Rectal Mucosal Proliferation and Risk of Colorectal Adenomas: Results from a Randomized Controlled Trial[1]

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Abstract

Although rectal mucosal labeling index is thought to be a useful surrogate biomarker for colorectal cancer, the ability of the index to predict future neoplasia is unclear. We obtained rectal mucosal biopsies from 333 participants of a randomized controlled chemoprevention trial of calcium supplementation to determine whether labeling index was correlated with concurrent or future colorectal neoplasms. Labeling index was measured using proliferating cell nuclear antigen immunohistochemistry. Adenomas were enumerated at the time of the biopsies (cross-sectional) and 3 years later (prospective). We used logistic regression to test for an association of adenoma occurrence with overall labeling index, the mean proliferative height, and labeling index in the upper 40% of colon crypts. In the cross-sectional analysis, we found indications that higher proliferation was associated with an increase in the prevalence of adenomas. The overall adjusted odds ratios (OR) (95% confidence interval) were 1.14 (0.90–1.45) per % crypt labeling index, OR 1.08 (0.99–1.19) for upper crypt proliferation, and OR 1.07 (1.03–1.12) for proliferative height. In contrast, individuals with higher labeling index at baseline were actually less likely to have adenomas in the prospective analyses: OR 0.80 (0.62–1.02) per % crypt labeling index, OR 0.86 (0.73–1.00) for upper crypt index, and OR 0.97 (0.93–1.01) for proliferative height. Proliferative index does not predict future colorectal neoplasia, although it may be weakly associated with the presence of current adenomas. These results have important implications for the design of future intervention studies. Although it may be attractive to include the measurement of intermediate markers in large controlled trials, until we have more confidence in their performance, we should rely on better proven and more reliable intermediates, such as adenomas.

Introduction

Colorectal cancer is thought to arise through a series of putative intermediate stages that can serve as surrogate biological markers for colorectal cancer risk. Because it may be possible to rapidly modify these intermediates, they are attractive for use as end points in colorectal cancer prevention studies.

Rectal mucosal proliferation has been extensively studied as a colorectal cancer risk biomarker both in laboratory animals and in humans. Numerous studies have demonstrated elevated proliferation in the normal-appearing mucosa of high-risk individuals, such as those with ulcerative colitis, family history of colorectal cancer, polyposis, sporadic adenomas, and large bowel cancer (1). Almost all of these studies have had a cross-sectional design, however, and the ability of these proliferation measures to predict future adenomas is not clear. Chemoprevention studies of calcium using proliferation markers have reached equivocal results (2). The discrepant results from these trials may have resulted, in part, from methodological differences in study design and execution. Moreover, measurement of proliferation is technically demanding. There are many sources of variation (within and between individuals) (3, 4). The size of the within-person variation may be nearly as large as between-person variation, making it difficult to separate signal from noise (5).

As part of a large, randomized, placebo-controlled study of calcium in the prevention of neoplastic polyps of the large bowel, we obtained rectal mucosal biopsies and calculated rectal mucosal proliferation index in 330 patients. Each of the study subjects underwent a careful colonoscopic examination at the conclusion of the study, 3 years after the rectal biopsies. We hypothesized that if individuals with higher proliferation indices were at increased risk for colorectal cancer and its precursors, we would find a direct association between proliferation index and adenoma risk. The purpose of this study was to test this hypothesis by examining the association between proliferation and adenomas at two time points, adenomas at the same time as the biopsies and adenomas in the 3 years after the proliferation measurement.

Materials and Methods

The methods have been described in detail elsewhere (6). The biopsy specimens for rectal mucosal proliferation in this study were obtained during a randomized, controlled, double-blind, placebo-controlled trial of calcium carbonate to prevent recurrent colorectal adenomas (7). The study was conducted in six medical centers and associated gastroenterology practices: the Cleveland Clinic; Dartmouth Hitchcock Medical Center; the University of California Los Angeles/Kaiser Hospital; the University of Iowa; the University of Minnesota; and the Univer-

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sity of North Carolina. Proliferation measurements were performed at the M. D. Anderson Cancer Center (Houston, TX). Institutional review boards at each of the participating medical centers approved the study.

Study participants had at least one adenomatous polyp of the large bowel removed endoscopically within 3 months of study entry and had all known polyps removed prior to enrollment. A total of 1118 patients were recruited, and 930 were randomized to either 3000 mg of calcium carbonate (1200 mg of elemental calcium) each day or identical-appearing placebo. Follow-up endoscopy occurred 1 year and 4 years after the qualifying exam.

Rectal biopsy specimens were obtained from consenting subjects during a colonoscopy scheduled 1 year after the qualifying colonoscopy. A total of 468 patients had year 1 colonoscopies at locations where biopsies could be processed, and of these, 401 were asked to participate in the rectal mucosal proliferation study. Only 39 (10%) refused; other problems prevented adequate biopsy specimens in 7 (2%). There were 22 (5%) who did not have an exit colonoscopy and therefore were not included in these analyses. Specimens from the 333 subjects (173 calcium treatment group and 160 placebo group) were processed according to a standard protocol for labeling by antibodies to PCNA[3] (8). Subjects were fasting for at least 8 h. Bowel preparation was performed using polyethylene glycol lavage in all subjects. One subject also had a tap water enema, one a saline enema, and one had Dulcolax pills in addition to polyethylene glycol. There were 46 subjects who also were given magnesium citrate, which did not have a significant impact on proliferation measures. The endoscopist obtained two rectal biopsies 8–10 cm from the anal verge at the start of the procedure. The specimens were flattened then immediately fixed in 70% alcohol and then shipped in batches to the laboratory center for proliferation scoring. Carefully oriented paraffin-embedded specimens were cut into 4-μm sections. To maximize antigenicity, the sections were prepared at 50–54°C, and serial sections were fixed to poly-L-lysine-coated slides prior to immunohistochemistry. Anti-PCNA (PC-10 clone; Signet Laboratories, Inc., Dedham, MA) monoclonal antibodies were used to detect proliferating cells. The Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA) was used for staining, and diluted hematoxylin (Fisher Scientific Co., Pittsburgh, PA) was used as a counterstain.

Strict rules were used to score the specimens. One technician, blind to treatment status, scored all of the specimens. The technician counted all well-oriented, U-shaped crypts that were open from the apical lumen to the base. A well-shaped crypt that did not contact the muscularis mucosa was acceptable if its height was similar to neighboring crypts; otherwise, we required that the crypt base contact the muscularis mucosa. Only the deepest staining or the next lighter shade of staining in a homogeneous nuclear pattern was scored as positive. The technician counted the number of cells in each half of the U-shaped crypt and recorded the number and position of each labeled cell. In a validation study, repeated PCNA scoring was performed on 20 patients by the study technician; the intraclass correlation was 0.40 for the overall labeling index (8)

The LI was calculated by dividing the number of labeled cells by the total number of crypt cells in scorable crypts that had at least one labeled cell or a crypt that was surrounded by crypts with at least one labeled cell. The crypt LIs were averaged to produce a patient average LI. In addition, we determined the proportion of labeled cells in the upper (luminal) 40% of the crypt (ϕ_{40}) and the PH, which is the mean position (in %) of labeled cells in the crypt, ordered from base to lumen.

[3] The abbreviations used are: PCNA, proliferating cell nuclear antigen; CI, confidence interval; OR, odds ratio; LI, labeling index; PH, proliferative height.

Table 1 Characteristics of study subjects

No. of patients	333
Mean age at randomization (± SD)	60.8 ± 9.4
% male	72.7
Mean no. of prior adenomas	2.18 ± 1.90
Mean no. of adenomas at year 1 exam (± SD)	0.42 ± 1.02
Mean no. of adenomas between year 1 and year 4 exams (± SD)[a]	0.56 ±1.12

[a] After year 1 exam up to and including the year 4 exam.

All scorable crypts were included in the analysis. ORs for adenoma recurrence (and 95% CIs) were calculated using logistic regression modeling in SAS (SAS Institute, Cary, NC). Age, race, sex, institution, and treatment group were included as covariates in these models. We examined trends by computing ORs per unit index. Because all subjects received polyethylene glycol preparation and because the preparation method did not have a significant impact on proliferation measures, we did not include type of bowel prep in the model. Analyses focused on two sets of relationships. In common with most prior studies, we investigated the relationship between the presence/absence of adenomas at the year 1 endoscopy and the proliferation measures from biopsies taken at that exam. We refer to these as cross-sectional analyses. We also conducted a prospective analysis investigating whether the proliferative measures at the year 1 exam predicted the occurrence of adenomas during the subsequent 3 years (*i.e.,* up to and including the year 4 exam).

To formally compare the adjusted relative risk coefficients for the baseline LIs as predictors of adenoma occurrence at years 1 and 4, longitudinal regression models (generalized estimating equations) were fit simultaneously, estimating the two coefficients. The coefficients were then compared with appropriate adjustment for the fact that they were fit using repeated observations on the same individuals.

Results

The characteristics of participants are shown in Table 1. There were 173 subjects who were randomized to calcium and 160 to placebo. Seventy % of the subjects were men, and the mean age of subjects was 60.8 years. There were fewer adenomas in the calcium group both at the first (year 1) colonoscopy and subsequently (up to and including the year 4 exam).

The mean LI for the 333 subjects was 3.88 (1.08 SD). Table 2 presents the adjusted ORs for adenoma development at the first follow-up examination, when the biopsies for proliferation measurement were obtained. If individuals with increased proliferation index were at higher risk for adenomas, we would expect to see an increased OR in the cross-sectional analysis. The ORs for the overall labeling index were modestly elevated, although the trend of risk with increasing proliferation was inconsistent, and the individual quartile estimates were not statistically significant. The adjusted ORs per % LI was 1.14 (95% CI, 0.90–1.45). The crude results were nearly identical to the adjusted ORs and are not presented in the table.

Because proliferation close to the lumen may better reflect risk for neoplasia (9), we also calculated the LI in the top 40% of the crypt, the ϕ_{40}, and PH. The mean ϕ_{40} among the 333 study subjects was 0.98% (2.57 SD). As shown in Table 2, subjects with $\phi_{40} > 0$ had a higher prevalence of adenomas than those with a $\phi_{40} = 0$, and the overall adjusted OR per % ϕ_{40} was increased with borderline statistical significance, OR

Table 2 Crude and adjusted ORs for adenomas by overall LI, ϕ_{40}, and PH in cross-sectional and prospective analyses in 333 study subjects

LI	Cross-sectional[a] adjusted OR[b] (95% CI)	Prospective[c] adjusted OR[b] (95% CI)	P^d
Whole-crypt LI			
Quartile 1	1.00	1.00	
Quartile 2	1.39 (0.66–2.90)	0.56 (0.28–1.13)	
Quartile 3	0.80 (0.36–1.82)	0.40 (0.19–0.84)	
Quartile 4	1.29 (0.61–2.71)	0.60 (0.30–1.20)	
Overall OR % LI	1.14 (0.90–1.45)	0.80 (0.62–1.02)	0.043
Upper crypt proliferation			
$\phi_{40} = 0$	1.00	1.00	
$\phi_{40} > 0$[e]	1.52 (0.84–2.74)	0.53 (0.28–1.00)	
Overall OR % ϕ_{40}	1.08 (0.99–1.19)	0.86 (0.73–1.00)	0.0003
PH			
Quartile 1	1.00	1.00	
Quartile 2	1.95 (0.85–4.47)	0.97 (0.48–1.95)	
Quartile 3	1.42 (0.61–3.32)	0.43 (0.20–0.89)	
Quartile 4	3.03 (1.38–6.64)	0.61 (0.29–1.26)	
OR per % crypt height	1.07 (1.03–1.12)	0.97 (0.93–1.01)	0.0002

[a] Biopsies and adenomas evaluated at the time of the year 1 colonoscopy.
[b] OR (95% CI) adjusted for treatment group, age, sex, institution, and time between exams.
[c] Proliferation measured at year 1 and adenomas evaluated 3 years later (year 4).
[d] General estimating equation-derived *P* for difference between cross-sectional and prospective ORs (see text for details).
[e] Compared to $\phi_{40} = 0$.

1.08 (95% CI, 0.99–1.19). Crude and adjusted ORs were similar. Results for PH were similar to the ϕ_{40}, with a modest increase in PH in the cross-sectional analysis, adjusted OR 1.07 (95% CI, 1.03–1.12). The mean PH in the study population was 6.21% (0.1 SD).

If proliferation index were predictive of adenoma risk, one might further hypothesize that a higher index at the first follow-up exam would be associated with the development of adenomas in the subsequent 3 years. However, as shown in Table 2, there was an inverse association of proliferation with subsequent adenoma risk. LIs in the second through fourth quartile were associated with a lower the risk of adenoma recurrence; the higher the LI, the lower the risk of subsequent adenomas (adjusted OR per % LI, 0.80; 95% CI, 0.62–1.02). For proliferation in the upper crypt, individuals with a $\phi_{40} > 0$ had a decreased risk of adenoma on follow-up (adjusted OR, 0.53; 95% CI, 0.28–1.00). The OR per % ϕ_{40} was 0.86 (95% CI, 0.73–1.00). PH showed similar findings with a borderline significant decreased risk of subsequent adenoma (Table 2). The ORs in the cross-sectional analysis were statistically different from those computed in the prospective analysis, as shown in Table 2.

To eliminate possible interaction between treatment and the ability of proliferation index to predict adenomas, we conducted a separate analysis in the placebo group. The results were similar to the overall analyses. The ORs in the cross-sectional analyses suggested increased risks. The adjusted ORs in the placebo group were 1.34 per % (95% CI, 0.92–1.96) for overall LI, 1.14 per % (95% CI, 0.97–1.35) for ϕ_{40}, and 1.06 per % (95% CI, 0.99–1.14) for PH. The adjusted ORs in the placebo group in the prospective analyses were decreased with borderline significance: 0.83 (95% CI, 0.59–1.18) overall LI, 0.87 (95% CI, 0.69–1.11) for ϕ_{40}, and 0.99 (95% CI, 0.92–1.05) for PH.

In contrast to proliferative measures, the presence of an adenoma at year 1 was a strong predictor of subsequent adenomas at year 4 (OR, 3.27; 95% CI, 1.84–5.82). This was true for the overall study and for the proliferation subset.

Discussion

In this randomized controlled clinical trial, there were suggestions that increasing proliferation was associated with an increased prevalence of adenomas in the bowel at the time the biopsies were taken. Contrary to expectation, however, higher proliferation was associated with a decreased risk of adenomas in the subsequent 3 years. The results were unchanged when we controlled for treatment group and other potentially confounding factors.

Rectal mucosal proliferation indices have attractive features for intervention studies. The LI has been shown to be increased in individuals who have a higher risk for cancer. For example, the labeling index has been found to increase with age (10–12) and to be higher in individuals with a family history of cancer (13, 14) and previous adenomas and cancer (15–17). The index seems to be lower in vegetarians, a group at lower risk of colorectal cancer (18). Medical conditions that are known to confer higher risk of cancer, such as familial polyposis (19) and ulcerative colitis (20), have been associated with higher LI.

Intervention studies to prevent colorectal cancer are complicated by the fact that it may take decades for colorectal cancer to develop. Using an intermediate biomarker, such as rectal mucosal proliferation, as an end point could substantially shorten an intervention trial. Previous studies have shown changes in LI within 1 month of an intervention (21), although small studies showed no effects after 5 days (22) and 2 weeks (23).

In a prospective study, Anti *et al.* (24) found that the baseline mean labeling index in upper crypt compartments was higher in 22 patients who developed recurrent adenomas after 24 months compared with 33 patients without recurrence. They concluded that an upward shift of the replicative compartment was associated with polyp recurrence. Bostick *et al.* (25) performed a cross-sectional analysis using baseline rectal mucosal biopsies in individuals participating in a cancer chemoprevention study similar to ours. They found lower proliferation index for individuals with high vegetable and fruit consumption and vitamin consumption.

How can we explain the lack of association between proliferation index and adenomas both cross-sectionally and after 3 years? Our study was restricted to individuals with previous adenomas. Several studies have shown that individuals with adenomas have a higher proliferation index (25). If the index were uniformly high in our subjects, we might not be able to discriminate those destined to develop adenomas. Although it is problematic to make comparisons of LI data between labs because of differences in techniques and scoring, it is relevant to note that the overall LI in our subjects was similar to that reported by McShane *et al.* (5). Bostick *et al.* (25) studied a similar population of patients with previous adenomas and found an association between calcium supplementation and proliferation index. Moreover, we found a difference in adenoma development between the calcium and placebo group, suggesting that there was sufficient diversity in the study population to detect a difference using another biomarker (adenomas). Another potential problem is that the intraclass correlation coefficient in our study (0.4) was lower than in some other reported studies (4). However, the lack of precision in our measurements could not possibly explain an inverse association between proliferation and adenomas in the prospective analysis, a direct association in the cross-sectional analysis, and a statistically significant difference between the two.

Our study was a multicenter study, and conducting the study in several locations could potentially have introduced variation. However, study site was not a significant predictor of proliferation

indices, and the proliferation measures were performed in a single laboratory with experienced personnel. Not all subjects had the same bowel preparation for their colonoscopy, although the majority used a balanced electrolyte lavage. Fireman *et al.* (26) found no difference in proliferative measures in patients prepared with a polyethylene glycol-electrolyte solution or extract of senna purgative. Although all of our biopsies were taken from the rectum, others have shown that the LI in the rectum is similar to that in the remainder of the large bowel (9, 27). Biopsies were taken at different times of day. In a large study by McShane *et al.* (5), the hour of biopsy did not influence the mucosal proliferation index. We used PCNA immunohistochemistry as our proliferation measure. Some authors have suggested that PCNA expression is not a sensitive marker of neoplasia risk in intestinal mucosa (28). Finally, despite various limitations, our study found an increased proliferation index among adenoma patients at the year 1 colonoscopy, as predicted. The fact that we found a direct association between proliferation index and adenomas in the cross-sectional analysis but an inverse association at follow-up cannot be attributed to systematic bias or lack of precision of the measurements.

There are several notable strengths of our study. Our study was very large. We measured proliferation in over 300 study subjects and carefully followed them for 3 years after their biopsies. The large size of the study increased our ability to detect small differences. The fact that we found no differences makes it unlikely that there is a strong association between proliferation index in rectal biopsies measured by PCNA immunohistochemistry in adenoma patients and the subsequent development of adenomas. The proliferation measurements were conducted in a laboratory with personnel of considerable experience, and we used a detailed protocol for processing specimens and counting labeled cells. The statistical analysis controlled for potentially confounding factors.

In conclusion, although on cross-sectional analysis we found indications of the expected association between proliferation and adenoma risk, on prospective analyses an inverse association emerged. These results have important implications for the design of future intervention studies. Measurement of rectal mucosal proliferation is tedious, expensive, and unreliable. Although it may be attractive to include the measurement of intermediate markers in large controlled trials, until we have more confidence in their performance, we should rely on better proven and more reliable intermediates, such as adenomas.

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