Reliability of Whole Crypt Mitotic Count as a Measure of Cellular Proliferation in Rectal Biopsies[1]

Tor D. Tosteson, Margaret R. Karagas, Richard Rothstein, Dennis J. Ahnen, and E. Robert Greenberg[2]

Norris Cotton Cancer Center and the Departments of Community and Family Medicine [T. D. T., M. R. K., E. R. G.] and Medicine [R. R.], Dartmouth Medical School, Hanover, New Hampshire 03755, and Department of Medicine and Denver Department of Veterans Affairs Medical Center, University of Colorado School of Medicine, Denver, Colorado 80220 [D. J. A.]

Abstract

We conducted a reliability study of whole crypt mitotic count, a measure of cellular proliferation with potential use as an intermediate marker in studies of colorectal cancer risk and prevention. The study involved biopsies taken from two distinct locations at 8–10 cm from the anal verge for 20 subjects scheduled to undergo routine endoscopy. In addition to the overall count of mitoses per crypt (mitotic count), we investigated two novel measures based on the percentages of heights of mitotic cells within crypts: the mean height, and the maximum minus minimum (max − min) height of mitoses. The max − min height was positively correlated with mitotic count ($r = 0.64$); however, there was little correlation between mitotic count and the mean height of mitotic cells ($r = 0.12$). Components of variance were estimated for the three measures; for mitotic count and max − min height, the variability between persons was substantially greater than that between locations within an individual. For mean height, the between-person and between-location variabilities were roughly equal. These results suggest that whole crypt mitotic count has promise as a reliable measure of rectal cellular proliferation, but further studies will be necessary to assess the utility of this assay.

Introduction

Several assays have been developed to measure cellular proliferation in the crypts of the normal mucosa lining the large bowel, and these assays are currently in use as markers of the risk or response to preventive interventions in human studies of colon and rectal cancer (1–3). Epidemiological studies demonstrating higher proliferation indices in association with colon cancer or adenoma and laboratory carcinogenesis experiments provide some rationale for using these markers as surrogates for neoplastic events (4–6), although there is still some disagreement as to whether increased cellular proliferation is a consistent early step in cancer development (7). The proliferation assays most commonly used in human studies involve biopsies from the rectum. Cells are labeled (either after incubation or following immediate fixation) using reagents such as tritiated thymidine or BrdUrd,[3] which specifically identify cells in the S-phase, or by immunostaining of proliferating cell nuclear antigen, an endogenous protein that is initially expressed in late G_1 and the S-phase. The assays are scored in terms of the proportion of proliferating cells (the labeling index) and their position in the crypts of the mucosa.

An alternative approach to measuring colorectal cellular proliferation is to dissect the crypts and count directly the number and position of mitoses, a technique termed WCMC (8). This technique has several practical advantages over other assays, but the reliability of WCMC has been examined in only one published study, which focused on variation between readers (9). We conducted a study that assessed some other possible sources of variation in the WCMC assay, and we also explored the reliability of two novel measures of proliferation location using WCMC.

Materials and Methods

The study included 20 patients, between the ages of 40 and 79 years, who were scheduled to undergo sigmoidoscopy or colonoscopy. Reasons for endoscopy included: symptoms of possible large-bowel disease; positive hemoccult test; prior history of polyps; family history of colorectal cancer; and a healthy screening examination. No phosphate-containing enemas were used to prepare patients for endoscopy.

During endoscopic entry into the rectum, biopsies were taken from two sites, both located 8–10 cm from the anal verge. Tissue specimens were fixed in Carnoy's fixative (60% ethanol, 30% chloroform, and 5% glacial acetic acid) for 4 h at room temperature, then transferred to 70% ethanol and stored at 4°C until shipped to the laboratory of one of us (D. J. A.). The tissue was then rehydrated in 50 and 25% ethanol (10 min each), hydrolyzed in hydrochloric acid at 60°C for 10 min, and placed in freshly made Schiff's reagent for 45–60 min. The biopsy was then transferred to a drop of 45% acetic acid on a microscope slide; with the aid of a dissecting microscope, the intact crypts were dissected from the biopsy using a 30-gauge hypodermic or microdissection needle. Individual or small clusters of crypts were teased away from the biopsy, covered with a coverslip and

Received 12/3/94; revised 1/23/96; accepted 3/1/96.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was funded by the National Dairy Promotion and Research Board in cooperation with the National Dairy Council, National Cancer Institute Grants CA-23108 and CA-61108, American Cancer Society Grant ACS SIG-17, and Department of Veterans Affairs Merit Review Grant 1312. D. J. A. is the recipient of a Department of Veterans Affairs Clinical Investigator Award. Presented at a special invited session at the Joint Statistical Meetings, Toronto, Ontario, Canada, August 1994.

[2] To whom requests for reprints should be addressed, at Norris Cotton Cancer Center, One Medical Center Drive, Lebanon, NH 03756.

[3] The abbreviations used are: BrdUrd, bromodeoxyuridine; WCMC, whole crypt mitotic count; max − min, maximum minus minimum; CI, confidence interval.

Downloaded from cebp.aacrjournals.org on October 25, 2021. © 1996 American Association for Cancer Research.

Table 1 Summary of WCMC measures[a]

	Mean	Median	SD
Overall MC	9.79	9.00	5.55
MC in compartment 1	3.54	3.00	2.68
MC in compartment 2	4.20	4.00	2.86
MC in compartment 3	1.67	1.00	1.84
MC in compartment 4	0.23	0.00	0.66
MC in compartment 5	0.01	0.00	0.16
Mean height (%)	27.4	27.0	7.2
Max − min height (%)	34.3	35.0	15.2

[a] Based on 1172 counted crypts (1171 crypts for mean height and max − min height); mean number of counted crypts per biopsy, 29.7; SD, 2.1.

Table 2 Variance components for WCMC: Estimate of component SD (95% CI)

Between	Individuals	Locations	Crypts
Overall MC	3.3 (2.1, 4.5)	1.8 (1.1, 2.4)	4.2 (4.0, 4.3)
Mean height (%)	2.3 (1.1, 3.6)	2.3 (1.4, 3.2)	6.4 (6.1, 6.7)
Max − min height (%)	7.1 (4.3, 10.0)	4.6 (2.8, 6.4)	12.7 (12.1, 13.2)

Table 3 Correlation between alternative proliferation measures: Spearman correlation coefficient (*P* value)

	Mean height (%)	Max − min height (%)
Overall MC	0.12 (0.0001)	0.64 (0.0001)
Mean height (%)		0.41 (0.0001)

examined under ×400 magnification light microscopy. Mitoses were identified as dark magenta-stained bodies with no apparent surrounding nuclear membrane; they could readily be distinguished from the pink-stained nuclei. The number of mitoses within the entire crypt was counted. The outline of each crypt was drawn with the aid of a drawing tube attached to the microscope, and the position of each mitosis within the crypt was recorded within the outline. Thus, both the total number of mitoses per crypt, a reflection of proliferation rates, and the spatial distribution of mitoses within the crypt were recorded.

For the validation study, we assessed measures of both overall and compartmental proliferation. Unlike other assays of colorectal cell proliferation, the WCMC assay does not involve counting nonlabeled cells. Therefore, we used total crypt and compartment-specific mitotic counts rather than the actual labeling indices in our analyses. Because we were interested in characterizing the variability between crypts, we defined each index on a crypt-by-crypt basis. We treated the mean height of each mitosis, expressed as a proportion of the total crypt height, as a direct measure of the location of mitotic cells. We also computed the range of heights of the mitoses in a crypt, *i.e.*, the max − min percentage height, as a measure of the width of the proliferative zone.

The primary goals of the statistical analysis were: (*a*) to summarize and compare alternative measures of proliferation in the WCMC assay; and (*b*) to estimate the sources of variability for each measure of proliferation between persons and between locations within persons. For each of these goals, we took the primary data to be the crypt-specific measures of mitotic count, mean height, and max − min height. Summary statistics and correlation coefficients were calculated using these measures. A nested random-effects ANOVA was performed for each proliferation measure to estimate variance components using the restricted maximum likelihood method implemented in the SAS procedure PROC MIXED (10). Approximate CIs were derived based on the asymptotic SDs for the variance components from the maximum likelihood estimation procedure.

Results

We obtained an average of 29.7 crypts per biopsy (the goal was 30) for our assays of WCMC. The average number of mitoses per crypt was 9.79, and all but one of the 1172 counted crypts contained at least 1 mitosis (Table 1). Mitoses were predominantly distributed in the bottom two compartments (lower 40%) of the crypts. The mean height of mitotic cells was 27% up the crypt, and the proliferative zone (max − min height) extended over 34% of the crypt length.

Random-effects ANOVA permits estimation of variance components for normal data. Based on histograms (not shown) of the three proliferation measures used in this study, the normality assumptions were reasonable for our WCMC data, and the means for each measure were roughly equal to the medians (Table 1). The estimated random effects are shown in Table 2, in terms of component SDs and 95% CIs. For all measures, there was substantial between-person variability beyond that explained by the between-location and between-crypt components of variance. Spearman correlation coefficients for the three measures were all positive and statistically significant, although there was a relatively low correlation between mitotic count and mean height (Table 3).

Discussion

Proliferation assays are relatively labor-intensive procedures, due to the need for counting and locating many cells in each biopsy. The proliferating cell nuclear antigen BrdUrd and tritiated thymidine assays necessitate proper orientation of biopsy specimens to permit visualization of entire crypt columns. Often, relatively few crypts per biopsy are appropriately oriented, resulting in imprecision of the estimated labeling indices (2, 11). In addition, BrdUrd and tritiated thymidine assays involve incubation of biopsies to ensure that proliferating cells can take up the label. WCMC eliminates these requirements for incubation and specimen orientation and provides greater numbers of countable crypts per biopsy. In our study, we had no difficulty obtaining data for 30 crypts per biopsy, and many more crypts could have been counted if necessary. Thus, considerably more crypts can be evaluated using WCMC than are usually available for analyses using assays that require specimen orientation. However, WCMC only measures cells in mitosis, a narrow segment of the cell cycle, and WCMC results thus may not correlate closely with the results of other proliferation assays that indicate cells in the S-phase, the period of DNA synthesis (9). The underlying rationale for the WCMC assay, however, is that the number of cells in mitosis at any given time should reflect the proliferative rate of the tissue and, therefore, should be proportional to percentage of cells in the S-phase. The mitotic index in tissues has been used extensively to characterize the rate of tumor cell proliferation, and the WCMC correlates well with tritiated thymidine labeling in animal studies (8).

Analysis of the results of other proliferation assays has focused on the labeling index and the distribution of labeling. The labeling index is usually defined as the number of labeled cells divided by the total number of cells in the crypts. Labeling distribution may be defined in terms of the proportion of labeled cells in compartments based on the percentage height

Downloaded from cebp.aacrjournals.org on October 25, 2021. © 1996 American Association for Cancer Research.

from the base of a crypt. An example is the ϕ_h statistic (12), *i.e.*, the proportion of the labeled cells that are located in the upper 40% of the crypt. Compartment-specific labeling indices are variations on this theme, usually with five compartments defined by the quintiles of cell heights. An alternative to the traditional labeling distribution statistics involves fitting smoothed distributions to compartmental indices (13).

The mean of the percentage heights of labeled cells and the max − min heights are novel and practical methods for summarizing the location distribution of labeled (or mitotic) cells in proliferation assays. A possible advantage of the mean height as a proliferation measure is that unlike compartmental labeling indices and measures such as ϕ_h, mean height summarizes all available information on the relative position of cells within the crypt. In our analysis of WCMC data, the high correlation between mitotic count and max − min height suggests that a potentially time-saving scoring procedure might consist of physically measuring the crypt height and the heights of the highest and lowest mitoses in a crypt. The relatively low correlation we observed between mitotic count and mean height, however, suggests that the location distribution may be an independent dimension of proliferation.

Our finding of a significant between-location component of variance is similar to prior reports involving other proliferation markers (14, 15). However, the CIs for the variance components in our analyses were relatively wide, reflecting the low power of a study with only 20 people. Also, we could not separate the effects of true biological variability in proliferation in adjacent tissues from possible differences in the reading of biopsy specimens as contributors to this component of variance. However, the one report that compared the results obtained from two readers of a single WCMC specimen noted close agreement (9). Another source of variability not assessed in the report could come from changes in proliferation rates over time. Regardless of the source of variability, sampling schemes that involve multiple biopsies should be given careful consideration as a way to improve precision when designing prevention studies.

WCMC shows promise as a reproducible measure of cellular proliferation. However, the relation of WCMC to risk of colorectal neoplasia in humans has not been characterized. Further studies should be helpful in determining the utility of this assay as an intermediate marker for cancer studies.

Acknowledgments

We are grateful for the assistance of Julia Weiss, Leila Mott, and Kim Seaver (Dartmouth-Hitchcock Medical Center) and Linda Drigers (Veterans Administration Medical Center).

References

1. Lipkin, M., Bhandari, M., Hakissian, M., Croll, W., and Wong, G. Surrogate endpoint biomarker assay in Phase II chemoprevention clinical trials. J. Cell. Biochem., *19:* 47-54, 1994.

2. Rozen, P. An evaluation of rectal epithelial proliferation measurement as biomarker of risk for colorectal neoplasia and response in intervention studies. Eur. J. Cancer Prev., *1:* 215-224, 1992.

3. Greenwald, P., Kelloff, G. J., Boone, C. W., and McDonald, S. S. Genetic and cellular changes in colorectal cancer: proposed targets of chemopreventive agents. Cancer Epidemiol, Biomarkers & Prev., *4:* 691-702, 1995.

4. Lipkin, M. Biomarkers of increased susceptibility to gastrointestinal cancer: new application to studies of cancer prevention in human subjects. Cancer Res., *48:* 235-245, 1988.

5. Cohen, S. M., and Ellwein, L. B. Genetic errors, cell proliferation, and carcinogenesis. Cancer Res., *51:* 6493-6505, 1991.

6. Preston-Martin, S., Pike, M. C., Ross, R. K., Jones, P. A., and Henderson, B. E. Increased cell division as a cause of human cancer. Cancer Res., *50:* 7415-7421, 1990.

7. Farber, E. Cell proliferation as a major risk factor for cancer: a concept of doubtful validity. Cancer Res., *55:* 3759-3762, 1995.

8. Pohl, A., Weyant, J., and Ahnen, D. J. Validation of the whole crypt mitotic count (WCMC) as a measure of proliferation in the rat colon (Abstract). Gastroenterology, *102:* A939, 1992.

9. Murray, S. C., Sandler, R. S., Keku, T. O., Lyles, C. M., Millikan, R. C., Bangdiwala, S. I., Kupper, L. L., Jiang, W., and Ulshen, M. H. Comparison of rectal mucosal proliferation measured by proliferating cell nuclear antigen (PCNA) immunohistochemistry and whole crypt dissection. Cancer Epidemiol, Biomarkers & Prev., *4:* 715-720, 1995.

10. SAS Institute, Inc. SAS User's Guide. Basic. Cary, NC: SAS Institute, Inc., 1990.

11. Baron, J. A., Wargovich, M. J., Tosteson, T. D., Sandler, R., Haile, R., Summers, R., van Stolk, R., Rothstein, R., and Weiss, J. Epidemiological use of rectal proliferation measures. Cancer Epidemiol, Biomarkers & Prev., *4:* 57-61, 1995.

12. Lipkin, M., Blattner, W. E., Fraumeni, J. F., Lynce, H. T., Deschner, E., and Winawer, S. Tritiated thymidine (ϕ_p,ϕ_h) labeling distribution as a marker for hereditary predisposition to colon cancer. Cancer Res., *43:* 1899-1904, 1983.

13. Bostick, R. M., Potter, J. D., Fosdick, L., Grambsch, P., Lampe, J. W., Wood, J. R., Louis, T. A., Ganz, R., and Grandits, G. Calcium and colorectal epithelial cell proliferation: a preliminary randomized, double-blinded, placebo-controlled clinical trial. J. Natl. Cancer Inst., *85:* 132-141, 1993.

14. Lyles, C. M., Sandler, R. S., Keku, T. O., Kupper, L. L., Millikan, R. C., Murray, S. C., Bangdiwala, S. I., and Ulshen, M. H. Reproducibility and variability of the rectal mucosal proliferation index using proliferating cell nuclear antigen immunohistochemistry. Cancer Epidemiol, Biomarkers & Prev., *3:* 597-605, 1994.

15. Freedman, L. S., and Schatzkin, A. Sample size for studying intermediate endpoints within intervention trials or observational studies. Am. J. Epidemiol., *136:* 1148-1159, 1992.

Downloaded from cebp.aacrjournals.org on October 25, 2021. © 1996 American Association for Cancer Research.

Cancer Epidemiology, Biomarkers & Prevention

AACR American Association for Cancer Research

Reliability of whole crypt mitotic count as a measure of cellular proliferation in rectal biopsies.

T D Tosteson, M R Karagas, R Rothstein, et al.

Cancer Epidemiol Biomarkers Prev 1996;5:437-439.

Updated version Access the most recent version of this article at:
http://cebp.aacrjournals.org/content/5/6/437

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
http://cebp.aacrjournals.org/content/5/6/437.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC)
Rightslink site.

Downloaded from cebp.aacrjournals.org on October 25, 2021. © 1996 American Association for Cancer Research.