol intake group, those who drunk more than 30g alcohol per day had significant higher risk of colorectal cancer.[12] The mechanism for this association is still unclear, but the antagonist effect between alcohol and folate has been received interest for years.

Association regarding a possible role of tobacco in colorectal carcinogenesis has evolved in past decades. In general, those who currently smoke or have a history of smoking 20-40 cigarettes pack per year had about twofold to threefold higher risk of colorectal adenoma.[13] This positive association is not surprising because during the cigarette burning, varieties of genotoxic compounds are generated. Another issue that need to be resolved is how quickly risk was drop after quitting smoking, or in other words, how long would risk persist among past cigarette smokers.

A family history of colorectal cancer in one or more first-degree relatives is associated with increased risk of colorectal cancer. Some investigators estimated those who had a family history of colon cancer were at approximately twofold risk of the disease.[14] Despite the difficulty in separating genetic effect from environmental factors, more studies was needed to fully understand this positive association.

Calcium and Colorectal Cancer

The results of animal studies have suggested that calcium may be involved in the etiology of colorectal cancer. This association between calcium and colorectal cancer is also largely reflected by anthropological facts.[15] Among homo-sapiens in Paleolithic times, the estimated calcium intake was far higher than modern times: 1,500-2,000 mg daily compared to current American

average intake of 740 mg.

An inverse association between calcium and colorectal cancer incidence has been widely accepted. There are two different hypotheses about how calcium may reduce risk for colorectal cancer. The first is the bile acid hypothesis,[16] which posits that calcium can bind bile acids and form insoluble soaps of these potentially toxic compounds in the lumen of the colon. The second theory is that calcium can directly lower cellular proliferation in the colonic mucosa;[17] in support of this that calcium can slow cell proliferation and speed up cell differentiation in cell culture.

Results from epidemiologic studies about the association between calcium and colorectal cancer are consistent. Although some of the studies do not support the preventive effect of calcium to colorectal cancer, most have found at least some weak association between high calcium intake and the risk of colorectal cancer. Among 13 analytical epidemiological studies earlier than 1990[18] (nine case-control studies and four cohort studies), three case-control studies and two cohort studies reported statistical significant adverse relationship between calcium and colorectal cancer incidence, while only two study indicated there was no direction of the association.

Some new prospective cohort studies with large study population and clinical trials need to be focused as well. Virtamo et al., conducted a prospective study in cohort with 27,111 Finnish male participants.[19] The study found the relative risk for men in the highest quartile of calcium intake compared with men in the lowest quartile was 0.6 (95% CI 0.4-0.9, P_{trend}=0.04). With respect of cohort's characteristic, the study indicated that among the men with traditional western life-style, high calcium intake was associated with lowered risk of colorectal cancer. Schatzkin et al., conducted a 8-year follow-up prospective cohort study among 45,354 women in

US, which separated the study population based on daily supplementation of calcium at the cut-points of 0, 400 and 800 mg/day.[20] This study found a statistical significant trend that with the increasing category of daily calcium supplement, the risk of colorectal cancer would decrease. Baron et al., conducted a placebo-controlled double-blinded randomized clinical trial to study the association between daily supplementary of 1200 mg of elemental calcium (with 3.0 g calcium carbonate) and recurrence of colorectal adenoma, among 930 subjects.[21] The adjusted risk ratio for any recurrence of adenoma with calcium as compared with placebo was 0.85 (95 percent confidence interval, 0.74 to 0.98; P=0.03), which indicated that calcium supplementation was association with a significant though moderate reduction in the risk of the recurrent colorectal adenomas.

Vitamin D and Colorectal Cancer

As a natural partner of calcium, investigating an association of vitamin D with colon cancer incidence has been increasing recently. Vitamin D can inhibit cell proliferation and increase apoptosis *in vitro*, and several tissues can locally produce the physiologically active form of vitamin D, which has anti-carcinogenic properties. In addition, many cell types, including colorectal epithelial cells, contain vitamin D receptors. These cells can convert circulating 25(OH)D into active 1-25(OH)D metabolites, which in turn bind to the cells' own vitamin D receptors to produce an autocrine effect of inducing cell differentiation and inhibiting proliferation, invasiveness, angiogenesis, and metastatic potential.[22] Therefore, low vitamin D levels may increase the risk of colorectal cancer through the above potential mechanisms

Direct and indirect associations between vitamin D and colorectal cancer have been founding variety of epidemiological studies. An ecologic study found higher age-adjusted

mortality rates of colorectal cancer in the northern and northeastern United States compared to the southwest, Hawaii, and Florida.[23] Since blood vitamin D level is largely affected by the exposure to sunlight, the differentiation of colorectal cancer mortality rates in different geographic regions indirectly supports an inverse association between vitamin D and colorectal cancer morality.

Several epidemiological studies that investigated the direct association between vitamin D and colon cancer were conducted as well. Of nine analytical epidemiological studies published from 1993 to 2010 (eight cohort studies and one nested case-control study), six suggested an inverse association and other two indicated a null association, although none of the findings was statistically significant association.[18] A meta-analysis that pooled those nine studies together found that a relative risk of colorectal cancer among those in highest vitamin D intake groups to be 0.88 fold (95%CI 0.80-0.96) when compared with the lowest intake group.[24]

Another important measurement of vitamin D is blood 25-(OH)-vitamin D concentration. The meta-analysis[24] mentioned above also analyzed nine published epidemiological studies (seven cohort studies and two nested case-control studies) that investigated the association between blood 25-(OH)-Vitamin D levels and colorectal cancer incidence. Among those nine studies, eight found an inverse association, although none was statistically significant. Those data from those nine studies were further pooled during a meta-analysis, finding that those in the highest blood vitamin D level group were at a statistically significant lower (Risk Ratio=0.68, 95% CI: 0.54-0.80) risk, relative to lowest level.

Biomarkers and Colorectal Cancer Prevention

Despite the development in cancer screening and treatment, the mortality rate from colon

cancer has declined only modestly in past few years. This situation recalls an analogous one with ischemic heart disease two decades ago. With several biological measurements as markers of risk for the disease, the population and individual use of specific 'biomarkers' led to a sharp decrease in mortality rate which continues today. If some biomarkers that directly related to the risk for colorectal cancer can be found, a decline of mortality rate for the disease must be accelerated.

As autocrine/paracrine growth factors, TGFα[25] and TGFβ_1[26] are classically thought of as potent promoters and inhibitors of cell growth in normal tissues, and likely contribute to or at least affect colorectal carcinogenesis. Within colon crypts, TGFα expression is correlated with the distribution of proliferating cells and mediated by its interaction with the epidermal growth factor receptor. Although limited *in vivo* evidence is available to state that TGFα or TGF$\beta_1$may be a potential marker of colorectal cancer risk, some epidemiological studies have observed a different expression of such genes between colorectal adenoma cases and control groups. Daniel et al., measured the expression of TGFα among 31 sporadic colorectal adenoma patients and 29 controls.[27] From this case-control study, it was tested that the average TGFα expression throughout the rectal crypts was 51% higher in the cases than in the control, after adjusted for age and sex (P=0.05). Another case-control study, the Markers of Adenomatous Polyps II (MAPII) study suggested that the expression of TGFα among 21 colorectal adenoma cases was 37% higher than the expression among 20 controls; and compared with 15 controls, the expression ratio of TGFα/ TGFβ_1 was 24% higher among 17 cases. Those results indicated that the expression of TGFα, TGFβ_1 and the ratio of TGFα/TGFβ_1 in normal-appearing rectal mucosa may be an early, potentially modifiable biomarker of risk for colorectal cancer.

To our knowledge, there is only one study in humans that assessed the separate and joint

effects of calcium and vitamin D supplementation on the expression of TGFα and TGFβ_1 in the human colorectal mucosa. A randomized controlled pilot trial was conducted on 92 colorectal adenoma patients, who were randomly assigned, stratified by sex and NSAID use, to four treatment groups receiving daily over six months: placebo; 2,000 mg elemental calcium (as carbonate in equal doses twice daily); 800 IU vitamin D_3 and 2,000 mg elemental calcium plus 800 IU vitamin D_3.[9] In the three active intervention groups, the mean overall expression of TGFβ_1 increased by 14%, 19% and 22%; the ratio of TGFα in the differentiation zone to that in the proliferation zone of the crypts decreased by 34%, 31% and 26%; and the TGFα/TGFβ_1 ratio in the differentiation zone decreased by 28%, 14% and 22%, respectively. Although those pilot results preliminary analysis was statistically significant, they were consistent with the hypothesis that calcium and vitamin D may increase the expression of TGFβ_1 and normalize the crypt TGFα expression, shifting it through proliferation zone. Further investigation on larger population is needed before one can conclude that supplementary calcium and vitamin D can favorably modify TGFα and/or TGFβ_1 expression and prevent the recurrence of adenomas in sporadic colorectal adenoma patients.

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Introduction

Colorectal cancer is the fourth most common incident cancer and the second most common cause of cancer deaths in the United States,[1] with more than 111,000 new cases and 51,000 deaths annually.[2] The incidence of colon cancer varies approximately 20-fold around the world.[3] Western countries have a significantly higher incidence rate, while the rates for developing countries are far lower. Migrants from low incidence to high incidence countries develop the incidence rates of their adopted countries within one to two generations.[4] Taken together, these ecologic studies indicate that colorectal cancer is heavily environmentally influenced and thus preventable.

An inverse association between calcium and colorectal cancer incidence[5] has been widely accepted. There are two different hypotheses about how calcium may reduce risk for colorectal cancer. The first is based the bile acid hypothesis,[6] which posits that calcium can bind bile acids and form insoluble soaps of these potentially toxic compounds in the lumen of the colon. The second theory is that calcium can directly lower cellular proliferation in the colonic mucosa; in support of this is that calcium can slow cell proliferation and speed up cell differentiation in cell culture.[7]

As a natural partner of calcium, investigating an association of vitamin D with colon cancer incidence has been increasing recently.[8] The role of vitamin D is linked with colon cancer because the active metabolite of vitamin D mediates intestinal calcium absorption. Despite its important effects in maintaining calcium homeostasis, vitamin D may have some direct genomic or non-genomic actions[9-10] that may be relevant to colon cancer. The biological plausibility of this theory is mainly based on the fact that the active metabolite of vitamin D in the colon exerts its

anti-neoplastic effects by genomic and non-genomic mechanisms, including regulation of vitamin D-responsive genes, activation of intracellular signaling pathways resulting in cell cycle modulation, degradation of bile acids, immune response, and growth factor signaling.[11]

The autocrine/paracrine growth factors TGFα and TGFβ_1 likely contribute to[12-13] or at least affect[14] colon carcinogenesis. To our knowledge, only one study[15] in humans has assessed the separate and joint effects of calcium and vitamin D supplementation on the expression of these two biomarkers in the human colorectal mucosa. We hypothesize that supplemental calcium and vitamin D_3 individually and combined can decrease the expression of TGFα, increase the expression of TGFβ_1, and decrease the ratio of TGFα to TGFβ_1 in the colorectal mucosa in sporadic colorectal adenoma patients.

Method

Participant Population

The participants in this study were all participating in a larger clinical trial in sporadic colorectal adenoma patients ("parent study"). The study was conducted in the state of Georgia and South Carolina, participated by several medical facilities. The criteria for eligibility in the parent study included age 30 to 75 years old, in general good health, capable of informed consent, a history of at least one pathology-confirmed sporadic colon or rectal adenoma with in past 36 months and no contradiction to calcium or vitamin D supplementation. An additional criterion for inclusion in adjunct biomarker study was no contraindications to biopsy procedures.

Clinical Trial Protocol

Before the conducting of treatment assignment, all potential participants attended an eligibility visit during interview process. Each participant completed a questionnaire about their

socio-demographics, medical history, lifestyle and habits. Baseline blood samples were collected. Diet was assessed with a food frequency questionnaire. Medical and pathology records were collected. All participants still eligible entered a 30-day placebo run-in trial. A total of 104 participants took more than 80% of assigned tablets and reported no side-effects during the placebo run-in, and thus were eligible for randomization.

Participants were randomly assigned into four treatment groups, receiving daily intervention over 1 year: placebo; 1,200 mg elemental calcium (1,000 mg twice a day as calcium carbonate); 1,000 IU vitamin D_3; and 1,200 mg elemental calcium plus 1,000 IU vitamin D_3. Some female participants were not able to stop calcium supplementation due to medical therapies. For those patients, they were randomly assigned with 1,200 mg elemental calcium or placebo. The randomization was stratified on sex and expected follow-up colonoscopy interval, and was conducted within study center. Corresponding supplement and placebo pills were identical in size, appearance and taste. Participants were instructed to maintain their usual diet and not take any nutritional supplementation that they were not taking at the time of study started.

Participants in the parent study were invited to also participate in the adjunct biomarker study in which they would undergo out-patient, non-prep rectal biopsies at baseline and 1-year follow up. A total of 10 potential pre-neoplastic molecular phenotypic biomarkers of risk for colorectal neoplasms, including TGFα and TGFβ_1, were measured in the biopsies of 104 adjunct biomarkers study participants using automated immunohistochemistry with image analysis.

Immunohistochemistry and Scoring protocol

From each block, five slides, with 3 levels of 3.0-mm thick biopsy sections (taken 40 mm apart) on each slide, were prepared for each antigen, yielding a total of 20 levels for each antigen.

Heat-mediated antigen retrieval was performed by steaming the slides in a preheated Pretreatment Module (Lab Vision Corp., Fremont, CA) with citrate Buffer (pH 6.0; DAKO S1699; DAKO Corp.) for 40 min. Then, the slides were immunohistochemically processed in a DAKO Automated Immunostainer (DAKO Corp., Carpinteria, CA) using a labeled streptavidin–biotin method (TGFα antibody manufactured by Calbiochem (KGaA, Darmstadt, Germany), catalog No. GF10, dilution 1:100; $TGF\beta_1$ antibody manufactured by Santa Cruz (Dallas, TX), catalog No. sc-146, dilution 1:75), but not counterstained. The processed slides were coverslipped with a Leica CV5000 Coverslipper (Leica Microsystems, Inc., Buffalo Grove, IL). Each staining batch contained approximately equal numbers of participants from each treatment group. Positive and negative control slides were included in each staining batch.

A quantitative image analysis method to quantify the labeling optical densities ("expression") of the immunohistochemically-detected biomarkers in normal rectal crypts was described in detail previously[16]. Briefly, the image analysis unit was a "hemicrypt", defined as one side of a rectal crypt bisected from base to rectal lumen surface. A "scorable" hemi-crypt was defined as an intact hemi-crypt extending from the muscular mucosa to the colon lumen. Before image analysis, staining adequacy was checked by examining the batch's negative and positive control slides. Before scoring, the scorer, who was blinded to the intervention assignment, selected the two of the three biopsies with the greatest number of scorable hemi-crypts, captured background correction images for each slide, and captured 16-bit grayscale images of crypts at 200 magnification. Then, the scorer traced the borders of the "hemi-crypt" in the image analysis program. The program then segmented the traced hemi-crypt, and the background-adjusted optical density of the labeling across the whole hemi-crypt and within each segment was

measured and exported to a SQL database. The goal was to score 16–20 hemi-crypts per biopsy visit for each biomarker. Reliability was assessed by selecting samples of previously analyzed slides (10%) to be re-analyzed by the same scorer. The scorer was blinded to the selection. Intra-reader reliability for TGFα and TGFβ_1 was above 0.90 throughout.

Statistical Analysis

All data analysis steps were conducted using Statistical Analysis System (SAS, version 9.4; SAS Institute Inc.). The baseline characteristics of the participants across treatment groups were compared using the Fisher exact test for categorical variables and analysis of variance or co-variance for continuous variables. Treatment effects were evaluated by analyzing the differences in the baseline to follow-up biomarker differences in the active treatment groups relative to that in the placebo groups (calcium vs. calcium placebo; vitamin D vs. vitamin D placebo; calcium plus vitamin D vs. calcium only) using a repeated-measures linear mixed-effects model, implemented using the *'Proc MIXED'* procedure in SAS. The model included the intercept, indicators for treatment group and visit, visit by treatment interaction term, and potential confounding factors sex, BMI, physical activity score, NSAID usage, race and dietary factors. A cutoff level of P-value ≤0.05 was used for evaluating statistical significance. Stratified analyses were conducted to investigate potential differential treatment effects by sex, age, BMI and NSAID usage. A final relative treatment effect was calculated to provide an estimate of the average proportional change in each treatment group relative to that in the placebo group and its interpretation is analogous to that of an odds ratio[17].

Results

Characteristics of Study Participants

The mean age of the study participants was 59 years, and approximately 80% were male. Six treatment groups were balanced on demographic characteristics that included age, sex, race, and education level (Table 1). There were no substantial differences across treatment groups in the proportions of participants who regularly took a NSAID or currently smoked, or in mean BMI. Dietary intakes were fairly equal across the treatment groups, although the placebo group in the full-factorial trial had a comparatively low intake of dietary fiber. Baseline serum 25-OH-vitamin D concentrations did not differ among the six treatment groups.

Effects on TGF-α

At baseline, TGF-α expression in the whole crypt, the crypt differentiation zone (upper 40%), and the proliferation zone (lower 60%) in the rectal mucosa did not differ substantially among treatment groups (Table 2). In those who received vitamin D relative to those who did not, TGF-α expression decreased by 1% in the whole crypt and in the differentiation zone, but did not change in the proliferation zone. Relative to those who did not receive calcium supplementation, the TGF-α expression in the calcium supplementation group decreased 2% in the whole crypt, the differentiation zone, and the proliferation zone. In the calcium plus vitamin D group relative to calcium only, TGF-α expression increased by 3% in the whole crypt and 8% in the proliferation zone, but did not substantially change in the differentiation zone. The ratio of the expression in the differentiation zone to that in the whole crypt (Øh) decreased by 1% and 4% in the vitamin D and the calcium plus vitamin D treatment groups, respectively, relative to their corresponding

control groups. No difference in the Øh was observed between the calcium and calcium placebo groups.

Effects on TGF-β_1

At baseline, mean TGF-β_1 expression in the whole crypt, in the crypt differentiation zone, and in the proliferation zone in the rectal mucosa did not differ substantially among treatment groups (Table 3). In those who received vitamin D relative to those who did not, TGF-β_1 expression decreased by 2% in the whole crypt and in the differentiation zone, and decreased by 3% in the proliferation zone. In those who were supplemented with calcium, TGF-β_1 expression decreased by 3% in the differentiation zone, 5% in the whole crypt, and 7% in the proliferation zone, relative to those who took the calcium placebo. In the calcium plus vitamin D group relative to those who were supplemented with calcium only, TGF-β_1 expression in the whole crypt, the differentiation zone, and the proliferation zone decreased by 7%, 8%, and 6%, respectively. The ratio of the expression in the differentiation zone to that in the whole crypt (Øh) increased by 4%, 11%, and 18% in the calcium, vitamin D, and calcium plus vitamin D treatment groups, respectively, relative to their corresponding control groups.

Effects on TGF-α/TGF-β_1 Ratios

The estimated treatment effects on the balance of TGF-α expression to TGF-β_1 expression (TGF-α/TGF-β_1 ratio) in the whole crypt are summarized in Table 4. In those who received vitamin D relative to those who did not, there was a 50% decrease in the TGF-α/TGF-β1 ratio. Among those who were supplemented with calcium, there was a 76% increase in the ratio relative to

those who took the calcium placebo. Relative to those who took calcium supplementation only, the ratio in those who received the calcium plus vitamin D intervention decreased by 50%.

Stratified Analysis

As shown in table 3, there were no meaningful differences in findings according to categories of age, sex, BMI, or NSAID use.

Discussion

Our results suggest that calcium 1,200 mg and/or vitamin D 1,000 IU supplementation over 12 months may not substantially affect TGF-α or TGF-β_1 expression, individually or in balance with one another, in the crypts of the normal-appearing colorectal mucosa of sporadic colorectal adenoma patients.

Currently, there are no widely accepted pre-neoplastic biomarkers of risk for colorectal neoplasms. One potential biomarker is colorectal epithelial cell proliferation,[18] which is regulated in part by growth factors. TGF-α was found to be one of the main survival factors for early adenoma cells against apoptosis,[19-21] suggesting that lower TGF-α expression may decrease the likelihood of developing colorectal adenomas. Previous studies reported that TGF-α expression was greater in the normal-appearing rectal mucosa of sporadic colorectal adenoma patients[22] than in adenoma-free patients, which made it a potential marker to aid identification of high colorectal cancer risk individuals. However, in our study, we found no substantial changes in TGF-α expression in whole crypts or within functional crypt zones in response to calcium and/or vitamin D supplementation.

TGF-β_1, which is a signaling protein that may play a complex role in carcinogenesis,[23] regulates cell proliferation, apoptosis, autophagy, inflammation, tumor angiogenesis, and metastasis.[24] A dual role of TGF-β_1 has been proposed[24] because, while it was found to suppress the growth of normal epithelial cells (suggesting that it may reduce risk for developing a cancer), its expression is increased in later stages of cancer (suggesting that it may promote metastasis). A pilot, randomized, double-blind, placebo-controlled, 2x2 factorial clinical trial based on 92 sporadic colorectal adenoma patients [15] indicated that calcium and/or vitamin D supplementation may increase TGF-β_1 expression in the normal-appearing colorectal mucosa where TGF-β_1 should function as a cell growth suppressor. However, in our present study we found no substantial changes in TGF-β_1 expression in whole crypts or within functional crypt zones in response to calcium and/or vitamin D supplementation. This result is not consistent with findings from previous epidemiologic, cell line, [25-26] and animal studies[27] that suggested that calcium and vitamin D could increase TGF-β_1 expression.

We previously conducted a pilot, randomized, double-blind, placebo-controlled, 2x2 factorial clinical trial (n=92; 23/treatment group) of calcium 2 g and/or vitamin D_3 800 IU/day versus placebo over 6 months.[15] In the calcium, vitamin D_3, and calcium plus vitamin D_3 groups relative to the placebo group 1) the mean overall expression of TGFβ_1 increased by 14% (P=0.25), 19% (P=0.17), and 22% (P=0.09); 2) the ratio of TGFα expression in the upper 40% (differentiation zone) to that in the lower 60% (proliferation zone) of the crypts decreased by 34% (P=0.11), 31% (P=0.22), and 26% (P=0.33); and 3) the TGFα/TGFβ_1 ratio in the upper 40% of the crypts decreased by 28% (P=0.09), 14% (P=0.41), and 22% (P=0.24), respectively. Differences between the present and the previous pilot study included 1) that the population for the present study

was drawn without knowledge of randomized assignment from a larger study that had a more complex randomization scheme, 2) the intervention duration, and 3) the intervention doses. These differences in study design and study populations as well as chance may have contributed to the differences in the results between the two studies.

Our study had several limitations and strengths. First, as noted above, the study population was recruited from a parent study population without knowledge of randomized assignment. This resulted in imbalances in the numbers and characteristics of the biomarker study participants across treatment groups; however, adjustment for differences in the baseline characteristics of study participants did not appreciably change the estimated treatment effects. Second, this study was a pilot study with limited statistical power, especially for stratified analysis. Third, we collected biopsies only from the rectum; however, previous studies found that levels of cell proliferation markers in the rectum reflected those in other areas of the colon[28-29]. Finally, our participants were limited to sporadic colorectal adenoma patients, so our results may not be generalizable to a broader population. Strengths of our study included the high protocol adherence, the automated immunohistochemical staining, the newly designed image analysis software to accurately measure the expression of the biomarkers overall and within functional zones of the crypts, and the collection of extensive clinical, lifestyle, and dietary information.

In conclusion, the results of this pilot clinical trial, unlike those in our previous trial, suggest that calcium and/or vitamin D supplementation over 12 months may not substantially shift the level of expression of TGF-α or TGF-β_1, individually or in balance with one another, in the normal colorectal mucosa of sporadic colorectal adenoma patients. Further investigations with a larger sample size are needed to assess whether (1) calcium and vitamin D supplementation can

substantially affect the expression of TGF-α and TGF-β_1 in the rectal mucosa, and (2) TGF-α and TGF-β_1 could be used as modifiable biomarkers of risk for colorectal neoplasms.

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Summary, Public Health Implications, Possible Future Directions

In this pilot trial we found no evidence to suggest that supplementation with 1,200 mg calcium and/or 1,000 IU vitamin D over 12 months can substantially change the expression of TGF-α, TGF-β_1, or their ratio in whole crypts or within the differentiation or proliferation zones of the crypts in the normal-appearing rectal mucosa of sporadic colorectal adenoma patients. These findings differ from those of a previous pilot trial, so whether or not calcium and/or vitamin D can modulate TGF-α or TGF-β_1 individually or in relation to one another remains unclear.

Further investigation is needed into whether 1) TGF-α or TGF-β_1 are valid, modifiable biomarkers of risk for colorectal neoplasms, and 2) calcium and/or vitamin D supplementation can substantially affect the expression of TGF-α and TGF-β_1 in the colorectal mucosa. These questions could be addressed via larger 1) cross-sectional or prospective studies of colorectal adenoma in which TGF-α and TGF-β_1 is measured in multiple levels of the colon, and 2) larger clinical trials of calcium and/or vitamin (possibly exploring multiple doses) to assess their effects on TGF-α and TGF-β_1 in multiple levels of the colon. Of additional interest for the former types of studies would be to assess the associations of the markers with modifiable risk factors to generate ideas/support for potential interventions for modulating the markers. Of particular interest for the latter types of studies would be whether any possible modulation of the biomarkers predicts colorectal adenoma occurrence or recurrence.

Table 1. Selected baseline characteristics of the study participants[a] (n=104), by treatment group;

	Full Factorial-Treatment Groups				Two-arm-Treatment Groups		
	Placebo	Calcium	Vitamin D	Ca+VD	Placebo	Vitamin D	
Sample Size	12	16	17	17	23	19	
Demographics, habits, anthropometrics							P-value
Age, years	59.9(7.2)	59.9(6.5)	59.2(7.8)	57.6(7.1)	58.2(5.3)	59.2(7.3)	0.91
Men (%)[c]	75	81	71	82			0.83
White (%)	83	75	71	94	70	84	0.66
College graduate (%)	67	38	65	53	48	37	0.41
Take NSAID regularly[d] (%)	58	69	59	71	63	74	0.89
Smoking History (%)	42	31	37	59	30	47	0.51
BMI, kg/m^2	29.4(4.9)	32.3(7.6)	28.7(5.5)	30.0(4.5)	29.7(5.6)	27.5(4.7)	0.23
Daily mean dietary intakes							
Total energy intake, kcal	1314(381)	1737(556)	1437(527)	1613(550)	1254(549)	1429(595)	0.10
Dietary calcium[f], mg	602(403)	707(186)	564(262)	557(234)	538(262)	636(265)	0.45
Dietary Vitamin D[f], IU	171(133)	160(83)	107(66)	123(85)	122(79)	132(63)	0.32
Total fat, g	57.1(22.3)	68.9(25.6)	60.5(27.3)	62.6(27.2)	50.3(25.9)	61.5(36.1)	0.48
Dietary fiber, g	9.5(4.1)	15.8(5.6)	13.7(6.2)	15.6(5.5)	13.8(5.4)	17.2(5.0)	0.01
Serum biomarker concentration							
Serum Vitamin D, ng/ml	22.4(8.2)	24.5(13.4)	23.1(8.7)	22.5(6.5)	24.8(8.9)	26.5(9.6)	0.78

[a]Data was given as means (SD) unless otherwise specified;

[b]P-values were generated via the Fisher exact test for categorical variables and ANOVA for continuous variables;

[c]Only applicable for Full-Factorial study design;

[d]Defined as taking Non-Aspirin NSAID (Non-steroidal anti-inflammatory drug) more than one day per week.

[e]Measured by international physical activity questionnaire (IPAQ).

[f]Sum of dietary and supplementary intake.

Tables 2: Expression of Transforming Growth Factor Alpha in the Normal-Appearing Colorectal Mucosa during the Clinical Trial (n=102)

		Baseline Visit			Follow-up Visit			Absolute Rx Effect			
Treatment Group	n	Mean	SE[1]	P-value	Mean	SE	P-value	Rx Effect[2]	SE	P-value[3]	Relative Effect[4]
Whole Crypts											
No Vitamin D	50	6340.2	298.1		6571.8	284.2					
Vitamin D	52	6684.1	302.1	0.42	6861.1	287.3	0.48	-54.6	326.6	0.87	0.99
No Calcium	29	7051.2	375.2		7204.2	407.9					
Calcium	32	6180.6	378.7	0.11	6185.5	327.8	0.05	-148.1	382.1	0.70	0.98
Calcium Only	38	6157.6	317.2		6299.0	295.0					
Joint Group	35	6460.2	403.4	0.55	6773.8	352.5	0.30	172.2	414.6	0.68	1.03
Upper 40% of Crypts											
No Vitamin D	50	3229.4	134.5		3385.2	135.3					
Vitamin D	52	3479.2	131.4	0.19	3604.2	125.6	0.24	-30.7	155.5	0.84	0.99
No Calcium	29	3582.1	166.0		3736.3	192.4					
Calcium	32	3244.6	175.9	0.17	3322.8	158.1	0.10	-76.0	195.1	0.70	0.98
Calcium Only	38	3161.3	148.4		3295.3	144.4					
Joint Group	35	3382.4	172.5	0.33	3517.3	145.8	0.28	0.8	195.2	1.00	1.00
Lower 60% of Crypts											
No Vitamin D	50	2677.6	163.4		2721.4	143.6					
Vitamin D	52	2720.4	171.9	0.86	2763.1	159.2	0.85	-1.0	183.6	0.99	1.00
No Calcium	29	2993.0	223.5		2958.7	205.8					
Calcium	32	2477.6	192.7	0.08	2401.0	166.8	0.04	-42.3	201.7	0.83	0.98
Calcium Only	38	2569.4	166.5		2541.1	146.7					
Joint Group	35	2597.4	226.1	0.92	2782.6	205.3	0.34	213.6	226.4	0.35	1.08

(Continued)

Top 40% to Whole Crypts											
No Vitamin D	50	0.518	0.007		0.522	0.007					
Vitamin D	52	0.536	0.010	0.16	0.537	0.008	0.19	-0.003	0.011	0.75	0.99
No Calcium	29	0.515	0.011		0.525	0.009					
Calcium	32	0.536	0.011	0.19	0.546	0.009	0.13	-0.001	0.012	0.97	1.00
Calcium Only	38	0.521	0.008		0.531	0.008					
Joint Group	35	0.543	0.013	0.14	0.532	0.010	0.90	-0.020	0.013	0.12	0.96

1. SE: standard error;

2. Rx effect= [(treatment group follow-up)(treatment group baseline)][(placebo group follow-up)(placebo group baseline)];

3. P value for difference between each active treatment group and placebo group from repeated-measures MIXED model;

4. Relative effect= [(treatment group follow-up)/(treatment group baseline)]/[(placebo follow-up)/(placebo baseline)]; interpretation similar to that for an odds ratio (e.g., a relative effect of 1.7 indicates a 70% proportional increase in the treatment group relative to that in the placebo group).

Tables 3: Expression of Transforming Growth Factor Beta$_1$ in the Normal-Appearing Colorectal Mucosa during the Clinical Trial (n=103)

		Baseline Visit			Follow-up Visit			Absolute Rx Effect			
Treatment Group	n	Mean	SE[1]	P-value	Mean	SE	P-value	Rx Effect[2]	SE	P-value[3]	Relative Effect[4]
Whole Crypts											
No Vitamin D	50	1943.4	324.4		1841.9	361.3					
Vitamin D	53	1645.7	328.8	0.52	1524.1	349.7	0.53	-20.1	171.7	0.91	0.98
No Calcium	29	1705.2	415.4		1651.1	479.1					
Calcium	33	1049.1	272.7	0.18	961.3	350.3	0.24	-33.7	197.9	0.87	0.95
Calcium Only	38	2061.9	390.7		1969.5	440.4					
Joint Group	36	1571.9	397.0	0.38	1393.1	392.7	0.33	-86.5	209.0	0.68	0.93
Upper 40% of Crypts											
No Vitamin D	50	736.6	136.8		735.7	152.8					
Vitamin D	53	628.0	137.8	0.58	612.4	148.5	0.56	-14.7	70.4	0.83	0.98
No Calcium	29	647.7	180.7		678.5	214.1					
Calcium	33	375.6	112.8	0.20	383.1	149.6	0.25	-23.3	85.5	0.79	0.97
Calcium Only	38	799.9	164.5		797.9	186.5					
Joint Group	36	581.6	160.9	0.35	534.6	156.7	0.29	-44.9	83.2	0.59	0.92
Lower 60% of Crypts											
No Vitamin D	50	1110.6	173.4		1005.7	189.8					
Vitamin D	53	938.5	177.4	0.49	827.5	184.4	0.50	-6.1	98.0	0.95	0.97
No Calcium	29	972.6	214.7		882.8	238.5					
Calcium	33	621.2	147.4	0.17	523.7	181.5	0.23	-7.7	102.6	0.49	0.93
Calcium Only	38	1157.3	207.9		1058.3	230.0					
Joint Group	36	919.1	221.5	0.44	786.8	219.8	0.40	-33.2	122.5	0.79	0.94

(Continued)

Top 40% to Whole Crypts											
No Vitamin D	50	0.295	0.014		0.305	0.015					
Vitamin D	53	0.292	0.016	0.91	0.313	0.015	0.71	0.010	0.021	0.63	1.04
No Calcium	29	0.306	0.021		0.314	0.017					
Calcium	33	0.268	0.018	0.16	0.305	0.020	0.71	0.028	0.030	0.35	1.11
Calcium Only	38	0.307	0.016		0.302	0.018					
Joint Group	36	0.268	0.020	0.12	0.312	0.019	0.70	0.049	0.024	0.04	1.18

1. SE: standard error;

2. Rx effect= [(treatment group follow-up)(treatment group baseline)][(placebo group follow-up)(placebo group baseline)];

3. P value for difference between each active treatment group and placebo group from repeated-measures MIXED model;

4. Relative effect= [(treatment group follow-up)/(treatment group baseline)]/[(placebo follow-up)/(placebo baseline)]; interpretation similar to that for an odds ratio (e.g., a relative effect of 1.7 indicates a 70% proportional increase in the treatment group relative to that in the placebo group).

Tables 4: Ratio of Transforming Growth Factor Expression to Transforming Growth Factor $Beta_1$ Expression in Whole Crypt of Normal-Appearing Colorectal Mucosa during the Clinical Trial (n=102)

		Baseline Visit			Follow-up Visit			Absolute Rx Effect			
Treatment Group	n	Mean	SE[1]	P-value	Mean	SE	P-value	Rx Effect[2]	SE	P-value[3]	Relative Effect[4]
No Vitamin D	50	41.0	12.2		91.9	40.2					
Vitamin D	52	127.6	44.4	0.07	128.3	40.7	0.53	-50.2	50.0	0.32	0.45
No Calcium	29	81.4	43.4		69.3	23.7					
Calcium	32	119.7	61.2	0.62	179.8	75.7	0.19	72.2	73.1	0.33	1.76
Calcium Only	38	44.2	15.5		107.2	52.6					
Joint Group	35	132.6	56.4	0.12	147.9	57.5	0.60	-48.7	63.5	0.46	0.46

1. SE: standard error;

2. Rx effect= [(treatment group follow-up)(treatment group baseline)][(placebo group follow-up)(placebo group baseline)];

3. P value for difference between each active treatment group and placebo group from repeated-measures MIXED model;

4. Relative effect= [(treatment group follow-up)/(treatment group baseline)]/[(placebo follow-up)/(placebo baseline)]; interpretation similar to that for an odds ratio (e.g., a relative effect of 1.7 indicates a 70% proportional increase in the treatment group relative to that in the placebo group).