Journal of Cellular Biochemistry 57:256–261 (1995)

Comodulation of Cellular Polyamines and Proliferation: Biomarker Application to Colorectal Mucosa

Carl M. Higuchi and Weiqun Wang

Prevention and Control Program, Cancer Research Center of Hawaii, University of Hawaii, Honolulu, Hawaii 96813

Abstract Polyamines are low molecular weight aliphatic amines required for normal cellular growth which are ubiquitously found in all living tissues. Polyamine biosynthesis is known to increase with mitogenesis, and elevated polyamine concentrations are found in hyperproliferative tissues. Quantitation of tissue polyamine content may thus provide a biochemical measure of proliferation, with potential biomarker application to the colonic mucosa where dysregulated epithelial proliferation is associated with cancer risk. This study was performed to validate polyamine analyses as a measure of cellular proliferation, and to preliminarily assess polyamine assay characteristics when applied to clinical samples. Using FHC, a human colonic epithelial cell line, for in vitro experimentation, deoxycholic acid or retinol was added to freshly passaged cultures to either stimulate or inhibit proliferation, respectively. Parallel cultures were then assayed for (1) proliferation by sulforhodamine B staining; and (2) polyamine content by a high-performance liquid chromatographic method. Deoxycholic acid stimulated, and retinol inhibited proliferation in dose-dependent fashion. Polyamine content, specifically the spermidine content and the spermidine/spermine ratio, also increased or decreased in response to culture with deoxycholic acid or retinol, respectively. Significant linear correlations between proliferation and spermidine ($r = 0.858$, $P < 0.001$), and with the spermidine/spermine ratio ($r = 0.574$, $P < 0.05$) were observed. When quantitative polyamine analyses were applied to human colonic specimens, replicate mucosal sampling revealed a high degree of intra-individual variability, indicating a heterogeneous distribution of polyamines within anatomically confined colonic segments. The results support a role for quantitative polyamine analyses as a correlative measure of colonic epithelial proliferation; however, intraindividual variability may limit the utility of colorectal biomarker measurements.

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

Examination of the grossly normal-appearing colonic mucosa of individuals with increased susceptibility to neoplasia by a microautoradiographic thymidine labeling technique has revealed subtle, but reproducible alterations in the normal compartmentalization of proliferating cells [Lipkin, 1988; Scalmati and Lipkin, 1992; O'Brien et al., 1992]. Whereas within the normal bowel, proliferating epithelial cells are confined to the bases of colonic crypts, the dysregulated epithelium of high-risk individuals is characterized by an expansion of the proliferative crypt compartment toward the lumenal surface, or an overall hyperproliferation. Measures of colonic mucosal proliferation have thus been proposed as biomarkers indicative of colorectal cancer risk, and as surrogate endopoints for cancer preventive interventions. Several alternative methods of assessing mucosal proliferation are in current investigational use, including immunohistochemical analyses for bromodeoxyuridine labeling or proliferating cell nuclear antigen (PCNA) staining, or enzymatic assays of ornithine decarboxylase activity [Risio et al., 1993; Luk and Baylin, 1984]. However, the clinical applicability of the available measures may be limited by technical factors, including labor intensity, lengthy procedure times, the requirement for radioisotope use, assay imprecision, and a limited individual predictive value [Scalmati and Lipkin, 1992].

An alternative measure of potential applicability to colorectal mucosa is the quantitative analysis of polyamine content. Polyamines are low molecular weight aliphatic amines which are required for normal cellular growth and function, and polyamine biosynthesis is highly regu-

Received May 25, 1994; accepted June 23, 1994.

Address reprint requests to Dr. Carl M. Higuchi, Prevention and Control Program, Cancer Research Center of Hawaii, University of Hawaii, Honolulu, HI 96813.

lated [Tabor and Tabor, 1984; Pegg, 1988]. Spermidine (Spd), spermine (Spm), and their precursor putrescine (Put) are the principal polyamines found within virtually all mammalian cells and tissues. Observations that cellular polyamine concentrations are correlated with cell cycle phase [Heby et al., 1973], that polyamine content increases when fibroblast monolayer cultures are stimulated to proliferate [Heby et al., 1975], and of elevated polyamine levels within hyperproliferative neoplastic epithelial tissues [Takenoshita et al., 1984; Kingsnorth et al., 1984] suggest that polyamine analyses may provide a biochemical index of cellular proliferation. Elevated polyamine levels have been reported within the flat colonic mucosa of individuals at increased risk for neoplasia [La-Muralgia et al., 1986; Upp et al., 1988; McGarrity et al., 1990], consistent with the proposed association between mucosal polyamines and proliferation.

To experimentally validate polyamine analyses as a quantitative measure of proliferation, comparative analyses of cellular polyamines and proliferation were conducted using an in vitro model. Polyamines were subsequently measured in human colonic mucosal samples to preliminarily assess the reliability of the polyamine assay when applied to clinical material.

METHODS

Reagents and Test Standards

All cell culture reagents and media, and all chromatographic standards were purchased from Sigma Chemical Company (St. Louis, MO).

Cell Culture

FHC, a human diploid colonic epithelial cell line, was obtained from American Type Cell Collection (Rockville, MD) and is maintained in continuous culture with weekly passage. Experimental cultures were initiated simultaneously in flasks for subsequent polyamine analyses, and in 96-well flat bottom microculture plates for assessment of proliferation. The culture media (45% Dulbecco's Modified Eagle + 45% Ham's F12 + 10% fetal bovine serum) was supplemented with deoxycholic acid (DCA) or retinol (RoL) at concentrations ranging from 10^{-8} to 10^{-5} M. RoL was solubilized in absolute ethanol, and final ethanol concentration within all RoL-containing cultures and the appropriate control cultures was adjusted to 0.5%. Cultures were incubated at 37°C in a 5% CO_2 atmosphere for 6–17 days, with supplementation of fresh media every 3–4 days. Cell proliferation within the microculture plates was assessed colorimetrically after staining with sulforhodamine B, and is expressed as optical density at 560 nm (OD_{560}). Cells for polyamine assay were removed from culture flasks after trypsinization.

Colonic Mucosal Tissues

Fresh human colonic mucosal tissue was obtained from patients within 1-h of bowel resection for colon cancer (6 patients) or pneumatosis cystoides intestinalis (1 patient). Samples were taken from the portion of the surgical specimen grossly unaffected by disease at a distance no less than 2 cm from the tumoral margin. The mucosa was dissected free from the underlying submucosa and muscularis, then sharply divided into approximately 1–4 mm^3 segments for subsequent homogenization and polyamine assay.

Polyamine Assay

Cultured cells or mucosal tissue was suspended in 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 diaminododecane 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 h. Dansylated polyamines were extracted in toluene, dried, then redissolved in acetonitrile (100 μl), and finally quantified using a published high-performance liquid chromatography method [Minocha et al., 1990], with slight modifications. A linear gradient of acetonitrile-heptanesulfonate (10 mM, pH 3.4), at a flow rate of 2.5 ml/min was used, with parameters as indicated in Table I. The chromatographic apparatus consisted of a Beckman Model 332 Gradient Liquid Chromatograph (Beckman Instruments, Berkeley, CA) fitted with a 10-μl injection loop, a Perkin-Elmer (Norwalk, CT) Pecosphere-3 × 3 CR C_{18}, 33 × 4.6 mm I.D. cartridge column (3 μm particle size), and a GTI/SpectroVision FD-100 fluorescence detector (Groton Technology,

TABLE I. HPLC Solvent Gradient Profile for the Quantitation of Dansyl-Polyamines*

Step	Time[a]	Solvent A (%)	Solvent B (%)
0	0.1	10	90
1	3.0	70	30
2	3.3	100	00
3	2.0	100	00
4	0.1	10	90
5	1.0	10	90

*Solvent A = 100% acetonitrile; solvent B = heptanesulfonate (10 mM, pH 3.4)-acetonitrile (90:10).
[a]Time is not cumulative from the start of the run.

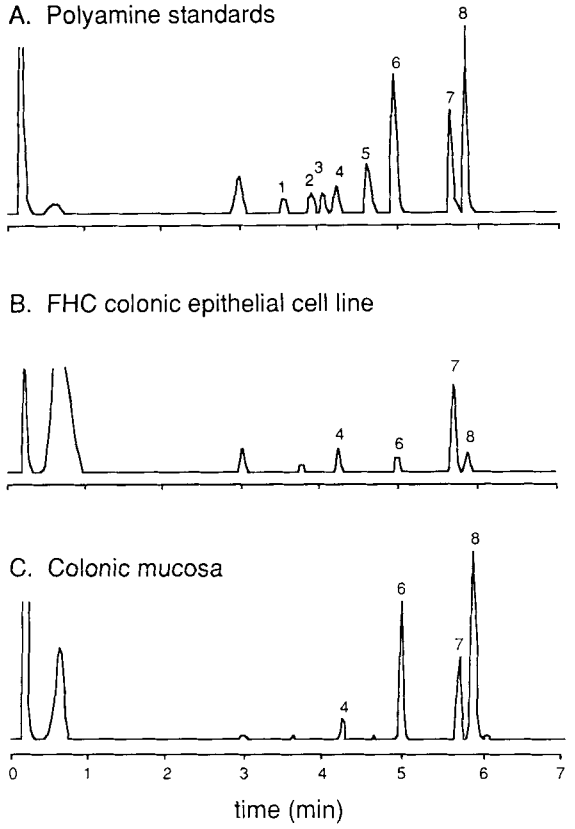

Fig. 1. Typical polyamine chromatograms obtained with standards (**A**), FHC cells (**B**), or colonic mucosa (**C**). 1, acetylspermidine; 2, cadaverine; 3, putrescine; 4, diaminohexane (internal standard I); 5, acetylspermine; 6, spermine; 7, diaminododecane (internal standard II); 8, spermine.

Inc, Concord, MA) equipped with a 330 nm excitation filter and a 470 nm cut-off emission filter. Peak areas were calculated using a Dynamax MacIntegrator (Rainin Instrument Co., Inc., Woburn, MA). Using this method, the limit of detection is ~0.2 nmol/mg protein for Put, Spd, Spm, and their acetyl-derivatives.

RESULTS

Polyamine Assay Characteristics

Typical polyamine chromatograms are shown in Figure 1. A set of 8 commercially purchased standards was used to demonstrate chromatographic separation, and to determine retention times of the individual polyamines (Fig. 1A). Following dansylation, the use of a high-efficiency column allowed rapid measurements, with a sample run time of only 9.5 min. Spd and Spm were readily quantified in the FHC cells (Fig. 1B) and in colonic mucosal samples (Fig. 1C). Assay results were found to be highly reproducible: the coefficient of variation (c.v.) of replicate measurements within a single batched assay (intra-assay precision), or assayed separately on different days (inter-assay precision) was consistently $\leq 6\%$. Put was detected inconsistently, and was not reliably quantified due to low concentrations within the examined specimens. Acetylated polyamines were not detected in either the cultured cells or the clinical samples.

Correlations Between Cellular Polyamines and Proliferation

FHC cells were cultured for 7 days with and without varied concentrations of DCA or RoL (10^{-8} to 10^{-5} M), then assessed for cellular polyamine content and proliferation. Table II shows representative results from 1 of 4 separate, but similarly designed experiments. DCA stimulated and RoL inhibited proliferation in dose-dependent fashion. A maximal stimulation of 27% above control was observed at the highest DCA concentration, and a maximal inhibition of 80% below control was observed at the highest RoL concentration. Cellular Spd content increased in response to DCA, and decreased with RoL, in dose-dependent fashion, and in parallel to the observed proliferation changes. By contrast, cellular Spm content remained unchanged irrespective of the addition of DCA or RoL to culture. As a result, the ratio of intracellular Spd to Spm (Spd/Spm) changed in direct parallel with Spd content and proliferation. Figure 2 graphically demonstrates the significant linear correlations observed between polyamines and proliferation.

Polyamine Analyses of Colonic Mucosa

Colonic mucosal polyamines were quantified using replicate samples taken from 7 surgical

TABLE II. Effect of DCA and RoL on the Proliferation and Polyamine Content of FHC Cells*

Culture supplement		Proliferation	Polyamines (nmol/mg protein)		
DCA	RoL	(OD560)[a]	Spd	Spm	Spd/Spm
0	—	.318 ± .008	1.87	2.41	0.78
10^{-8} M	—	.319 ± .006	2.53	2.27	1.11
10^{-7} M	—	.335 ± .006	3.63	2.46	1.48
10^{-6} M	—	.365 ± .030	3.70	2.17	1.71
10^{-5} M	—	.404 ± .008	3.77	2.06	1.83
—	0	.317 ± .004	1.81	2.31	0.78
—	10^{-8} M	.236 ± .014	1.53	2.15	0.71
—	10^{-7} M	.102 ± .003	1.19	2.27	0.52
—	10^{-6} M	.087 ± .008	1.17	2.23	0.52
—	10^{-5} M	.062 ± .003	0.16	2.58	0.06

*Representative data from 1 of 4 separate experiments are shown.
[a]Mean ± SEM of triplicate cultures.

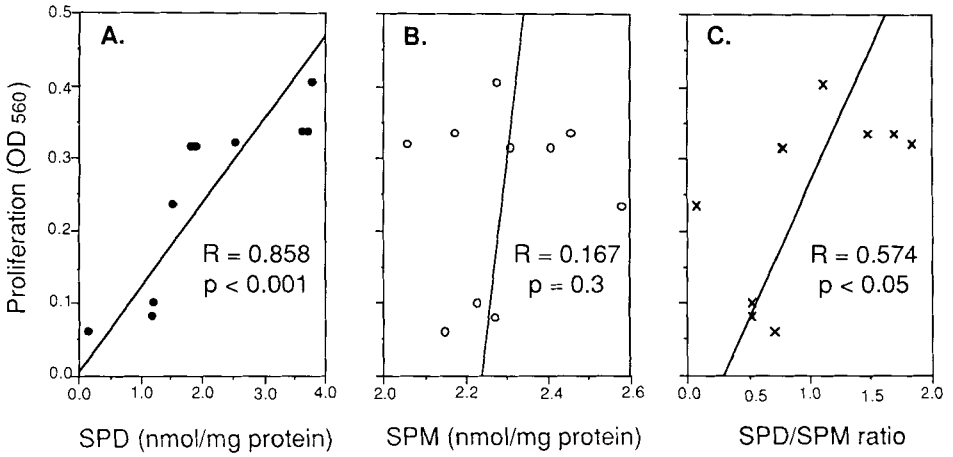

Fig. 2. Correlations between FHC proliferation and cellular content of SPD (**A**), SPM (**B**), or the SPD/SPM ratio (**C**). FHC cells were cultured with varying concentrations of DCA or RoL, then proliferation and polyamine content were determined as described in Materials and Methods. Representative data from a single experiment are shown.

specimens. The results are presented in Table III. Two processing protocols, A and B, were used to ascertain assay precision, and intraindividual variation, respectively. In Protocol A (specimens 01, 05, 06, and 07), mucosal tissue samples were pooled together for homogenization, then the resultant extracts were aliquoted for replicate polyamine assay. In Protocol B (specimens 02, 03, 04, 05, 06, and 07), replicate mucosal samples were individually homogenized, then independently assayed for polyamine content. Protocol A confirmed a high degree of assay precision (c.v. of replicate measurements $\leq 6\%$); however, protocol B demonstrated a considerable degree of intraindividual variability (c.v. of replicate samples 9–22%).

DISCUSSION

Technical factors, including labor-intensity, subjectivity, and assay variability preclude the microautoradiographic and enzymatic methods of assessing colonic mucosal proliferation from routine, non-investigative clinical application. Newer immunohistochemical methods of assessing mucosal proliferation are under investigation, with apparent correlations with the thymidine-labeling method; however, the immunohistochemical measures have not been demonstrated to be directly associated with cancer risk, and are themselves not exempt from technical problems of subjectivity and assay imprecision. Quantitative analysis of colonic mucosal polyamines

TABLE III. Quantitative Assay of Colonic Mucosal Polyamines

Specimen no.	Processing protocol[a]	No. of replicates	Mucosal polyamine content (nmol/mg protein)			
			Spd[b]	cv (%)	Spm[b]	cv (%)
01	A	7	3.63 ± 0.21	5.8	8.09 ± 0.34	4.2
05	A	6	3.29 ± 0.09	2.7	4.27 ± 0.25	5.9
06	A	3	7.25 ± 0.22	3.0	8.92 ± 0.42	4.7
07	A	6	4.62 ± 0.26	5.6	7.09 ± 0.34	4.8
02	B	12	5.35 ± 1.43	26.7	10.53 ± 2.13	20.2
03	B	11	4.77 ± 0.47	9.9	8.70 ± 1.13	13.0
04	B	6	4.11 ± 0.81	19.7	7.69 ± 1.71	22.2
05	B	6	3.22 ± 0.57	17.7	4.83 ± 0.86	17.8
06	B	3	5.89 ± 0.86	14.6	7.80 ± 0.88	11.3
07	B	6	5.05 ± 0.79	15.6	7.97 ± 0.74	9.3

[a]See text for protocol descriptions.
[b]Mean ± SD of replicates.

is proposed as an alternative, biochemical measure of epithelial proliferation offering technical advantages of simplicity, rapidity, and high assay reproducibility. A method of analysis is presently reported which results in highly precise polyamine measurements within cultured human cells, as well as in mucosal tissue.

A prerequisite to the use of polyamine analyses as a proliferative measure is a demonstration that polyamine concentrations correlate with cellular growth rate. Other investigators have previously noted that cellular Put, Spd, and Spm concentrations increased when a cultured human fibroblast cell line was stimulated to proliferate by replenishment of exhausted culture media [Heby et al., 1975]. The present study confirms a direct relationship between proliferation and cellular polyamines, and extends the finding to an epithelial cell line derived from human colon. Parallel changes of Spd concentration and proliferation were demonstrated in the cultured FHC cells, and comodulation occurred in both stimulatory and inhibitory culture conditions (cultures supplemented with DCA and RoL, respectively). The observed relationship is therefore unlikely to be spurious. Although cellular Spm concentration increased with proliferation in the earlier published study, no relationship between Spm content and proliferation was noted in the present study. This disparity might be explained by differences in cell lineage (i.e., fibroblast vs. epithelial cell), or to differing culture conditions. A short culture duration (48 h) was used in the earlier study to investigate the kinetics of polyamine concentration changes, whereas a longer culture duration (7 days) was used in the present study in an attempt to reflect steady-state conditions. Since Spm remained relatively constant, the Spd/Spm ratio varied in proportion to Spd and also correlated with proliferation. Note that Spd/Spm ratio measurements are subject to less procedural error than polyamine quantitation because the use of exogenously added assay standards and indexing to protein content are not needed. The potential utility of Put quantitation as a measure of proliferation was not assessable because Put concentrations were below the limit of the assay sensitivity.

Other investigators have previously studied polyamine measurements as candidate colorectal biomarkers with provocative, but inconclusive published results. Two studies utilizing surgical colectomy specimens reported increased levels of Spd and Spm, but not of Put, within the colonic mucosa of patients with carcinoma compared against cancer-free controls [LaMuralgia et al., 1986; Upp et al., 1988]. One study utilizing multiple rectal biopsies from patients undergoing colonoscopy reported higher Put, but no differences in Spd or Spm content, when patients with colonic polyps were compared against polyp-free controls [McGarrity et al., 1990]. Finally, one study utilizing single rectal biopsies taken at colonoscopy found no differences in mucosal levels of Put, Spd, or Spm between controls and patients with colorectal carcinomas or adenomas [Hixson et al., 1993]. The reasons underlying the disparate results are unknown; however, confounding variables in the above-referenced studies might have included methodo-

logic problems of assay precision, measurement accuracy, and biologic variation.

The methodologic issue of assay precision was addressed in this study by repeated polyamine measurement using pooled mucosal extracts (Table III, protocol A). The analysis method, which was based on a prior publication [Minocha et al., 1990], yielded highly reproducible replicate measurements (c.v. $\leq$ 6%). An acceptable degree of assay precision was thus demonstrated; however, the separate issue of measurement accuracy was not specifically addressed in this study. Accuracy can only be assured by comparison against defined standards which were not immediately available. Nevertheless, it should be noted that in preliminary studies conducted to determine the optimal method for polyamine extraction, the different extraction solutions used in the previous published studies were tested, and major differences in the extraction efficiency of polyamines were noted (unpublished observations). It is clear that the adoption of stringent quality control measures to assure measurement accuracy, and to allow interinvestigator comparisons, is of major importance in the further development of biomarkers in cancer risk assessment.

Another critical issue in biomarker development is one of biologic variation. Biologic variation includes interindividual differences, and intraindividual variation attributable to either temporal or spatial fluctuation of the measured variable. Intraindividual variation of polyamine measurements due to spatial fluctuation of polyamines within the colonic mucosa was presently investigated by independently assaying replicate tissue samples taken from single surgical specimens. Considerable differences were noted in the measured polyamine contents, even with anatomic separations between samples of less than 1 cm. Since the samples were from resection specimens of cancer patients, the heteregeneous polyamine distribution may have resulted from a local effect of the malignant tumors upon the adjacent mucosa. Alternatively, the results suggest a focal heterogeneity of epithelial proliferation which may also be manifest throughout the colon. If mucosal heterogeneity is indeed widespread, then multiple sampling will be required for accurate biomarker measurements. The uniformity of distribution of colorectal mucosal polyamines and of colonic epithelial proliferation requires further systematic study.

ACKNOWLEDGMENTS

We thank the Pathology Staff of the Queen's Medical Center, Honolulu, Hawaii, for their assistance in providing the colonic mucosal samples. This investigation was supported in part by a Public Health Service Preventive Oncology Academic Award (5-K07-CA01686) from the National Cancer Institute.

REFERENCES

Heby O, Sarna GP, Marton LJ, Omine M, Perry S, Russell DH (1973): Polyamine content of AKR leukemia cells in relation to the cell cycle. Cancer Res 33:2959–2964.

Heby O, Marton LJ, Zardi L, Russell DH, Baserga R (1975): Changes in polyamine metabolism in WI38 cells stimulated to proliferate. Exp Cell Res 90:8–14.

Hixson LJ, Garewal HS, McGee DL, Sloan D, Fennerty MB, Sampliner RE, Gerner EW (1993): Ornithine decarboxylase and polyamines in colorectal neoplasia and mucosa. Cancer Epidemiol Biomarkers Prevention 2:369–374.

Kingsnorth AN, Lumsden AB, Wallace HM (1984): Polyamines in colorectal cancer. Br J Surg 71:791–794.

LaMuralgia GM, Lacaine F, Malt RA (1986): High ornithine decarboxylase activity and polyamine levels in human colorectal neoplasia. Ann Surg 204:89–93.

Lipkin M (1988): Biomarkers of increased susceptibility to gastrointestinal cancer: New application to studies of cancer prevention in human subjects. Cancer Res 48:235–245.

Luk GD, Baylin SB (1984): Ornithine decarboxylase as a biologic marker in familial colonic polyposis. N Engl J Med 311:80–83.

McGarrity TJ, Peiffer LP, Bartholomew MJ, Pegg AE (1990): Colonic polyamine content and ornithine decarboxylase activity as markers for adenomas. Cancer 66:1539–1543.

Minocha SC, Minocha R, Robie CA (1990): High-performance liquid chromatographic method for the determination of dansyl-polyamines. J Chromatogr 511:177–183.

O'Brien MJ, O'Keane JC, Zauber A, Gottlieb LS, Winawer SJ (1992): Precursors of colorectal carcinoma: Biopsy and biologic markers. Cancer 70:1317–1327.

Pegg AE (1988): Polyamine metabolism and its importance in neoplastic growth and as a target for chemotherapy. Cancer Res 48:759–774.

Risio M, Candelaresi G, Rossini FP (1993): Bromodeoxyuridine uptake and proliferating cell nuclear antigen expression throughout the colorectal tumor sequence. Cancer Epidemiol Biomarkers Prevention 2:363–367.

Scalmati A, Lipkin M (1992): Intermediate biomarkers of increased risk for colorectal cancer: Comparison of different methods of analysis and modifications by chemopreventive interventions. J Cell Biochem (Suppl) 16G:65–71.

Tabor CW, Tabor H (1984): Polyamines. Ann Rev Biochem 53:749–790.

Takenoshita S, Matsuzaki S, Nakano G, Kimura H, Hoshi H, Shoda H, Nakamura T (1984): Selective elevation of the N1-acetylspermidine level in human colorectal adenocarcinomas. Cancer Res 44:845–847.

Upp JR, Saydjari R, Townsend CM Jr, Singh P, Barranco SC, Thompson JC (1988): Polyamine levels and gastrin receptors in colon cancers. Ann Surg 207:662–669.