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Serum polyamines in pre- and post-operative patients with breast cancer corrected by menopausal status

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ABSTRACT

Polyamines are essential for cell growth and differentiation of cells and its increased production is observed in many cancers. Due to one of key enzymes in polyamine synthesis ornithine decarboxylase is stimulated by estrogens in breast cancer cells, the polyamine levels in serum samples obtained from patients with breast cancer (BCa; pre-menopause: 45.6 ± 0.8 years old, $n = 58$; post-menopause: 55.2 ± 1.1 years old; $n = 11$), who were also classified as pre- and post-surgery, and from normal controls (pre-menopause: ages 43.6 ± 0.9 years, n = 45, post-menopause: ages 55.2 ± 1.0 years, n = 18) were determined by liquid chromatography–mass spectrometry. In pre-menopausal patients, the concentrations of 1,3-diaminopropane, *N*-acetyl putrescine (*N*-actPut), *N*-acetyl spermidine and spermidine (Sp) were higher ($p < 0.05$) in pre-surgery breast cancer patients than in normal controls, with *N*-actPut ($p < 0.0005$) and Sp ($p < 0.005$) levels markedly increased. All polyamine levels were slightly decreased post-surgery, and were comparable to levels in normal subjects. Based on menopausal-dependent data, the results imply that serum polyamines levels may correlate with estrogen levels in association with estrogen-induced cell growth and ornithine decarboxylase activity.

1. Introduction

Polyamines play an important role in cell growth, proliferation, and the synthesis of proteins and nucleosides [1]. In mammalian cells, polyamine biosynthesis begins with putrescine, which is derived from ornithine catalyzed by ornithine decarboxylase (ODC). Subsequent addition of an aminopropyl group to putrescine leads to the synthesis of spermidine, and further addition of another aminopropyl group forms spermine [2,3]. In addition, the catabolic enzymes spermidine/spermine-N^1-acetyltransferase (SSAT) and polyamine oxidase h1 (PAOh1/SMO) are responsible for conversion of polyamines into their acetylated molecules (Fig. 1) [4,5].

Polyamines are scavengers of reactive-oxygen species and thereby protect DNA, protein, and lipids from oxidative damage [6]; thus, they have been investigated as anti-cancer agents and as tumor-markers [7–10]. Quantitative analysis of major polyamines (putrescine, spermidine, spermine) and their acetyl forms in various biological specimens of many cancers explains their altered biosynthesis and accumulation [7,11,12]. These altered polyamines can be useful molecular markers in the evaluation of treatment efficacy, as well as in the diagnosis of malignant tumor activity [13,14].

In general, the extensive exposure to ionizing radiation incurs an increased risk of breast cancer (BCa), and reproductive and hormonal history also play a role. Women who started menstruating at an early age or go through menopause are at slightly increased risk and estrogen replacement therapy after menopause has also been linked to small increases in BCa risk [15]. ODC is stimulated by

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estrogens in estrogen-responsive tissues [16,17]. The mitogenic effect of estrogen may require the synthesis of polyamines in BCa cells [18], and polyamines are required in estrogen stimulation of human BCa cells [19].

Based on the hypothetical association between estrogen-induced cell growth and ODC activity, this study was designed to evaluate serum polyamine levels along with operative conditions in both pre- and post-menopausal BCa patients compared to healthy subjects using a validated liquid chromatography–tandem mass spectrometry (LC–MS/MS).

2. Materials and methods

2.1. Chemicals

1,3-Diaminopropane [1,3-Dap], putrescine [Put], cadaverine [Cad], *N*-acetylputrescine [*N*-actPut], *N*-acetylcadaverine [*N*-actCad], spermidine [Spd], *N*-acetylspermidine [*N*-actSpd], spermine [Sp], N^1-acetylspermine [N^1-actSp], and 1,6-diaminohexane (an internal standard, [ISTD]) were purchased from Sigma (St. Louis, MO, USA). HPLC-grade ethyl ether, *n*-pentane and methanol were obtained from J.T. Baker (Phillipsburg, NJ, USA). Isobutyl chloroformate (IBF) was also purchased from Sigma. Each polyamine stock solution was prepared at 1000 μg/mL in methanol, and working solutions were made with methanol at varying concentrations from 0.01 to 10 μg/mL. All standard solutions were stored at −20 °C until use.

2.2. Sample collection

Serum samples of both BCa patients (pre-menopause: age 45.6 ± 0.8, $n = 58$, post-menopause: age 55.2 ± 1.1, $n = 11$) and healthy female subjects (pre-menopause: age 43.6 ± 0.9, $n = 45$, post-menopause: age 55.2 ± 1.0, $n = 18$) were collected from Samsung Hospital (Seoul, Korea) and Hanyang University (Seoul, Korea). All serum samples were collected in the morning after a 12 h fast. The subjects were not treated with, or exposed to, any drugs or chemicals for a defined period, before the serum was collected. The Ethics Committee of both medical centers approved and all patients provided written informed consent prior to this study. All samples were stored at −20 °C until analyzed.

All study subjects underwent the same diagnostic procedures, i.e., a breast physical examination, mammography, and ultrasonography as devised by the American Joint Committee on Cancer staging. All BCa patients were in the stage II and they underwent surgery of the modified radical mastectomy (MRM) or lumpectomy with an auxillary lymph node dissection. Pre- and post-surgery samples were collected before or 2-weeks after surgery. The sex- and age-matched normal controls had no evidence of benign or malignant breast disease. Menopausal conditions were determined based on both no menses for a half year and the blood test for follicle-stimulating hormone and 17β-estradiol. All menopausal volunteers experienced natural menopause. Information concerning known risk factors for BCa is summarized in Table 1 for both BCa patients and the controls. Pre-menopausal patients had slightly higher age and body mass index than the controls, and post-menopausal controls had slightly higher weight and body mass index; however, possibly because of lack of statistical power, none of the considered risk factors differed significantly between patients and control groups.

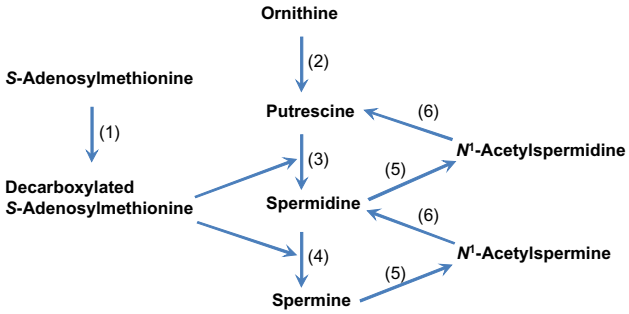

Fig. 1. The polyamine metabolic pathway in mammalian cells. (1) *S*-Adenosylmethionine decarboxylase, (2) ornithine decarboxylase, (3) spermidine synthase, (4) spermine synthase, (5) spermidine/spermine-N^1-acetyltransferase, (6) polyamine oxidase (modified from Refs. [4,5]).

2.3. Sample preparation

The serum polyamine levels were measured by our validated analytical method [20]. In brief, a 200 μL of serum was diluted with 1 mL of deionized water and mixed for 1 min. After heating at 60 °C for 20 min to precipitate the proteins, 50 μL ISTD (0.1 μg/mL) was added and the pH was adjusted to pH 9.0 by adding 25 μL sodium–carbonate buffer (1.0 M, pH 9.0). Amino groups were carbamoylated by addition of 20 μL isobutyl chloroformate and incubation at 35 °C for 15 min. After cooling, the solution was extracted twice with 2 mL of diethyl ether, and the combined organic solvent was evaporated under a gentle nitrogen steam at room temperature. The residue was reconstituted with 100 μL of a mixture of 0.2% acetic acid and 0.2% acetic acid/acetonitrile (50:50, v/v) and then a 30 μL aliquot was injected into the LC–MS system.

2.4. Liquid chromatography–mass spectrometry

Chromatographic separation was performed with a Shiseido Nanospace SI-2 HPLC system (Shiseido Co., Tokyo, Japan) coupled to a Shiseido MG C18 column (5 μm, 150 × 1.5 mm i.d.). A gradient eluent (0.2% acetic acid and 0.2% acetic acid in acetonitrile) was used at 100 μL/min.

All data were analyzed on a Thermo LCQ Advantage ion-trap MS equipped with electrospray (ESI) (Thermo, San Jose, CA, USA) operated in the positive ionization mode. The operating conditions were set as follows: spray voltage, 6 kV; capillary voltage, 4 V; tube lens offset voltage, 40 V; sheath gas flow rate, 30 U; and capillary temperature, 250 °C. In MS/MS analysis, the protonated molecular mass as precursor ions were dissociated by helium gas.

3. Results

Serum polyamine levels in pre-menopausal BCa patients, who were classified as pre- and post-surgery, were first compared to age-matched the controls. The polyamine concentrations in sera of 58 pre-menopausal

BCa patients and 45 healthy subjects are shown in Table 2. Most polyamine levels were higher in pre-menopausal BCa patients than in the controls, except Cad and N^1-actSp. The polyamine levels of pre- and post-surgery pre-menopausal patients were also compared. All polyamines levels were slightly decreased post-surgery, but they were not significant to the levels of normal controls. Statistical analyses for the level of significance between pre- and post-surgery were conducted by *t*-tests, and the differences were not significant. The results show that 1,3-Dap, *N*-actPut, *N*-actSpd, and Sp were significantly higher ($p < 0.05$) in pre-surgery BCa patients than in the controls; *N*-actPut ($p < 0.0005$) and Sp ($p < 0.005$) levels were markedly increased (Table 3). In comparing of post-surgery patients with the controls, 1,3-Dap and Sp were higher in post-surgery patients ($p < 0.05$), whereas Cad and N^1-actSp were higher in the controls ($p < 0.05$; Fig. 2).

In addition, the serum polyamine levels were measured in both 18 post-menopausal patients and 11 age-matched healthy subjects; these BCa patients were also classified as pre- and post-surgery (Table 4). In contrast to the pre-menopausal data, 1,3-Dap, Put, Cad, and *N*-actPut levels were higher in post-menopausal BCa patients than in the controls. *N*-actCad, Spd, *N*-actSpd, Sp, and N^1-actSp levels in the controls were higher than those of post-menopausal patients. However, the differences in the polyamine levels among the three groups (pre-surgery patients, post-surgery patients, and healthy subjects) were not statistically significant.

4. Discussion

In our previous reports, urinary and serum polyamine levels were closely associated with breast cancer [10,20]. Since age at menopause may be one of the most important occasions in breast cancer [21], we classified the patients as pre- and post-menopausal. Serum polyamine levels of pre-menopausal BCa patients were increased, except Cad and N^1-actSp, while those of post-menopausal patients were not altered compared to control subjects in this study. In general, menopause is responsible for decreasing ovarian estrogen production and the menopausal transition is accompanied by a number of biological changes. In post-menopausal women, estradiol (E2) is no longer a circulating hormone, although it continues to be formed in many extragonadal sites including breast, bone, vascular smooth muscle, and various sites in the brain [22]. This indicates that alterations in hormonal compounds could be detected along with the menopausal status. Although pre-surgery patients have slightly higher BMI values than the control, we do not believe this to be a factor based on our analytical results.

Tumor regression induced by ovariectomy is associated with a decline in the polyamines putrescine (Put), spermidine (Spd), and spermine (Sp). At the same time, E2 affects the levels of ornithine decarboxylase (ODC) and *S*-adenosylmethionine decarboxylase (SAMDC); E2 has been shown to increase the levels of ODC and polyamines in male hamsters [23]. In MCF-7 BCa cells, polyamines were stimulated by E2 in presence of growth factor [19], and ODC activity was greater in estrogen-treated cells [24]. Also, the polyamine pathway plays an essential role in the growth-promoting effect of E2-regulated growth factors in *N*-nitrosomethylurea (NMU)-induced rat mammary tumor cultures [25]. These reports explain the association of E2 and polyamines in BCa, in which E2 increases polyamine levels or stimulates polyamine biosynthesis. Therefore, the objective of this study was to evaluate serum polyamine levels in BCa patients classified both pre- and post-menopausaland to compare those with healthy subjects.

Table 1
Mean values of common risk factors in both patients with beast cancer and healthy subjects as the control

Gender	Pre-menopause Mean ± SEM, range					Post-menopause Mean ± SEM, range				
	Controls ($n = 58$)		Patients ($n = 45$)		*P* value	Controls ($n = 18$)		Patients ($n = 11$)		*P* value
	Female					Female				
Age (y)	43.6 ± 0.9	34–51	45.6 ± 0.8	34–57	0.066	55.2 ± 1.1	46–66	55.2 ± 1.0	48–60	0.992
Weight (kg)	59.7 ± 7.4	42.8–75.3	59.8 ± 7.5	45.0–77.4	0.172	60.7 ± 15.2	52.4–74.7	58.8 ± 17.7	51.8–67.6	0.074
Height (cm)	157.8 ± 24.9	149.8–167.0	157.8 ± 20.9	149.5–165.6	0.910	155.9 ± 39.0	156.4–160.7	158.3 ± 47.7	148.0–163.0	0.166
Body mass index*	23.2 ± 3.7	18.0–31.3	24.0 ± 3.2	19.2–31.2	0.159	25.0 ± 6.24	20.8–30.7	23.5 ± 7.08	20.1–27.5	0.130

* Weight in kg/squared height in m.

Table 2
Serum polyamine concentrations (ng/mL) pre- and post-surgery in pre-menopausal patients with breast cancer and in the controls

Polyamines	Control (n = 45)		Pre-surgery (n = 58)		Post-surgery (n = 58)	
	Mean ± SEM	Range	Mean ± SEM	Range	Mean ± SEM	Range
1,3-Dap	5.48 ± 0.98	0.23–24.49	11.67 ± 2.06	0.11–61.75	11.84 ± 2.36	0.12–83.58
Put	30.44 ± 2.89	3.46–71.73	40.02 ± 4.49	10.19–163.86	35.66 ± 7.67	3.75–108.25
Cad	5.96 ± 0.55	1.21–14.51	4.65 ± 0.50	0.04–17.47	4.22 ± 0.45	0.19–15.90
N-actPut	10.23 ± 1.60	0.33–60.97	16.66 ± 3.12	0.60–109.04	12.09 ± 1.53	0.54–53.80
N-actCad	1.12 ± 0.19	0.03–4.20	2.04 ± 0.28	0.14–8.67	1.38 ± 0.12	0.28–2.77
Spd	11.76 ± 1.2	3.23–40.27	15.72 ± 2.09	1.33–82.50	14.83 ± 2.05	0.75–107.40
N-actSpd	8.59 ± 0.41	3.64–18.61	10.94 ± 0.79	2.49–32.87	9.44 ± 0.68	0.55–23.88
Sp	3.19 ± 0.41	0.16–13.58	6.23 ± 0.79	0.03–29.52	4.75 ± 0.62	0.08–21.83
N^1-actSp	3.30 ± 0.14	1.70–5.28	2.80 ± 0.20	0.00–7.09	2.45 ± 0.61	0.04–4.86

Table 3
The P values between pre- and post-surgery of pre-menopausal patients with breast cancer and the controls

Polyamines	Normal vs. pre-surg.	Pre- vs. post-surg.	Normal vs. post-surg.
1,3-Dap	0.05	NS	0.05
Put	NS	NS	NS
Cad	NS	NS	0.05
N-actPut	0.0005	NS	NS
N-actCad	NS	NS	NS
Spd	NS	NS	NS
N-actSpd	0.05	NS	NS
Sp	0.005	NS	0.05
N^1-actSp	NS	NS	0.05

Pre-surg., pre-surgery; Post-surg., post-surgery; NS, not significant.

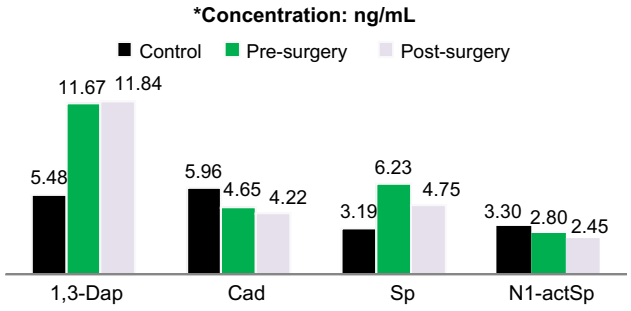

Fig. 2. The altered polyamine levels in serum obtained from control and pre- and post-surgery patients with breast cancer in pre-menopausal conditions. 1,3-Dap, 1,3-diaminopropane; Cad, cadaverine; Sp, spermine; N1-actSp, N^1-acetylspermine.

Based on estrogen-induced cell growth and ODC activity, serum levels of both polyamine and estrogen could be associated. The results that the increased polyamines in pre-menopausal BCa patients return to the levels of the controls after surgery and it may support our hypothesis on the correlation of polyamine and estrogen levels along with menopausal status. In particular, Sp levels can be a positive parameter for breast cancer diagnosis [26]. In addition, all BCa patients were not taking any hormone replacements during defined periods, suggesting these results may not be affect by exogenous estrogens. The lack of clinical implications in this study may be, however, addressed. One limitation of the study is that urine samples were not collected on all patients so an association between urine and serum levels was not able to be determined.

In conclusion, this study compared serum polyamine levels in both pre- and post-menopausal BCa patients to the controls. Serum polyamines were higher in pre-menopausal BCa patients, while those in post-menopausal patients were similar to the controls. Also, all polyamines levels decreased slightly in post-surgery patients and they were comparable to healthy subjects. The result suggests that serum polyamine levels correlate with estrogen levels, along with menopausal status, due to the association of estrogen-induced cell growth and ODC activity. In this study, we could not give an answer about a risk factor for future occurrences of BCa, but the quantitative results in various biological conditions tested may be useful for evaluating the efficacy of medical surgery and chemoprevention in BCa.

Table 4
Serum polyamine concentrations (ng/mL) between pre- and post-surgery in post-menopausal patients with breast cancer and the controls

Polyamines	Control (n = 18)		Before-surgery (n = 11)		After-surgery (n = 11)	
	Mean ± SEM	Range	Mean ± SEM	Range	Mean ± SEM	Range
1,3-Dap	6.67 ± 1.29	0.89–18.71	11.41 ± 3.69	0.12–34.13	4.33 ± 1.48	0.04–13.78
Put	38.76 ± 3.73	4.43–70.03	39.70 ± 10.53	12.09–135.33	43.56 ± 9.26	11.99–118.92
Cad	5.41 ± 0.94	1.56–14.60	9.53 ± 4.19	0.73–50.27	4.88 ± 0.89	1.11–9.94
N-actPut	16.66 ± 3.06	5.06–53.98	19.30 ± 4.77	1.31–46.45	16.46 ± 3.86	1.23–40.26
N-actCad	1.25 ± 0.42	0.25–5.17	0.46 ± 0.10	0.26–0.84	0.37 ± 0.12	0.00–2.35
Spd	22.19 ± 3.86	4.45–33.97	9.04 ± 2.18	0.53–20.93	12.26 ± 3.37	2.77–39.37
N-actSpd	9.48 ± 0.57	2.43–13.24	9.51 ± 1.08	5.87–16.02	9.25 ± 1.45	1.58–16.23
Sp	4.85 ± 1.66	0.18–28.39	4.68 ± 0.58	2.46–7.95	2.63 ± 0.57	0.22–5.11
N^1-actSp	3.12 ± 0.23	0.99–4.81	2.85 ± 0.40	0.81–4.28	2.81 ± 0.47	0.23–5.51

Conflict of interest

All authors does not conflicts of interest.

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