A randomized clinical trial of the effects of supplemental calcium and vitamin D₃ on the APC/β-catenin pathway in the normal mucosa of colorectal adenoma patients

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Abstract

APC/β-catenin pathway perturbation is a common early event in colorectal carcinogenesis and is affected by calcium and vitamin D in basic science studies. To assess the effects of calcium and vitamin D on APC, β-catenin, and E-cadherin expression in the normal appearing colorectal mucosa of sporadic colorectal adenoma patients, we conducted a randomized, double-blinded, placebo-controlled 2×2 factorial clinical trial. Pathology-confirmed colorectal adenoma cases were treated with 2 g/day elemental calcium and/or 800 IU/day vitamin D₃ versus placebo over 6 months (N=92; 23/group). Overall APC, β-catenin, and E-cadherin expression and distributions in colon crypts in normal-appearing rectal mucosa biopsies were detected by standardized automated immunohistochemistry and quantified by image analysis. In the vitamin D₃-supplemented group relative to placebo, the proportion of APC in the upper 40% of crypts (ϕh APC) increased 21% (p=0.01), β-catenin decreased 12% (p=0.18), E-cadherin increased 72% (p=0.03), and the ϕh APC/β-catenin ratio (APC/β-catenin score) increased 31% (p=0.02). In the calcium-supplemented group ϕh APC increased 10% (p=0.12), β-catenin decreased 15% (p=0.08), and the APC/β-catenin score increased 41% (p=0.01). In the calcium/vitamin D₃ supplemented group β-catenin decreased 11% (p=0.20), E-cadherin increased 51% (p=0.08), and the APC/β-catenin score increased 16% (p=0.26). These results support 1) that calcium and vitamin D modify APC, β-catenin, and E-cadherin expression in humans in directions hypothesized to reduce risk for colorectal neoplasms, 2) calcium and vitamin D as potential chemopreventive agents against colorectal neoplasms, and 3) the potential of APC, β-catenin, and E-cadherin expression as modifiable, pre-neoplastic risk biomarkers for colorectal neoplasms.

Keywords

calcium; vitamin D; colonic neoplasms; biomarkers; clinical trial

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Introduction

Colorectal cancer (CRC), the second leading cause of cancer deaths in the United States (1), is responsible for approximately 8% of all cancer deaths worldwide (2, 3). The etiology of sporadic CRC is predominately rooted in dietary and lifestyle behaviors (2, 4), suggesting that it may be preventable. The molecular basis of colorectal carcinogenesis is becoming clearer (4); however, there are no validated, treatable, pre-neoplastic biomarkers of risk for colorectal neoplasms.

Malfunction of the APC/β-catenin signaling pathway is an early and common event in the pathogenesis of colorectal neoplasms. Impaired APC function occurs in approximately 80-90% of sporadic CRCs(5), resulting in the increased potential of β-catenin to translocate to the nucleus and activate target genes responsible for promoting cell proliferation and inhibiting differentiation (5-7). E-cadherin may also antagonize β-catenin nuclear expression by sequestering β-catenin to its cytoplasmic tail, linking E-cadherin to actin filaments and promoting cell adhesion and differentiation (5, 7). We reported that APC expression (especially the proportion of APC in the upper 40% of colorectal crypts (φh APC)), β-catenin expression, and the φh APC/β-catenin ratio (APC/β-catenin score) in normal colorectal mucosa may be valid, potentially modifiable, pre-neoplastic biomarkers of risk for colorectal neoplasms (8).

Convincing evidence from experimental and observational studies and randomized, placebo-controlled clinical trials suggests that calcium and vitamin D have chemopreventive effects against colorectal neoplasms (9). The beneficial effects of calcium may partially be attributed to its binding of toxic secondary bile acids and ionized fatty acids, and/or by directly inhibiting proliferation and promoting differentiation and apoptosis (9). Vitamin D signaling may induce cell-cycle arrest and promote differentiation and apoptosis directly through vitamin D-mediated gene transcription and indirectly through modifying growth factors, and play roles in promoting oxidative DNA damage repair, inhibiting angiogenesis, and regulating immune cell function (9). Also, prospective cohort studies have consistently found higher total calcium intake to be associated with lower risk for colorectal neoplasms (9), calcium supplementation reduces colorectal adenoma recurrence (modified by vitamin D status) (10), and higher circulating 25(OH)-vitamin D (25(OH)D) is inversely associated with colorectal neoplasms (9, 11, 12).

Evidence from animal models (13, 14) and *in vitro* (15-17) studies suggest that the chemopreventive effects of calcium and vitamin D may, in part, include modification of the APC/β-catenin signaling pathway. However, to our knowledge there are no reported human *in vivo* investigations on the effects of supplemental calcium and vitamin D_3 on the expression of APC, β-catenin, and E-cadherin in the normal colorectal mucosa. To address this, as reported herein, we conducted a pilot, randomized, double-blind, placebo-controlled 2×2 factorial chemoprevention clinical trial of supplemental calcium and vitamin D_3, alone and in combination, versus placebo over 6 months, to estimate the efficacy of these agents on APC, β-catenin, and E-cadherin expression in the normal colorectal mucosa.

Study Participants and Methods

Participant population

A detailed description of the study protocol for recruitment procedures and detailed specific exclusions was published previously (18). Briefly, eligible participants were 30 to 75 years of age, in general good health, and had a history of at least one pathology-confirmed adenomatous colorectal polyp within the past 36 months. Exclusions from participation included contraindications to calcium or vitamin D supplementation or rectal biopsy

procedures, and medical conditions, habits, or medication usage that potentially could interfere with the study. Participants were recruited from patients attending the Digestive Diseases Clinic of the Emory Clinic, Emory University.

Clinical trial protocol

Between April 2005 and January 2006, 522 potentially eligible patients were identified through initial screening of electronic medical records; of these, 244 (43%) were contacted, and of these 105 (47%) attended the eligibility visit to be interviewed, sign a consent form, complete questionnaires, and provide blood samples (18). Diet was assessed using a semi-quantitative Willett Food Frequency Questionnaire (19). Medical and pathology records were reviewed. Following a 30-day placebo run-in trial, 92 (88%) participants with no significant perceived side effects and who took at least 80% of their assigned tablets underwent a baseline rectal biopsy were randomly assigned, stratified on sex and nonsteroidal anti-inflammatory drug (NSAID) use, to the following four treatment groups: placebo ($n = 23$), 2.0 g elemental calcium supplementation (as calcium carbonate in equal doses twice daily; $n = 23$), 800 IU vitamin D_3 supplementation (400 IU twice daily; $n = 23$), and 2.0 g elemental calcium plus 800 IU vitamin D_3 supplementation ($n = 23$). Additional details and rationale for the doses and forms of calcium and vitamin D_3 supplements were previously published (18). Participants were instructed to maintain their usual diet and not take any new nutritional supplements they were not taking at the time of entry into the study. All aspects of the trial were approved by the Institutional Review Board of Emory University.

During the 6-month treatment period, participants attended follow-up visits 2 and 6 months after randomization. At follow-up visits participants completed questionnaires and were interviewed about adherence and adverse events. At the 6-month follow-up, participants again underwent a venipuncture and rectal biopsy. All visits for a given participant were scheduled for the same time of day to control for potential circadian variation. Dietary, lifestyle, and other factors hypothesized to modify biomarker expression in normal colon mucosa were assessed at baseline and at 6-months follow-up. Participants were asked to abstain from aspirin use seven days prior to each biopsy. Participants were not required to be fasting for their visits and did not take a bowel cleansing preparation or enema.

Six approximately one millimeter thick biopsy specimens were taken from the normal-appearing rectal mucosa 10 cm above the level of the external anal aperture through a short rigid sigmoidoscope using a jumbo cup flexible biopsy forceps mounted on a semi-rigid rod. No biopsies were taken within 4.0 cm of a polypoid lesion. Biopsies were placed onto a strip of bibulous paper and immediately placed in phosphate buffered saline (PBS), oriented, transferred to 10% normal buffered formalin for 24 hours, and then transferred to 70% ethanol. Then, within a week, the biopsies were processed and embedded in paraffin blocks (2 blocks of 3 biopsies per participant, per biopsy visit).

Immunohistochemistry protocol

Five slides with 4 levels of 3 micron-thick biopsy sections taken 40 microns apart were prepared for each antigen, yielding a total of 20 levels for each antigen. Antigen retrieval was performed by placing the slides in a preheated Pretreatment Module (Lab Vision Corp.) with 100x Citrate Buffer (pH 6.0; DAKO S1699, DAKO Corp.) and steaming them for 40 min. Following antigen retrieval, slides were immunohistochemically processed in a DAKO Automated Immunostainer (DAKO Corp.) using a labeled streptavidin-biotin method for APC (Calbiochem, OP80; 1:70 dilution), β-catenin (Transduction Laboratories 610154; 1:300 dilution), and E-cadherin (Zymed 33-4000; 1:50 dilution). No slides were counterstained. After processing, slides were coverslipped with a Leica CV5000

Coverslipper (Leica Microsystems, Inc.). The negative and positive control slides were treated identically to the patients' slides except that antibody diluent was used rather than primary antibody on the negative slides.

Protocol for quantifying labeling densities of immunohistochemically detected biomarkers in normal colon crypts ("scoring")

A detailed description of the protocol used to quantify biomarker labeling optical densities ("biomarker expression") in normal colon crypts was previously described (18). Briefly, a scorable crypt was defined as an intact crypt extending from the muscularis mucosa to the colon lumen (20). Prior to "scoring", the negative and positive control slides were checked for staining adequacy. The major equipment and software for the image analysis procedures included: 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).

Evaluation of biomarker expression consisted of the same technician cleaning all slides, selecting the two of the three biopsies with the most scorable crypts per biopsy, creating background correction images for each slide scored, capturing 16-bit grayscale images of crypts at 200x magnification, and tracing the border of the "hemicrypt" (one half of the crypt). The program then divided the outlined hemicrypt into equally spaced segments that corresponded to the average width of colonocytes, and measured the optical density of the labeling across the entire hemicrypt and within each segment, adjusting for the background. The technician then repeated this process for the adjacent hemicrypt, and proceeded to the next crypt, level, biopsy, and/or slide. The goal was to score 16 to 20 hemicrypts per biopsy visit for each biomarker (Figure 1).

Reliability control was performed by selecting samples of previously analyzed slides to be re-analyzed by the technician, who was blinded to the selection. Intra-reader reliability was greater than 0.90 for APC, β-catenin, and E-cadherin.

Protocol for measuring serum vitamin D levels

Serum 25-OH-vitamin D and 1,25-$(OH)_2$-vitamin D were measured by Dr. Bruce W. Hollis at the Medical University of South Carolina using a RIA method as previously described (21, 22). 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 was 2.3% and 6.2% for serum 25-OH-vitamin D and for 1,25-$(OH)_2$-vitamin D, respectively.

Statistical analysis

All statistical analyses were performed using SAS 9.3 statistical software (SAS Institute Inc.). A *P* value 0.05 (two-sided) was considered statistically significant. 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.

The mean labeling optical density expression of each biomarker on each study participant, at baseline and 6-month follow-up, was calculated by summing the biomarker's expression for all analyzed crypts and dividing by the total number of analyzed crypts. Biomarker

expression was transformed to adjust for possible staining batch effects by dividing an individual's mean biomarker expression by their batch mean biomarker expression (18). To evaluate distinct functional zones of crypts, measures of crypt biomarker distribution selected *a priori* were the upper 40% of the crypts (differentiation zone), the lower 60% of the crypts (proliferation zone), and the ratio of the upper 40% of crypts to the whole crypt to (φh). An APC/β-catenin score was calculated by dividing an individual's φh APC by their β-catenin expression in the whole crypt (φh APC expression/β-catenin expression). E-cadherin was not included in the APC/β-catenin score because during carcinogenesis malfunctioning regulation of β-catenin by APC occurs most often earlier than E-cadherin down-regulation (23). We hypothesize that a higher score is associated with reduced potential of β-catenin to promote proliferation.

The distributions of batch standardized APC, β-catenin, and E-cadherin labeling optical densities along the full length of the crypts were graphically plotted and evaluated using the LOESS procedure. First, each hemicrypt was standardized to 50 sections. Then, the average of each section across all crypts was predicted by the LOESS model separately for each patient and then for each treatment group by visit. The results were graphically plotted along with the smoothing lines. Although the plots illustrate the distribution of expression, they do not provide a complete analysis of treatment effects because they do not account for changes in the placebo group.

Primary analyses were based on assigned treatment at the time of randomization, regardless of adherence status (intent-to-treat analysis). Treatment effects were evaluated by assessing the differences in the transformed biomarker expression from baseline to the 6-month follow-up between participants in the active treatment groups and those in the placebo group by a repeated-measures linear MIXED effects model. The model included the intercept, follow-up visit effects (baseline and follow-up), treatment groups, and interactions between treatment groups and the follow-up visit effect. Because optical density is measured in arbitrary units, to provide perspective on the magnitude of the treatment effects, we also calculated the relative effect. The relative effect was calculated as the (treatment group at follow-up/treatment group at baseline)/(placebo group at follow-up/placebo group at baseline). The relative effect provides a conservative estimate of the proportional change in the treatment group relative to that in the placebo group, and its interpretation is somewhat analogous to that of an odds ratio (e.g., a relative effect of 2.0 would mean that the proportional change in the treatment group was two times that in the placebo group).

Sensitivity analysis

To investigate the sensitivity of our analyses to missing data, we used multiple imputation to impute biomarker expression for study participants who did not have scorable crypts or were lost to follow-up. To create a monotone missing pattern, we used a Markov Chain Monte Carlo method to impute a value for the few observations that were missing on biomarkers measured at baseline, based on an assumption of multivariate normality (SAS, V 9.3, Proc MI). Once a monotone missing pattern was created, we used a regression approach to impute the remaining values(24). Age and sex were included as covariates for imputation to create six completed data sets that we then analyzed as previously described.

Results

Characteristics of study participants

Baseline characteristics of study participants did not significantly differ by treatment group (Table 1). The mean age of study participants was 61 years, 70% were men, 71% were

white, and 20% had a family history of colorectal cancer in a first degree relative. Most participants were non-smokers, college graduates, and overweight.

Adherence to visit attendance averaged 92% and did not significantly differ among the four treatment groups. On average, at least 80% of pills were taken by 93% of participants at the first follow-up visit and by 84% of participants at the final follow-up visit. No adverse events were attributed to study procedures or treatments. Seven participants (8%) were lost to follow-up. Dropouts included one person from the vitamin D_3 supplementation group and two from each of the other three groups (18).

Baseline serum 25-OH-vitamin D and 1,25-$(OH)_2$-vitamin D levels did not differ between the four treatment groups. At the conclusion of the study, serum 25-OH-vitamin D levels had increased 60% (p<0.0001) and 56% (p<0.0001) in the vitamin D_3 and calcium/vitamin D_3 groups, respectively, relative to placebo; however, mean serum 25-OH-vitamin D concentrations remained below 32 ng/ml in all treatment groups (18). There was no evidence of treatment effect modification by obesity status (body mass index   30).

APC

A graphical comparison of APC crypt expression distribution at baseline and 6-month follow-up indicated that APC expression decreased in approximately the lower 40% of the crypt and increased in the upper 60% of the crypt (Figure 2A). As shown in Table 2, following 6 months of treatment, APC expression increased in the vitamin D_3 treatment group 25% (p=0.14) in the full length of crypts, 48% (p=0.03) in the upper 40% of crypts, 11% in the lower 60% of crypts (p=0.47), and 21% (p=0.01) in the ϕh of crypts, relative to the placebo group. In the calcium group APC expression decreased 2% (p=0.91) in the full length of crypts, increased 7% (p=0.66) in the upper 40% of crypts, decreased 10% (p=0.51) in the lower 60% of crypts, and increased 10% (p=0.12) in the ϕh of crypts, relative to the placebo group. APC expression tended to increase in the calcium/vitamin D_3 less than in the vitamin D_3 group, and these findings were not statistically significant (Table 2A).

β-catenin

A graphical evaluation of β-catenin crypt expression distribution at baseline and 6-month follow-up indicated that β-catenin expression did not change in approximately the lower 20% of the crypt, but steadily decreased towards the crypt apex (Figure 2B). As shown in Table 2, following 6 months of treatment, β-catenin expression decreased along the full length of crypts by 15% (p=0.08), 12% (p=0.18), and 11% (p=0.20) in the calcium, vitamin D_3 and calcium/vitamin D_3 groups, respectively, relative to the placebo group. The findings in the upper 40% and lower 60% of crypts did not appreciably differ from those observed in the full length of crypts. There were no apparent treatment effects on β-catenin expression in the ϕh of crypts.

E-cadherin

A graphical evaluation of E-cadherin crypt expression distribution at baseline and 6-month follow-up indicated that E-cadherin expression uniformly increased along the full length of the crypt (Figure 2C). As shown in Table 2, following 6 months of treatment, E-cadherin expression increased in the vitamin D_3 group 72% (p=0.03) in the full length of crypts, 78% (p=0.02) in the upper 40% of crypts, 68% (p=0.05) in the lower 60% of crypts, and 14% (p=0.10) in the ϕh of crypts. E-cadherin expression also increased in the calcium/vitamin D_3 group, but less so than in the vitamin D_3 group, except in the ϕh of crypts where E-cadherin expression increased 18% (p=0.03). In the calcium group E-cadherin did not appreciably change relative to the placebo group (Table 2C).

APC/β-catenin score

The APC/β-catenin score increased 41% (p=0.01), 31% (p=0.02), and 16% (p=0.26) in the calcium, vitamin D_3, and calcium/vitamin D_3 groups, respectively, relative to the placebo group (Table 2D).

Sensitivity analysis

There were no apparent differences in findings following imputation of missing observations (Supplemental material Table 1).

Discussion

The results of this pilot, randomized, placebo-controlled clinical trial provides the first human *in* vivo evidence that supplemental calcium and vitamin D_3, alone or in combination, may increase APC and E-cadherin expression and the APC/β-catenin score and decrease β-catenin expression in the normal colorectal mucosa of sporadic adenoma patients. These finding support the hypothesis that the anti-carcinogenic effects calcium and vitamin D, alone or in combination, may in part operate by modifying the APC/β-catenin signaling pathway. These findings are relevant because, in light of our previous report of differences in APC and β-catenin expression between persons with incident sporadic adenomas and persons with no past or current adenomas, they 1) provide further support that APC and β-catenin expression and the APC/β-catenin score in the normal colorectal mucosa may be modifiable, pre-neoplastic biomarkers of risk for colorectal adenomas, and 2) provide human *in vivo* mechanistic evidence of the possible protective effects of calcium and vitamin D_3 against colorectal neoplasms.

APC and β-catenin are appealing candidates for being pre-neoplastic biomarkers of risk because malfunctioning of the APC/β-catenin signaling pathway is a common and early event in the colorectal neoplastic transition (5). In normal colorectal mucosa APC, axin, glycogen synthase kinase 3, and casein kinase negatively regulate Wnt signaling by forming the "β-catenin destruction" complex, and, in the absence of Wnt signaling, phosphorylate and promote the degradation of free β-catenin (5). Normal functioning of the *APC* gene is inhibited in approximately 80-90% of sporadic CRC, resulting in increased potential for β-catenin to translocate to the nucleus and activate Wnt target genes (5). In normal colorectal mucosa APC, β-catenin, and E-cadherin are all strongly expressed—APC primarily in the cytoplasm, and E-cadherin and β-catenin primarily at the cell membrane. During the adenoma-carcinoma sequence APC and E-cadherin expression markedly decrease (although the decrease in E-cadherin tends to occur in later stages) (25-27), and β-catenin expression appears to steadily increase and translocate from the membrane to the cytoplasm and eventually into the nucleus (25, 28). We previously proposed that the APC/β-catenin score may represent the potential of β-catenin to translocate to the nucleus and promote proliferative signaling (8). We found the APC/β-catenin score in the normal colorectal mucosa of sporadic colorectal adenoma patients to be statistically significantly lower than in the normal colorectal mucosa of healthy controls; and that φh APC and β-catenin expression and the APC/β-catenin score may be modifiable as suggested by their being associated with lifestyle and dietary risk factors for colorectal neoplasms (8).

The etiology of CRC is heavily influenced by modifiable dietary and lifestyle behaviors. Dietary-induced epigenetic modifications to the APC/β-catenin signaling pathway may initiate or be a "second hit" in the adenoma-carcinoma pathway. Calcium and vitamin D are two promising chemopreventive agents that may act against colorectal neoplasms; however, the mechanisms by which they operate are not entirely clear (9). Colorectal cancer cell line studies suggest that calcium and 1,25$(OH)_2$D up-regulate E-cadherin expression and

promote the translocation of β-catenin from the nucleus and cytoplasm to the plasma membrane (15-17, 29). Mice fed a diet comparable to a typical "Western" diet had increased *β-catenin* and *Tcf* gene expression and decreased *APC* gene expression; however, supplementation of the "Western" diet with increased dietary calcium and vitamin D decreased *β-catenin* and *Tcf* gene expression, but had no apparent effect on *APC* gene expression (13). In a transmissible murine colonic hyperplasia model high dietary calcium modestly reduced total β-catenin expression (14). A diet supplemented with the vitamin D analog 1α(OH)D$_5$ inhibited β-catenin nuclear expression in azoxymethane-treated mice (30), and endometrial E-cadherin expression increased in mice fed a diet high in vitamin D$_3$ (31). Investigations of the *in vivo* effects of calcium and/or vitamin D on APC expression are limited (13), but dietary modification of APC expression is supported by reports that diets moderately deficient in B-vitamin methyl donors reduced APC expression and increased β-catenin/Tcf signaling in rodents (32, 33).

Our results are consistent with the hypothesis that calcium and vitamin D reduce cell proliferation and promote differentiation in the colorectal mucosa. To our knowledge this is the first human *in vivo* study to suggest that calcium and/or vitamin D$_3$ may affect APC expression in the normal colorectal mucosa; however, the mechanism by which calcium and/or vitamin D$_3$ may modify APC expression remains unclear. We did not evaluate β-catenin localization, but consistent with the observed increase in APC expression, particularly ϕh APC, we observed decreased β-catenin expression and an increased APC/β-catenin score in all three active treatment groups, suggesting that calcium and vitamin D$_3$ treatment may decrease the potential of β-catenin to promote proliferative signaling. These results are in line with our reports that suggested that calcium and/or vitamin D$_3$ treatment reduced hTERT expression (a potential cofactor in the β-catenin transcriptional complex (34)) and increased p21 expression (which is negatively regulated by β-catenin/TCF signaling (35)) (36). These results also corroborate our report that the APC/β-catenin score may be a valid, potentially modifiable, pre-neoplastic biomarker of risk for colorectal neoplasms, providing *in vivo* evidence that supplemental calcium and/or vitamin D$_3$ may promote a greater APC/β-catenin score in the normal rectal mucosa.

Conflicting with previous *in vitro* studies (15, 16) we did not observe increased E-cadherin expression in the calcium group. The explanation for this lack of consistency in findings is not clear and may be a chance finding; however, the difference may be attributed to our investigation evaluating the effects of calcium on E-cadherin expression in the normal mucosa of free living humans rather than *in vitro*, or that the ability of calcium to increase E-cadherin expression may be limited to neoplastic mucosa.

Contrary to our hypothesis and what has been reported in some studies (10, 37), the estimated treatment effect of calcium plus vitamin D$_3$ was not greater than that of either the calcium or vitamin D$_3$ alone in increasing ϕh APC, E-cadherin, or the APC/β-catenin score, or in decreasing β-catenin. We previously reported that calcium combined with vitamin D$_3$ may mitigate treatment effects of calcium and vitamin D$_3$ alone on colorectal mucosa markers of apoptosis and differentiation (18, 36). There are several plausible explanations for why this was observed, the first being that these could have been chance findings given the small sample size of our study. At least one study reported that calcium and vitamin D individually suppressed tumorigenesis in rodents, but the combination of the two was ineffective (38). We previously observed that calcium combined with vitamin D$_3$ increased CYP24A1 expression (39), which may reduce the effects of 1,25(OH)$_2$D in the colorectal mucosa. A large clinical trial of colorectal adenoma recurrence suggested that calcium supplementation was primarily effective among people with 25(OH)D concentrations greater than the median in the study population (29.1 ng/ml) (10). In our study population

only participants in the vitamin D_3 group reached 25(OH)D concentrations greater than 29.1 ng/ml (18), suggesting the possibility of a threshold effect.

We previously reported that calcium and vitamin D_3 supplementation in this same trial favorably modified the expression of markers of calcium and vitamin D metabolism (39), proliferation (36), differentiation (36), apoptosis (18), mismatch repair (40), and oxidative DNA damage (41) in the normal human colorectal mucosa. Our current results, taken together with our previous findings, support the hypothesized effects of calcium and vitamin D on favorably modulating the molecular phenotype of the normal colorectal mucosa and reducing risk for colorectal neoplasms.

Our study had several limitations. First, it was a pilot study with a relatively small sample size, increasing the role of chance observations and limiting our ability to perform stratified analyses. We were unable to evaluate β-catenin sub-cellular localization; however, our previous findings (8) suggested that sporadic colorectal adenoma cases relative to normal controls may have greater total β-catenin expression in the normal colorectal mucosa. We propose that the APC/β-catenin score may represent the potential of β-catenin to promote proliferative signaling, and needs to be investigated in basic science studies. Also, we only examined the rectal mucosa and therefore treatment effects in other parts of the colon remain unknown. Another limitation is that we measured protein expression but not protein activity, and, therefore, could not correlate changes in expression with changes in protein activity. Finally, these markers are not proven biomarkers of risk; however, evidence from our pilot case-control study suggests that APC, β-catenin expression, and the APC/β-catenin score may be pre-neoplastic biomarkers of risk.

The strengths of this study include 1) that it is, to our knowledge, the first randomized, double-blind, placebo-controlled clinical trial to test the effects of supplemental calcium and vitamin D_3, alone and in combination, on components of the APC/β-catenin signaling pathway in the normal colorectal epithelium in sporadic adenoma patients, 2) the high protocol adherence by study participants, and 3) the automated immunostaining and newly-designed image analysis software to quantify the crypt distribution of the expression of APC, β-catenin, and E-cadherin, resulting in high biomarker measurement reliability.

In summary, the results of this pilot, randomized, placebo-controlled clinical-trial provide human *in vivo* evidence that supplemental calcium and vitamin D_3, alone and in combination, may increase APC and E-cadherin expression and the APC/β-catenin score and decrease β-catenin expression in the normal colorectal mucosa of sporadic colorectal adenoma cases. These results suggest that the anti-carcinogenetic effects of supplemental calcium and vitamin D_3 may, in part, depend on the ability of these agents to favorably modulate the expression of APC, β-catenin, and E-cadherin and thus, possibly, inhibit proliferative β-catenin signaling. Taken together with our previous findings, APC (especially φh APC) and β-catenin expression, the APC/β-catenin score, and E-cadherin may be modifiable, pre-neoplastic biomarkers of risk for colorectal neoplasms and warrant further investigation. Finally, our results support further investigation of calcium and vitamin D_3 as chemopreventive agents against colorectal neoplasms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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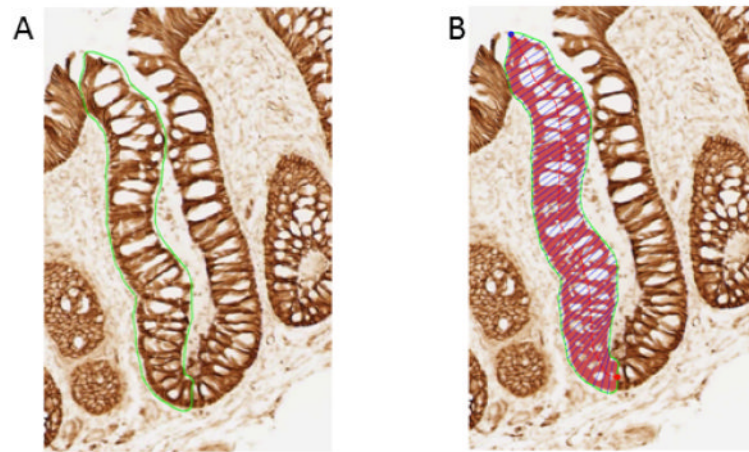
Figure 1.
Quantitative image analysis. A, finding and tracing the hemicrypt; B, automated sectioning and quantification of β-catenin labeling optical density (figure adapted from reference 8).
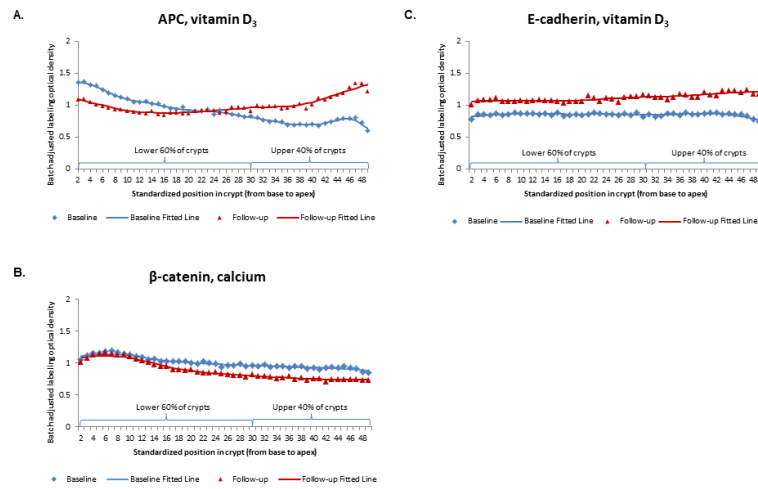
Figure 2.
Representative examples of labeling expression distribution of (A) APC, (B) βcatenin, and (C) E-cadherin along normal colorectal crypts by treatment group at baseline and 6-month follow-up.

Table 1

Selected baseline characteristics of the study participants[a] (n=92)

Characteristics	Placebo (n=23)	Calcium (n=23)	Vitamin D (n=23)	Calcium + Vit. D (n=23)	P-value[b]
Demographics, medical history, habits, anthropometrics					
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	0.39
College graduate (%)	65	61	57	44	0.53
History of colorectal cancer in 1° relative (%)	17	30	17	13	0.60
Take NSAID[c] regularly[d] (%)	22	13	9	22	0.60
If woman (n = 28), taking estrogens (%)	4	9	4	4	1.00
Current smoker (%)	9	4	0	0	0.61
Take multivitamin (%)	30	30	26	39	0.86
Body mass index (BMI), kg/m²	30.6 (7.2)	29.4 (5.5)	28.9 (5.56)	31.6 (6.0)	0.44
Mean dietary intakes[e]					
Total energy intake, kcal/d	1,596 (528)	1,788 (691)	1,848 (821)	1,845 (752)	0.59
Total[f] calcium, mg/d	625	678	753	733	0.75
Total[f] vitamin D, IU/d	279	326	348	401	0.50
Total fat, gm/d	66	66	61	65	0.59
Dietary fiber, gm/d	15	16	16	15	0.97
Alcohol intake, gm/d	8	10	13	9	0.84
Total serum vitamin D					
25-OH-vitamin D, ng/mL	20.4 (7.6)	25.7 (7.6)	21.0 (8.3)	20.9 (9.7)	0.12

[a] Data are given as means (SD) unless otherwise specified.

[b] By Fisher's exact χ² test for categorical variables, and ANOVA for continuous variables.

[c] Nonsteroidal anti-inflammatory drug.

[d] At least once a week.

[e]All nutrients energy adjusted using residual method.

[f]Diet plus supplements.

Table 2

Expression of APC, β-catenin, E-cadherin, and the APC/β-catenin score[a] in the normal-appearing colorectal mucosa

Treatment Group	n	Baseline			6-mo follow-up				Absolute Rx effect			Relative Effect[d]
		Mean	Std Err	P	n	Mean	Std Err	P	Rx effect[b]	Std Err	P[c]	
A. APC												
Whole crypts												
Placebo	22	1.09	0.08	.	21	0.97	0.08	.	0.00	.	.	1.00
Calcium	23	1.07	0.08	0.90	21	0.94	0.08	0.80	−0.02	0.15	0.91	0.98
Vitamin D	23	0.90	0.08	0.10	22	1.00	0.08	0.80	0.22	0.14	0.14	1.25
Ca + Vit. D	23	0.97	0.08	0.29	21	0.91	0.08	0.61	0.06	0.15	0.68	1.06
Upper 40% of crypts												
Placebo	22	0.39	0.04	.	21	0.41	0.04	.	0.00	.	.	1.00
Calcium	23	0.40	0.04	0.91	21	0.44	0.04	0.51	0.03	0.06	0.66	1.07
Vitamin D	23	0.30	0.04	0.07	22	0.46	0.04	0.31	0.15	0.06	0.03	1.48
Ca + Vit. D	23	0.36	0.04	0.47	21	0.41	0.04	0.39	0.04	0.06	0.58	1.10
Lower 60% of crypts												
Placebo	22	0.68	0.05	.	21	0.57	0.05	.	0.00	.	.	1.00
Calcium	23	0.70	0.05	0.81	21	0.53	0.05	0.53	−0.06	0.09	0.51	0.90
Vitamin D	23	0.60	0.05	0.24	22	0.56	0.05	0.84	0.07	0.09	0.47	1.11
Ca + Vit. D	23	0.60	0.05	0.24	21	0.52	0.05	0.49	0.03	0.09	0.73	1.04
φh[e]												
Placebo	22	0.35	0.01	.	21	0.42	0.01	.	0.00	.	.	1.00
Calcium	23	0.34	0.01	0.06	21	0.46	0.01	0.06	0.04	0.03	0.12	1.10
Vitamin D	23	0.31	0.01	0.10	22	0.46	0.01	0.05	0.07	0.03	0.01	1.21
Ca + Vit. D	23	0.35	0.01	0.86	21	0.44	0.01	0.30	0.01	0.03	0.64	1.03
B. β-catenin												
Whole crypts												
Placebo	21	0.99	0.04	.	18	0.98	0.04	.	0.00	.	.	1.00
Calcium	22	1.01	0.04	0.69	19	0.85	0.05	0.04	−0.15	0.08	0.08	0.85
Vitamin D	21	0.94	0.04	0.45	21	0.82	0.04	0.03	−0.11	0.08	0.18	0.88
Ca + Vit. D	22	1.04	0.04	0.38	17	0.92	0.05	0.37	−0.11	0.08	0.20	0.89

Treatment Group	Baseline				6-mo follow-up				Absolute Rx effect			Relative Effect[d]
	n	Mean	Std Err	P	n	Mean	Std Err	P	Rx effect[b]	Std Err	P[c]	
Upper 40% of crypts												
Placebo	21	0.38	0.02	.	18	0.37	0.02	.	0.00	.	.	1.00
Calcium	22	0.40	0.02	0.52	19	0.33	0.02	0.07	−0.06	0.03	0.06	0.85
Vitamin D	21	0.36	0.02	0.36	21	0.33	0.02	0.12	−0.02	0.03	0.61	0.95
Ca + Vit. D	22	0.39	0.02	0.69	17	0.36	0.02	0.61	−0.02	0.03	0.50	0.94
Lower 60% of crypts												
Placebo	21	0.61	0.02	.	18	0.62	0.03	.	0.00	.	.	1.00
Calcium	22	0.63	0.02	0.44	19	0.56	0.02	0.11	−0.08	0.05	0.09	0.87
Vitamin D	21	0.58	0.02	0.38	21	0.54	0.02	0.02	−0.05	0.05	0.27	0.91
Ca + Vit. D	22	0.63	0.02	0.58	17	0.59	0.03	0.46	−0.05	0.05	0.36	0.93
ϕh [e]												
Placebo	21	0.39	0.01	.	18	0.38	0.01	.	0.00	.	.	1.00
Calcium	22	0.38	0.01	0.87	19	0.36	0.01	0.16	−0.01	0.01	0.31	0.97
Vitamin D	21	0.39	0.01	0.94	21	0.38	0.01	0.81	0.00	0.01	0.80	1.01
Ca + Vit. D	22	0.38	0.01	0.88	17	0.37	0.01	0.67	0.00	0.01	0.81	0.99
C. E-cadherin												
Whole crypts												
Placebo	21	1.13	0.12	.	19	1.04	0.13	.	0.00	.	.	1.00
Calcium	21	1.23	0.12	0.69	21	1.09	0.12	0.78	−0.05	0.25	0.85	0.96
Vitamin D	21	0.82	0.12	0.05	18	1.29	0.13	0.17	0.56	0.25	0.03	1.72
Ca + Vit. D	22	0.91	0.12	0.14	19	1.26	0.13	0.23	0.46	0.25	0.08	1.51
Upper 40% of crypts												
Placebo	21	0.44	0.05	.	19	0.42	0.05	.	0.00	.	.	1.00
Calcium	21	0.47	0.05	0.70	21	0.45	0.05	0.70	0.00	0.10	0.99	1.01
Vitamin D	21	0.33	0.05	0.09	18	0.56	0.05	0.06	0.25	0.10	0.02	1.78
Ca + Vit. D	22	0.37	0.05	0.26	19	0.55	0.05	0.09	0.20	0.10	0.05	1.56
Lower 60% of crypts												
Placebo	21	0.64	0.07	.	19	0.60	0.08	.	0.00	.	.	1.00
Calcium	21	0.69	0.07	0.68	21	0.63	0.07	0.78	−0.01	0.15	0.93	0.98
Vitamin D	21	0.48	0.07	0.12	18	0.75	0.08	0.19	0.31	0.16	0.05	1.68

Treatment Group	n	Baseline			6-mo follow-up				Absolute Rx effect			Relative Effect[d]
		Mean	Std Err	P	n	Mean	Std Err	P	Rx effect[b]	Std Err	P[c]	
Ca + Vit. D	22	0.55	0.07	0.38	19	0.70	0.08	0.39	0.19	0.15	0.23	1.35
φh [e]												
Placebo	21	0.41	0.02		19	0.40	0.02		0.00	.	.	1.00
Calcium	21	0.41	0.02	0.92	21	0.41	0.02	0.66	0.01	0.03	0.71	1.03
Vitamin D	21	0.40	0.02	0.61	18	0.45	0.02	0.06	0.05	0.03	0.10	1.14
Ca + Vit. D	22	0.40	0.02	0.60	19	0.46	0.02	0.01	0.07	0.03	0.03	1.18
D. APC/β-catenin Score												
Placebo	21	0.37	0.03		18	0.44	0.03		0.00	.	.	1.00
Calcium	22	0.35	0.03	0.49	18	0.58	0.03	0.003	0.16	0.06	0.01	1.41
Vitamin D	21	0.36	0.03	0.83	20	0.57	0.03	0.004	0.13	0.06	0.02	1.31
Ca + Vit. D	22	0.37	0.03	0.84	17	0.50	0.03	0.18	0.07	0.06	0.26	1.16

[a] APC/β-catenin score = φh APC/β-catenin expression in the whole crypt, where φh APC = ratio of APC expression in the upper 40% of the crypts to the whole crypt.

[b] Rx effect (treatment effect) = [(treatment group follow-up) – (treatment group baseline)] – [(placebo group follow-up) – (placebo group baseline)].

[c] P value for difference between each active treatment group and placebo group from repeated-measures MIXED model.

[d] 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).

[e] φh = proportion of expression in the distribution zone (i.e. ratio of expression in upper 40% to expression in whole crypt)