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Polyamine Measurements in the Uterine Cervix

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Abstract Cervical cancer remains a significant health problem. New strategies based on the molecular aspects of cervical carcinogenesis are needed. Chemoprevention represents a novel strategy for cervical cancer prevention. Our group plans phase I and II trials using α-difluoromethylornithine, a suicide inhibitor of ornithine decarboxylase and potent antiproliferative chemopreventive agent. We conducted a study to identify which polyamines in tissue could best serve as surrogate endpoint biomarkers for future trials. Thirty patients with biopsy-proven cervical intraepithelial neoplasia grade 3 underwent colposcopically directed biopsies of normal and abnormal areas of the uterine cervix for analysis of polyamine synthesis biomarkers. Statistically significant differences were found in the ornithine decarboxylase value and the spermidine:spermine ratio between normal and abnormal areas of the cervix. In general, the ranges in measurements varied widely. Differences in polyamine synthesis biomarkers between colposcopically normal and abnormal areas can be demonstrated. However, studies using polyamine synthesis biomarkers in the cervix would require large numbers of patients to achieve significance. J. Cell. Biochem. Suppls. 28/29:125–132. © 1998 Wiley-Liss, Inc.†

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Despite the advent of the Papanicolaou (Pap) smear, cervical cancers and precancers remain important health problems for women, especially underserved women in the United States (US) and women in underdeveloped countries [1]. In the (US), an estimated 2,500,000 women actually have abnormal Pap smears demonstrating atypical cells of uncertain significance and low-grade intraepithelial lesions (lesions of HPV and cervical intraepithelial neoplasia [CIN 1]) annually [2]. The exact number of patients with high-grade squamous intraepithelial lesions (CIN 2 and 3) not classified as carcinoma in situ is unknown. The incidence rates of both invasive cervical cancer and carcinoma in situ are increasing in the (US); 15,900 cases of invasive cancer and 65,000 cases of carcinoma in situ are expected in 1996 [3–6]. While the reasons for this increase are unknown and must be viewed cautiously, they may include the rise in human papillomavirus (HPV) and human immunodeficiency virus (HIV) infection or, alternatively, changes in the way these diseases are reported [7]. The most important risk factor for cervical cancer is infection with HPV, whose high-risk types include types 16, 18, 45, and 56. This association has been consistent and independent of the HPV-assay method employed or of epidemiologic study design [8]. Despite the accessibility of the cervix and the existence of the Pap smear, a relatively good screening test, the overall 5-year survival rate for women who have invasive cervical cancer remains a dismal 40% worldwide [1].

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Novel strategies that block cervical carcinogenesis are desperately needed. One such novel approach is chemoprevention, the use of chemical agents (e.g., micronutrients, pharmaceuticals) to prevent or delay the development of cancer in healthy populations [9,10]. Because most current chemopreventive agents have side effects, they are used in women who have a high risk of developing cancer (for example, those with premalignant lesions). It is hoped that intervention in the precancerous stage will prevent a lesion from becoming invasive [11]. The advantage of chemoprevention in treating a preneoplastic condition is that its effects are systemic and, thus, it treats preneoplastic cells in all areas of the body.

As a more convenient alternative to the endpoint of cancer incidence reduction, chemoprevention studies can use surrogate endpoint biomarkers (SEBs) as intermediate measures of cancer development. These markers should be differentially expressed in normal and high-risk tissue, be highly correlated with cancer incidence, be measured with acceptable sensitivity and specificity, and be modulated by the chemopreventive under study. SEBs provide a glimpse of cancer biology and its modulation [12]. Polyamines (putrescine, spermidine, and spermine) and their precursors (arginine and ornithine) (Fig. 1) are believed to play critical roles in cellular maintenance, proliferation, differentiation, and transformation [13,14]; thus, polyamines might be considered SEBs of carcinogenesis. Polyamines are differentially expressed in normal and high-risk tissue, measured with acceptable sensitivity and specificity, and can be modulated by α-difluoromethylornithine (DFMO). Polyamines and their precursors can be measured in tissue, red blood cells, plasma, and urine. Ornithine decarboxylase (ODC), a key enzyme in polyamine biosynthesis, is considered a proto-oncogene that is crucial for the regulation of cellular growth and transformation and is irreversibly inhibited by

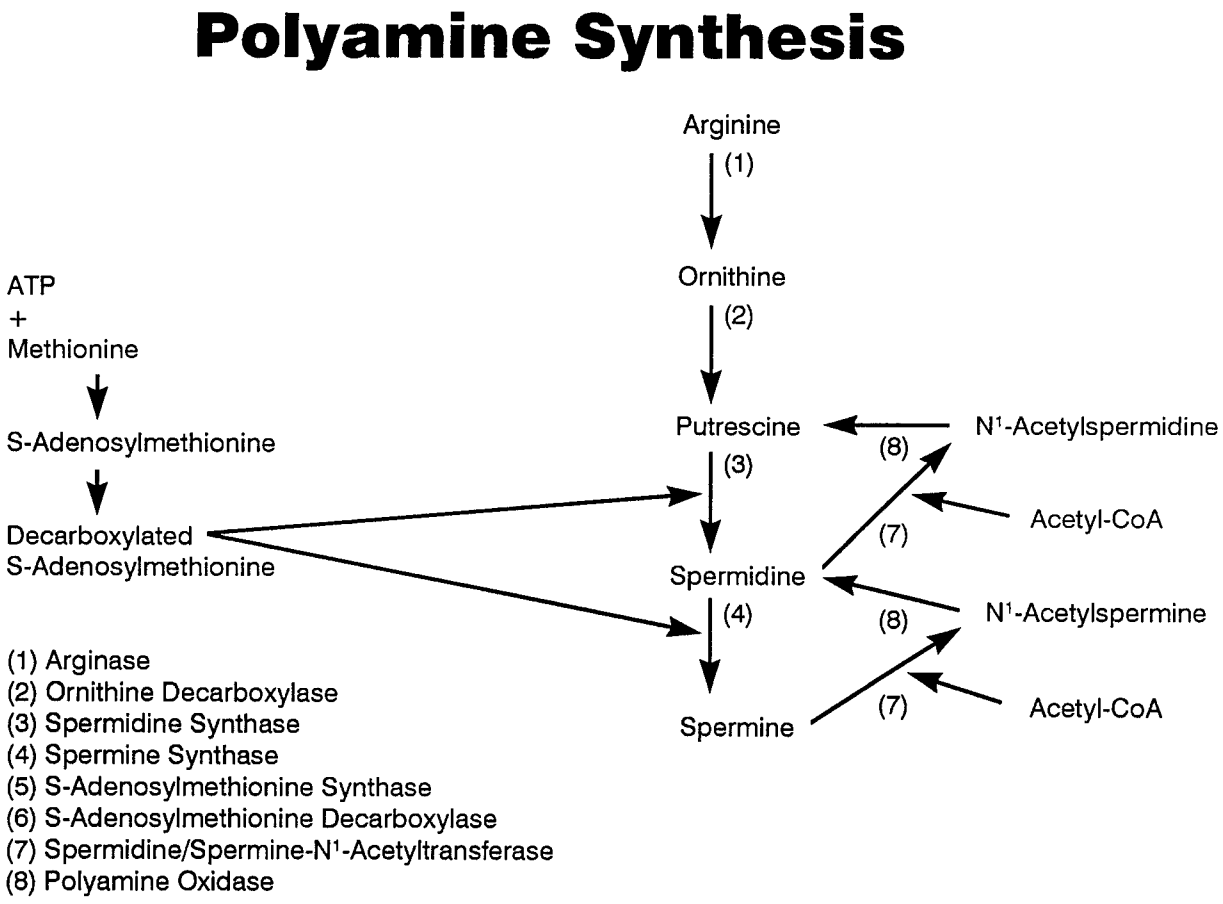

Fig. 1. Schema of polyamine synthesis. After reference [54].

DFMO. DFMO is considered a potent antiproliferative chemopreventive agent and has been studied in other organ sites but not previously in the cervix [12].

In this study, we chose patients with CIN 3 as the high-risk cohort for polyamine measurement. The objective of the study was to identify which polyamines in cervical tissue could best serve as SEBs for CIN 3 in subsequent phase I and II clinical trials of DFMO.

MATERIALS AND METHODS

Patient Eligibility

Patients were identified among women attending the Colposcopy Clinic of the University of Texas M.D. Anderson Cancer Center Department of Gynecologic Oncology. Eligible patients were nonpregnant women aged 18 years and older with a biopsy-confirmed diagnosis of CIN 3, a lesion involving at least one-third the surface area of the cervix, and no history of prior malignancy.

Baseline Evaluation

Prior to enrollment, all participants were evaluated by a complete medical history; physical examination; pelvic examination; Pap smear; gonorrhea and chlamydia cultures; HPV testing; colposcopic examination of the vulva, vagina, and cervix; risk-factor and dietary interview; and counseling regarding smoking cessation, nutrition, and sunscreen use.

Colposcopically directed biopsies from normal and abnormal areas were taken for permanent section and snap-frozen for studies of synthesis of polyamines, including ODC, putrescine, spermine, and spermidine. HPV testing was performed by dot-blot hybridization (ViraPap/ViraType®, Digene Diagnostics, Washington, DC); HPV-negative specimens were subjected to polymerase chain reaction analysis for confirmation. This protocol was reviewed by the Cancer Center's Institutional Review Board (Surveillance Committee), and each woman signed an informed consent form.

Polyamine Analysis

Polyamine analysis in tissue and blood was performed by one of the authors (K.N.). Tissues and blood samples were frozen at −70°C until analyses were performed. Each sample was analyzed in duplicate simultaneously. Samples consisted of a 25% tissue homogenate prepared in ornithine decarboxylase (ODC) buffer using a Polytron homogenizer (Brinkman Instruments, Westbury, NY) as described previously [15]. A portion of the homogenate (20 ml) was mixed with 80 ml of 5% sulfosalicylic acid, sonicated, and microcentrifuged (13,000*g*) for 15 minutes at room temperature to obtain a clear supernatant for polyamine analysis. The remaining portion of the homogenate was centrifuged (700*g*) for 15 minutes at 4°C, and the supernatant was analyzed for ODC activity and protein levels. Protein concentrations were determined using Bio-Rad (Richmond, CA) protein assay kits. A new procedure, using ophthalaldehyde in a Dionex BioLC high-performance liquid chromatograph equipped with a HPLC-CS2 column and postcolumn detection system (Dionex, Inc., Sunnyvale, CA), was used to determine levels of free polyamines [13]. Levels of arginine, ornithine, and DFMO were measured by the method of Grove et al. [16] using the same equipment. Tissue polyamine values are reported in nmol/mg soluble protein.

The ratio of spermidine (SPD) to spermine (SPM) was also calculated as a possible SEB; this parameter is a well-accepted mathematical method of compensating for variability of the measurements [17].

Statistical Analysis

Baseline polyamine values of colposcopically normal and abnormal tissue areas were compared among all patients. Because of the large variations in all polyamine measurements, data were analyzed by the Wilcoxon matched-pairs signed-rank test. Statistical significance was set at an alpha of 0.05 based on a two-sided test.

For the polyamine markers that were statistically significant, sample sizes for a clinical study using those markers as biomarkers were calculated using STPlan software. The distribution was plotted and found to be normal. A two-sided test was used, assuming an alpha of 0.05 and a power of 0.80.

RESULTS

The demographic characteristics of the study population were consistent with those of our colposcopy clinic population. The median age of the study group was 27 years (range 20 to 41 years); 70% were non-Hispanic whites, 23%

Hispanics, and 7% African-Americans. In 59% of the women, cervical lesions were no more than one-third the size of the surface area of the cervix; in 34%, lesions measured greater than one-third and less than two-thirds of the surface area; and in 7%, they covered greater than two-thirds of the cervix surface. Eighty-three percent of the women tested positively for HPV by dot-blot hybridization or polymerase chain reaction.

Baseline Polyamine Levels in Normal and Abnormal Tissue

To determine whether polyamine levels differ between normal and abnormal tissues, we measured baseline differences in tissue polyamines between normal and abnormal tissue areas sampled by colposcopically directed biopsy (Table I). Values of ODC, putrescine, spermidine, and spermine were higher in abnormal tissue areas than in normal tissue areas, whereas the SPD:SPM ratio was slightly lower in abnormal tissue areas. Only the differences in ODC value and SPD:SPM ratio were in the expected direction (ODC value higher and SPD:SPM ratio lower in abnormal tissue). The differences in ODC and SPD:SPM ratio between baseline normal and abnormal tissue areas were statistically significant ($P < 0.05$).

Since only the ODC value and SPD:SPM ratio were statistically significantly different between normal and abnormal tissue, those two markers would be suitable candidates for a clinical study examining the differences between normal and abnormal tissue. We, therefore, calculated sample sizes needed for a clinical study using a two-sided test with an alpha of 0.05 and a power of 0.80. The mean difference in ODC values was 55.7 nmol/ml (standard deviation [s.d.] 174.2); the corresponding calculated sample size is 79. The mean difference for the SPD:SPM ratio was 0.065 (s.d. 1.3); the calculated sample size is 3261. After the elimination of an outlier value, the mean difference for the SPD:SPM ratio was 0.30 (s.d. 0.33); the calculated sample size is 12.

TABLE I. Baseline Differences in Polyamine Values Between Colposcopically Normal and Abnormal Tissue†

Polyamine	Normal tissue	Abnormal tissue	Difference	*P* *
ODC (pmol/mg soluble protein/hr)				
Mean	282.6	338.3	55.7	0.03
SE	48.4	64.4		
Median	188.6	214.8		
Minimum	26.8	65.6		
Maximum	1151.3	1811.7		
Putrescine (pmol/mg soluble protein)				
Mean	505.0	1664.2	1159.2	0.97
SE	245.4	328.4		
Median	948.5	1462.0		
Minimum	364.0	68.0		
Maximum	5265.0	9144.0		
Spermidine (pmol/mg soluble protein)				
Mean	5105.5	5220.4	114.9	0.99
SE	735.9	657.1		
Median	3562.0	3802.0		
Minimum	1737.0	812.0		
Maximum	19108.0	15400.0		
Spermine (pmol/mg soluble protein)				
Mean	4299.7	5426.4	1126.7	0.13
SE	646.8	765.4		
Median	3342.0	3969.0		
Minimum	957.0	846.0		
Maximum	18197.0	16831.0		
SPD:SPM ratio				
Mean	1.26	1.19	−0.07	0.0012
SE	0.07	0.23		
Median	1.23	0.97		
Minimum	0.70	0.55		
Maximum	2.15	7.46		

†SE, standard error.
*Wilcoxon's signed-rank test.

DISCUSSION

The accessibility of the cervix allows clinicians to observe cervical lesions over time with colposcopy and Pap smears, making the cervix uniquely well suited to the development of chemoprevention strategies. Additionally, carcinogenesis in the cervix is cited by pathologists as an example of multistep tumorigenic progression from mildly dysplastic lesions to severely dysplastic lesions to invasive cancer [18]. Studies that focus on the pathobiology of cervical carcinogenesis will contribute to our understanding of the neoplastic process and allow the development of new preventive and therapeutic strategies. Thus, the lessons learned from the

TABLE II. Studies of Polyamine Levels: Normal Tissue Vs. Cancer

Marker	Positive study showing statistically significant differences (reference)	Negative study showing no statistically significant differences (reference)
RBC polyamines	Takami et al., 1979 [21] Solid tumor patients Takami and Nishioka, 1980 [22] Solid tumor patients	None
Plasma polyamines	Takami et al., 1979 [21] Solid tumor patients Loser et al., 1990 [23] Colon cancer patients	None
Bone marrow plasma polyamines	Nishioka et al., 1980 [24] Leukemia patients	None
Urine polyamines	Russell, 1971 [25] Cancer patients Loser et al., 1990 [23] Colon cancer patients	None
Tissue polyamines	Dimery et al., 1987 [26] Oral cavity tissue Upp et al., 1988 [27] Colon tissue Loser et al., 1990 [23] Colon tissue Hixson et al., 1993 [28] Colon tissue	Gray et al., 1993 [29] Esophagus cancer
Tissue ODC	Kingsnorth et al., 1983 [30] Colon tissue Koo et al., 1988 [31] Colon tissue LaMuraglia et al., 1986 [32] Colon tissue Moorehead et al., 1987 [33] Colon tissue Narisawa et al., 1989 [34] Colon tissue Porter et al., 1987 [35] Colon tissue Upp et al., 1988 [27] Colon tissue Nishioka et al., 1991 [36] Colon, rectal tissue Berdinskikh et al., 1991 [37] Stomach, colon tissue Hixson et al., 1993 [28] Colon tissue	None
Serum polyamines	Nishioka and Romsdahl, 1974 [38] Solid tumor patients Nishioka and Romsdahl, 1977 [39]; Nishioka et al., 1977 [40] Colorectal cancer patients	None
Cerebrospinal fluid polyamines	Takaue et al., 1986 [41] Pediatric brain tumor patients	None

cervix may provide useful paradigms for other less accessible sites.

There have been several well-designed chemoprevention trials for cervical lesions using topical retinoic compounds and micronutrients; most of these trials were considered negative [19]. However, Meyskens et al. demonstrated statistically significant histological regression in patients with CIN 2, but not CIN 3, using topical *trans*-retinoic acid [20]. The rate of re-

TABLE III. Studies of Polyamine Levels: Normal Tissue Vs. Dysplasia or Tissue at Risk*

Marker	Positive study showing statistically significant differences (reference)	Negative study showing no statistically significant differences (reference)
RBC polyamines	None	None
Plasma polyamines	None	None
Urine polyamines	None	None
Tissue polyamines	McGarrity et al., 1990 [42] Colon polyps	Garewal et al., 1988 [43] Barrett's esophagus tissue
	Meyskens et al., 1994 [17] Colorectal mucosa	Gray et al., 1993 [29] Barrett's esophagus tissue
Tissue ODC	Luk and Baylin, 1984 [44] Familial polyposis, partial colorectal biopsies	Lawson et al., 1989 [47] Transitional area beside colon cancers
	Garewal et al., 1988 [45] Esophagus Barrett's biopsies	Love et al., 1992 [48] Colon cancer vs. NPHCC and adenomas
	McGarrity et al., 1990 [42] Colon polyps	Braverman et al., 1990 [49] Colon cancer vs. NPHCC and adenomas
	Arlow et al., 1991 [46] Adenomatous polyps	Desai et al., 1992 [50] Adenomatous polyps
	Nishioka et al., 1991 [36] Adenomatous polyps	

*NPHCC, nonpolyposis hereditary colon cancer.

gression in the treated CIN 2 group was 43%, compared with a 27% rate in the placebo group. The cervix appears to be well suited to chemoprevention trials with pharmaceuticals.

Polyamine levels have been compared in cancerous lesions and normal tissues in other sites and have been demonstrated to differ significantly (Table II). Fewer studies have focused on precancerous lesions, in which differences from normal tissue in polyamine levels are less marked than those for cancers (Table III). In this study, polyamine values were noted to differ between colposcopically normal and abnormal areas and thus, were differentially expressed. Detecting polyamine value differences in precancerous lesions suggests that polyamines are altered at an early step in carcinogenesis.

Although the sensitivity and specificity of polyamine measurements are dependent on the laboratory, they may also differ by organ site. Several research groups have written extensively about polyamine measurement issues in other organs, especially the colon [28, 46]. A notable feature of polyamine measurement is the inter-individual variation in measurement; studies require large numbers of patients to achieve statistical significance. Despite their variability, polyamine markers have been shown by several investigators to be modulated by DFMO [17, 51–53].

There are several reasons why chemoprevention is attractive as a treatment for cervical lesions. These reasons reflect the belief that precancers, like cancers, represent a systemic process [11]. Many of the patients who undergo colposcopy smoke (44% of patients in our population), and many patients also have metaplastic and neoplastic lesions of the aerodigestive tract [19]. Infection with HPV affects the entire squamous epithelium of the female genital tract, and up to 40% of patients with CIN have multifocal lesions of the vagina, vulva, and perianal area [19]. Chemoprevention may become a treatment of choice for the woman who smokes, has HPV infection, or has multifocal intraepithelial neoplasia of the cervix, vagina, and vulva. Another group for which chemoprevention may be an excellent choice is women who would tolerate surgical procedures poorly, for example, those who are immunodepressed owing to HIV infection, rheumatologic disease, renal failure, or the use of immunosuppressive medications.

Clinical chemoprevention trials using surrogate endpoint biomarkers that reflect the process of carcinogenesis will contribute to our understanding of the multistep neoplastic process. Lessons learned from the cervix may well unravel some of the mystery of squamous carcinogenesis and provide insight into new molecular therapies for other squamous neoplasms.

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