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Mucosal Polyamine Measurements and Colorectal Cancer Risk

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Abstract Polyamines are short-chain aliphatic amines required for normal cellular growth that are ubiquitously found in all living tissues. Polyamine content has been shown to correlate with cellular proliferation. Quantitation of polyamines may thus provide a biochemical measure of proliferation in the colorectal mucosa where dysregulated epithelial proliferation is associated with colorectal cancer risk. A case–control study was conducted to validate the hypothesized association between mucosal polyamine measurements and colorectal cancer risk. Polyamines were measured in 4–6 multiple rectal mucosal biopsies from 11 normal control subjects and seven case patients with colon cancer. Compared with the controls, mean polyamine measurements, after adjustment for age and sex, were significantly increased for spermidine ($P < 0.003$) and spermine ($P < 0.017$). Subsequent analyses indicated that in controls 1–4 biopsies appeared adequate to characterize an individual. However, mucosal polyamines in the cases exhibited more sampling variability, requiring 4–8 biopsies to achieve an acceptable level of reliability. After adjustment for age and sex, the odds ratios for spermidine and spermine levels, compared to the controls, were 4.8 (95% confidence interval: 1.6–33.7) and 2.3 (1.2–6.3), respectively. The results of this study indicate that increases of mucosal polyamine measurements, after taking the sampling and methodological variability into account, are significantly associated with colorectal cancer risk, and suggest that polyamine measurements in rectal mucosa may play an important role as biomarkers for identifying high-risk individuals and/or for using as intermediate endpoints in prevention trials.

Key words: polyamines, biomarkers, proliferation, colorectal cancer risk, mucosa, biopsy

Approximately 150,000 new cases of colorectal cancer are diagnosed annually in the U.S. [Boring et al., 1994; DeCosse et al., 1994]. Although colorectal cancer is curable in 60–90% of patients diagnosed with clinically localized disease, nearly two-thirds of patients have disease involving regional lymph nodes or have distant metastases at the time of clinical presentation. With cure rates for disease involving regional nodes of 60% or less, and with treatment for metastatic disease remaining unsatisfactory, an annual U.S. death rate of 60,000 is attributable to colorectal cancer. In view of the disappointing statistics with regard to colorectal cancer treatment, attention continues to focus on screening and early detection as a strategy for reducing colorectal cancer morbidity and mortality.

The development of biological measures of cancer risk offers a potential for the prevention of colorectal cancer [Lipkin, 1988; O'Brien et al., 1992]. Measures of dysregulated proliferation within the grossly normal flat colorectal mucosa, including crypt thymidine-labeling index (TLI) and ornithine decarboxylase (ODC) activity, have been investigated in this context [Lipkin, 1988; O'Brien et al., 1992]. These putative biomarkers appear to correlate with risk of colorectal neoplasia; however, inherent technical limitations with the TLI and ODC assays, including labor intensity, lengthy procedure times, the requirement for radioisotope use, assay imprecision, and/or a limited individual predictive value, may ultimately preclude the large-scale applications of these measures [Braverman et al., 1990; Scalmati and Lipkin, 1992].

Measurement of mucosal polyamines could be an alternative measure of dysregulated colorectal proliferation. Polyamines are ubiquitous

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short-chain aliphatic amines that are required for cellular growth and function [Pegg, 1988]. The polyamine biosynthetic enzymes, ODC and S-adenosylmethionine decarboxylase, are highly regulated in the cell and respond to a wide variety of growth-promoting stimuli. A link between polyamine metabolism and colorectal cancer seems reasonably well established in view of the reported association between colorectal mucosal ODC activity and cancer risk [Luk and Baylin, 1984; Koo et al., 1988; Narisawa et al., 1989; McGarrity et al., 1990], and since polyamine biosynthesis inhibition by difluoromethylornithine (DFMO), a specific inhibitor of ODC, is protective against colorectal carcinogenesis in animal models [Pegg, 1988]. Moreover, we currently observed a direct correlation between cellular polyamines and proliferation in a human colonic epithelial cell line, providing in vitro evidence of polyamine levels associated with colorectal cancer risk [Higuchi and Wang, 1995].

However, a few past case–control studies conducted on polyamine measurement in human colorectal specimens have provided conflicting results [LaMuralgia et al., 1986; Upp et al., 1988; McGarrity et al., 1990; Hixson et al., 1993; Hixson et al., 1994]. Although the reasons underlying the disparate results are unknown, the biological variations of polyamines and/or the methodological problems of assay precision might be involved. Here we conducted a case–control study to validate the hypothesis that the polyamine levels are associated with colorectal cancer risk. To avoid the more obvious analytic pitfalls, multiple biopsies were obtained from each subject and statistic analyses estimated for the first time to show the reliability and variability of the polyamines in human rectal mucosa.

MATERIALS AND METHODS

Subjects and Biopsy Procedures

Eleven normal volunteers without significant disease (four males, seven females; mean age, 62.2 years) and seven patients with colon cancer (three males, four females; mean age, 57.4 years) were included in this study. Details on characteristics of subjects are provided in Table I. No subject had evidence of other severe metabolic or life-threatening acute or chronic disease. Prior to study entry, all subjects signed an informed consent form approved by the University of Hawaii's Committee for Human Studies.

Each health volunteer underwent flexible sigmoidoscopy to obtain multiple biopsies of the rectal mucosa at a distance of 10 cm from the anal verge. They received two 133 ml of phosphate enemas (Chester Labs Inc., Erlanger, KY) prior to removal of up to six superficial mucosal biopsy specimens. A standard 7 mm flexible endoscope biopsy forceps was directed perpendicular to the rectal mucosa to obtain avulsion mucosal biopsies. For patients with colon cancer, multiple biopsies were obtained indirectly from fresh surgical specimen within 1 hr of bowel resection. Only normal-appearing mucosa separated from the portion of the surgical specimen grossly unaffected by disease at a distance no less than 2 cm from the tumoral margin was used to remove biopsies. Immediately after removal, all the biopsy samples were transported to the laboratory in ice-cold phosphate-buffered saline. Each assay was conducted to the case–control blind status of the subjects.

TABLE I. Characteristics of Subjects and Their Mean of Colorectal Mucosal Polyamine Values (nmol/mg protein)

	No.	Age (yr)	Sex	Spermidine[a]	Spermine[a]
Control	1	54	F	1.17 ± 0.12	3.62 ± 0.32
	2	65	F	2.62 ± 0.31	6.02 ± 0.45
	3	78	M	3.07 ± 0.11	8.37 ± 0.19
	4	50	M	2.30 ± 0.21	5.17 ± 0.53
	5	49	F	5.23 ± 0.67	8.49 ± 1.57
	6	76	F	3.56 ± 0.29	5.68 ± 1.40
	7	71	F	2.70 ± 0.33	5.82 ± 0.82
	8	53	M	1.34 ± 0.23	4.92 ± 1.59
	9	59	M	2.37 ± 0.47	5.56 ± 0.94
	10	62	F	2.43 ± 0.39	4.53 ± 0.92
	11	67	F	2.87 ± 0.25	4.44 ± 0.58
Cases	1	47	M	3.65 ± 0.25	8.10 ± 0.33
	2	53	F	5.34 ± 1.43	10.52 ± 2.13
	3	61	M	4.70 ± 0.60	8.87 ± 1.14
	4	55	F	4.11 ± 0.81	7.69 ± 1.71
	5	64	F	3.22 ± 0.57	4.83 ± 0.86
	6	61	M	5.89 ± 0.86	7.80 ± 0.87
	7	61	F	5.05 ± 0.79	7.98 ± 0.74

[a]Mean ± SD of 4–6 biopsy replicates.

Polyamine Assay

Each mucosal biopsy was suspended in 510 μl of ice-cold phosphate-buffered saline and homogenized using a Polytron Homogenizer (Brinkman Instruments, Inc., Westbury, NY). After removal of gross debris by centrifugation, protein content was measured using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA). Diaminohexane and diaminodo-

decane were next added as internal standards, then proteins were precipitated with perchloric acid (5%). Protein-free supernatants (500 μl) were admixed with saturated sodium carbonate (350 μl) and 1% dansyl chloride in acetone (400 μl), then the mixtures were incubated at 60°C for 1 hr. Dansylated polyamines were extracted in toluene, dried, redissolved in acetonitrile (100 μl), and finally quantified using our established HPLC method [Higuchi and Wang, 1995; Wang et al., 1996]. The HPLC assay results were found to be highly reproducible: the coefficient of variations (CV) of replicate measurements within a single batched assay or assayed separately on different days was consistently less than 6%. Using this method, the limit of detection is about 0.2 nmol/mg protein for putrescine (Put), spermidine (Spd), spermine (Spm), and their acetyl derivatives.

Statistical Analysis

In the analysis of the data, all the polyamine measures were log transformed, as log (x + 1), in order for the distributions to approximate normality. The multiple biopsy values for each individual were modeled with a one-way random effect ANOVA to obtain estimates of intra- and interindividual variance [Snedecor and Cochran, 1989].

The estimated number of biopsy measurements necessary to rank each individual correctly to his or her possible heterogeneous analyte levels was computed using the formula proposed by McAvay et al. [McAvay and Rodin, 1988]:

$$\left(\frac{E_\beta}{1 - E_\beta}\right)\frac{S_W^2}{S_W^2}$$

where E_β is error term for the regression coefficient between the measured and the true underlying values for an individual, and s_W^2 and s_B^2 are the observed within- and between-subject variances computed by ANOVA. A value of $E_\beta = 0.90$ was chosen because the observed regression coefficient would be kept no more than 10% different from the true regression coefficient. The extent of within-subject reproducibility was assessed by the intraclass correlation (ICC). ICC was computed using the formula as follows [Snedecor and Cochran, 1989]:

$$ICC = \frac{S_B^2}{S_B^2 + S_W^2}$$

In essence, ICC quantifies the extent of overall agreement between the repeated biopsy measurements. Power to detect differences in variables is increased if the ICC is high. If ICC were equal to one (maximum values), then there would necessarily be exact agreement between the repeat measurements for all the subjects.

The mean of polyamine levels for each subject was computed and modeled with a linear regression model adjusted by sex and age to explore mean differences between cases and controls. In order to test for significant associations between levels of mucosal polyamines and colon cancer risk, multiple logistic regression models were used to compute odds ratio (OR) and 95% confidence interval (95% CI) after adjustment with sex and age [Breslow and Day, 1980].

RESULTS

Polyamines were measured in 4–6 biopsy replicates in 11 normal subjects and seven patients with colon cancer. In addition to listing the age and sex distributions, Table I shows the individual mean of mucosal polyamine levels and their standard deviation, suggesting sampling variation in the levels of mucosal polyamines, especially in the cases. Spd and Spm were detectable in all biopsy samples, and the Spd levels were always less than the levels of Spm. The levels of Put were detected only in 30% of samples and thus were not presented for comparison in this study. Acetylated polyamines were not detectable in all the biopsy samples.

To visualize the variations suggested in Table I, the variability of mucosal polyamine measures was further examined. Table II presents the estimated within- and between-subject variations, within- to between-subject variance ratios, and the estimated biopsy number of repeat measurements required to characterize an individual with respect to the group. For all analytes, intraindividual variation was smaller than that for interindividual. In controls, 1–4 biopsies appeared adequate to characterize an individual. However, mucosal Spd in the cases exhibited more variability, requiring eight biopsies to achieve an acceptable level of reliability. The reproducibility of mucosal polyamine measurements was additionally assessed by the ICC. The ICC ranged from 0.7 to 0.9 in all study groups, excepting moderately high Spd in the cases, indicating a good reproducibility for intraindividual measures. However, the high intraindividual variation relative to interindividuals for

TABLE II. Variability of Mucosal Polyamine Levels Among Cases and Controls

Variables	Risk groups	Variance		W/B[a]	No. of measurements required[b]	ICC[c]
		Within	Between			
Spermidine[d]	Control	0.008	0.085	0.09	0.9	0.91
	Case	0.022	0.026	0.85	7.6	0.54
Spermine[d]	Control	0.021	0.045	0.47	4.2	0.68
	Case	0.021	0.047	0.45	4.0	0.69

[a]Ratio of within (W) to between (B) subject variability.
[b]Represents the number of repeat measurements needed to correctly rank at least 90% of the true regression coefficient of individuals as described in Materials and Methods.
[c]Intraclass correlation indicates the extent of within-subject reproducibility of mucosal polyamine measurements as described in Materials and Methods.
[d]Log transformed as log (x + 1).

TABLE III. Age- and Sex-Adjusted Mean of Mucosal Polyamine Levels for Cases and Controls

Variables	Cases (n = 7)	Controls (n = 11)	P[a]
Spermidine[b]	4.53	2.48	0.003
Spermine[b]	7.86	5.55	0.017

[a]P value for a difference between cases and controls after age and sex adjustment via regression.
[b]Log transformed as log (x + 1).

TABLE IV. Associations of Mucosal Polyamine Measurements With Colorectal Cancer Risk

Variables	No.[a]	OR[b]	95% CI[c]	P
Spermidine[d]	7/11	4.8	1.6–33.7	0.03
Spermine[d]	7/11	2.3	1.2–6.3	0.04

[a]Number of cases/number of controls.
[b]Odds ratio adjusted for age and sex using multiples logistic regression model.
[c]Ninety-five percent confidence interval.
[d]Log transformed as log (x + 1).

Spd in the cases resulted in more biopsies required.

Table III lists the sex- and age-adjusted means of mucosal polyamine levels among cases and controls using linear regression analysis. Compared with the controls, mean polyamine measurements were significantly increased for Spd ($P < 0.003$) and Spm ($P < 0.017$).

Table IV gives the associations of mucosal polyamines with colon cancer. After adjustment for age and sex, both Spd and Spm were associated with significantly higher risk. The OR for Spd levels, compared to the controls, was 4.8 (95% CI = 1.6–33.7). The corresponding OR for Spm was 2.3 (95% CI = 1.2–6.3). There were significant trends in which higher levels of Spd ($P < 0.03$) and Spm ($P < 0.04$) predicted cancer risk.

DISCUSSION

Recent increase in research concerning prevention and early detection of cancer risk in unaffected individuals has generated a parallel increase in the search for biomarkers of cancer risk. The rectal mucosal proliferation has been proposed and widely employed as an intermediate endpoint for assessing associations with colon cancer risk [Lipkin, 1988; O'Brien et al., 1992; Scalmati and Lipkin, 1992; Farber, 1995]. Although a number of methods have been proposed to measure cellular proliferation in the large bowel, quantitative analysis of colonic mucosal polyamines appears to be a promising alternative offering the technical advantages of simplicity, rapidity, and high assay reproducibility [Higuchi and Wang, 1995; Wang et al., 1996].

Previous studies measuring polyamine levels in colorectal mucosa as candidate biomarkers for cancer risk have had provocative but inconsistent results. Two studies reported increased levels of Spd and Spm, but not of Put, within the colonic mucosa of patients with carcinoma compared to controls [LaMuralgia et al., 1986; Upp et al., 1988]. One study found increased Put, but no differences in Spd or Spm content when mucosal samples from patients with colorectal polyps were compared with controls [McGarrity et al., 1990]. One more study showed elevated levels of Spd, Spm, and Put in cancer patients compared with control patients with diverticular disease [Kingsnorth et al., 1984]. Another study reported significant increases of Put, Spd, and Spm levels in patients with ulcerative colitis [Tonelli et al., 1991]. Finally, one study group recently claimed no significant differences in mucosal polyamine levels between controls and

patients with colorectal carcinomas or adenomas [Hixson et al., 1993; Hixson et al., 1994]. The reasons underlying the disparate reports are unknown, but negative results of some past studies might have resulted from misclassification due to methodological and biological variations.

The methodological issue of assay precision has been discussed in our previous reports [Higuchi and Wang, 1995]. In brief, we have conducted preliminary experiments to delineate reasons for the apparent discrepancies in the mean polyamine levels reported, and have noted markedly divergent polyamine extraction efficiencies with the different buffers used in the above-reported literature. It is clearly necessary to standardize procedures and techniques in order to assure measurement accuracy and to allow study comparisons.

The issue of sampling variation was addressed in this study by repeated polyamine measurements using multiple biopsies from each individual. Four to six replicated measures in each subject were analyzed to assess the intra- and interindividual variations of the mucosal polyamines (Table II). Interestingly, a high ratio of intra- to interindividual variability was observed in Spd levels in high-risk individuals. We have previously observed that intracellular Spd was more correlated than the Spm with cellular proliferation [Higuchi and Wang, 1995]. The considerable sampling variability of Spd in cases may thus suggest a focal heterogeneity of epithelial proliferation that may also be manifest throughout the colon. Alternatively, since the case samples were from resection specimens of cancer patients, the heterogeneous polyamine distribution may have resulted from a local effect of the malignant tumors upon the adjacent mucosa, that is, the diffuse "field effect" in colon mucosa from which adjacent cancers arise. If mucosal heterogeneity is indeed widespread, then multiple sampling appears required for accurate biomarker measurements. The estimated number of samples required to accurately characterize mucosal polyamine levels for a control individual generally ranged from one to four. However, up to eight biopsies seem required to characterize the mucosal levels in the case individuals. To our knowledge, this is the first published study documenting the reproducibility of mucosal polyamine measurements and the number of biopsies required to achieve the indicated level of accuracy.

Taking the methodological and sampling variability into account during analysis of individual mucosal polyamine levels, we compared mucosal polyamines between cases and controls. The significant increases in the levels of Spd and Spm within the normal-appearing flat mucosa of patients with colon cancer were observed when comparing against mucosa of controls without such neoplasms (Table III). These increases were associated with significantly higher colon cancer risk, with OR of 4.8 for Spd and 2.3 for Spm (Table IV). There were significant trends in which higher Spd ($P < 0.03$) or Spm ($P < 0.04$) predicted colon cancer risk. However, it should be noted that the number of subjects is relatively small in the present study, and the other relative variables such as race, cancer stage, family history, and/or polyps cannot be specified. Therefore, our results require confirmation in larger studies, by which stringent clinical criteria could be developed in an attempt to maximize accuracy of risk classification and to further assure practicality in the development of polyamine biomarkers.

In conclusion, this case–control study demonstrates that the mucosal polyamine levels, taking the sampling and methodological variability into account, are significantly associated with the colorectal cancer risk, and suggests that rectal mucosal polyamines, as a measure of epithelial proliferation, may be useful in future studies for identifying high-risk individuals and/or for use as intermediate endpoints in prevention trials.

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