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ORIGINAL ARTICLE

Altered urinary polyamine patterns of cancer patients under acupuncture therapy

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Abstract The reduction of elevated polyamine (PA) levels in biological fluids of cancer patients were known to be correlated with remission following diverse therapeutic treatments. In this study, altered urinary PA levels from three different cancer cases were monitored at different intervals during the long-term weekday acupuncture treatments. Nine urinary PA levels from 16 normal and three cancer patients with different types were measured by gas chromatography–mass spectrometry in selected ion monitoring mode as *N*-ethoxycarbonyl-*N*-pentafluoropropionyl derivatives. Their levels measured at three follow-up stages for each patient were then normalized to the corresponding normal group means and plotted into star symbol patterns. Large alterations of PA levels were observed for each patient. Each normalized concentration displayed elevation of the PA levels in multiples (0.0–57.7) of the respective normal mean values. The normalized PA values were transformed into distorted star patterns which were characteristic of each follow-up stage and of cancer type.

Keywords Cancer · Acupuncture · Urinary polyamines · Ethoxycarbonyl-pentafluoropropionyl derivatives · Gas chromatography–mass spectrometry in selected ion monitoring · Star pattern recognition

Abbreviations

PAs	Polyamines
N-EOC-*N*-PFP	*N*-Ethoxycarbonyl-*N*-pentafluoropropionyl
GC–MS	Gas chromatography–mass spectrometry
SIM	Selected ion monitoring
ECF	Ethyl chloroformate
PFPA	Pentafluoropropionyl anhydride
ANOVA	Analysis of variance
MANOVA	Multivariate analysis of variance

Introduction

The endogenous aliphatic polyamines (PAs) and their *N*-acetylated PAs occurring as cellular constituents are essential for the regulation of cell proliferation and differentiation (Morgan 1999; Igarashi and Kashiwagi 2000; Gugliucci 2004; Moinarda et al. 2005). Their levels are maintained by regulating the activity levels of the biosynthetic and catabolic reactions in polyamine metabolism. However, the levels of PAs in body fluids were significantly higher in various cancer patients as compared to normal controls (Wallace et al. 2000; Choi et al. 2001; Khuhawar and Qureshi 2001; Criss 2003; Lee et al. 2003; Thomas and Thomas 2003; Inoue et al. 2005; Gerner et al.

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2007). Increased polyamine synthesis has been linked to cell growth and cancer since the 1960s, but the mechanistic basis of this association has only been elucidated during the past decade (Criss 2003; Thomas and Thomas 2003; Gerner and Meyskens 2004). New advances in understanding the roles of polyamines in cancer have been discussed in recent years (Gerner and Meyskens 2004). PA depletion with the improvement in the patient's conditions on multiple therapies was noticed in cancer patients (Davidson et al. 1999; Devens et al. 2000; Khuhawar and Qureshi 2001; Seiler 2003; Thomas and Thomas 2003; Basuroy and Gerner 2006; Gerner et al. 2007). The accurate determination of the PAs as cancer markers has thus become an important task in timely diagnosis of cancer, therapeutic monitoring during the polyamine depletion therapy and development of anticancer drugs as well.

Acupuncture has been practiced as a relatively safe treatment of cancer with a low side effect profile in palliative cancer care (Filshie 2001; MacPherson et al. 2001; White et al. 2001; Samuels 2002; Cohen et al. 2005; Filshie and Hester 2006; Price et al. 2006). Expert-reviewed information summary about acupuncture as a treatment for cancer or cancer-related disorders is richly available in the literature (Cohen et al. 2005). Moreover, aspects of its safe treatment modality in cancer patients have been extensively surveyed (MacPherson et al. 2001; White et al. 2001). In recent year, clinical guidelines for the safe practice of acupuncture have been systematically developed to provide acupuncture treatment for cancer patients (Filshie and Hester 2006). Acupuncture works by modulating several endogenous analgesic mechanisms and also works via multiple central connections and descending pain inhibition via noradrenergic and serotinergic pathways to give pain relief throughout the body (Filshie and Thomson 2004). Acupuncture causes the release of a number of endogenous substances including beta-endorphin, met-enkephalin and dynorphins (Pomeranz 2001). Serotonin (Han and Terenius 1982), oxytocin (Uvnas-Moberg 1997) and endogenous steroids (Roth et al. 1997) released following acupuncture treatment were reported as potential substances related with analgesia. Acupuncture can also enhance the immune system by increasing the white blood cells that have been depleted through chemotherapy (Wei 1998; Ye et al. 2002). In addition, the immunomodulatory effects of acupuncture, both via the release of pituitary beta-endorphin and adrenocorticotropic hormone as well as alleviating patient stress through relief of symptoms, are now appreciated to be anti-carcinogenic (Pomeranz 2001). All these findings support that acupuncture definitely affects a complex network of metabolic inter-relationships, leading to significant changes in the levels of other metabolites such as PAs which are biomarkers for cancer. No approaches with metabolic profiling analyses were, however, attempted to measure PA levels related with various clinical stage, remission and relapse following acupuncture treatment in cancer patients.

This work discusses our recent investigation on the monitoring of urinary levels of nine PAs such as putrescine, cadaverine, spermidine, spermine, N^1-acetylputrescine, N^1-acetylcadaverine, N^1-acetylspermidine, N^8-acetylspermidine and N^1-acetylspermine which were available as their pure standards in three cancer patients at irregular intervals for the long duration of weekday acupuncture therapy. The patients were selected based on their positive responses to the acupuncture treatment. The age-matched eight healthy males and eight females were served as the normal groups. The target profiling analysis of the nine PAs as *N*-ethoxycarbonyl-*N*-pentafluoropropionyl (*N*-EOC-*N*-PFP) derivatives was achieved by gas chromatography–mass spectrometry (GC–MS) in selected ion monitoring (SIM) mode according to our previous method (Paik et al. 2006). Gender difference of each PA in normal subjects was examined by one-way analysis of variance (ANOVA) and one-way multivariate analysis of variance (one-way MANOVA) (Krzanowski 1988). *F*-test using ratio of variances of two groups was performed to access the equality of the two standard deviations (Snedecor and Cochran 1989). The star symbol plotting as the visual pattern recognition tool (Paik et al. 2006, 2007) was also applied to the changes in the urinary PA levels of each cancer patient induced by the acupuncture treatment as referenced to the normal group average.

Materials and methods

Chemical and reagents

Putrescine, cadaverine, spermidine, spermine, N^1-acetylputrescine, N^1-acetylcadaverine, N^1-acetylspermidine, N^8-acetylspermidine, N^1-acetylspermine, 1,6-diaminohexane, ethyl chloroformate (ECF) and pentafluoropropionyl anhydride (PFPA) were purchased from Sigma-Aldrich (St Louis, MO, USA). Diethyl ether, ethyl acetate, dichloromethane and sodium chloride of pesticide grade were obtained from Kanto (Tokyo, Japan). Sodium hydroxide was from Duksan (Seoul, Republic of Korea) and all other chemicals were of analytical grade.

Urine samples

Urine specimens from the following three cancer patients were collected thrice at individually different intervals when positive effects of acupuncture were significantly noticed: a rectal cancer patient (male, 55 years) as the Case 1, a lung cancer patient (female, 52 years) as the

Case 2 and a breast cancer patient (female, 59 years) as the Case 3. Each patient had been receiving a weekday acupuncture therapy without any medication under strict diet. The urine samples from age-matched 16 normal controls (aged from 50 to 58 years; eight males and eight females) were provided by Hanaro Clinic Laboratories, Hanaro Medical Institute (Seoul, Republic of Korea). All urine samples were immediately stored at −70°C until being analyzed.

Gas chromatography–mass spectrometry

GC–MS analyses in both scan and SIM modes were performed using an Agilent 6890 gas chromatograph, interfaced with an Agilent 5973 mass-selective detector (70 eV, electron impact mode) and installed with an Ultra-2 (5% phenyl–95% methylpolysiloxane bonded phase; 25 m × 0.20 mm id, 0.11 μm film thickness) cross-linked capillary column (Agilent Technologies, Atlanta, GA, USA). The temperatures of injector, interface and ion source were 260, 300, and 230°C, respectively. Helium was used as the carrier gas at a flow rate of 0.5 mL min^{-1} with constant flow mode. Samples were introduced in the split-injection mode (10:1), and the oven temperature was initially maintained at 100°C for 2 min and then programmed to 300°C (5 min) at a rate of 10°C min^{-1}. In the scanning mode, the mass range was 50–800 U at a rate of 0.42 scans s^{-1}.

Sample preparation for assay of urinary polyamines

According to the previous method (Paik et al. 2006), aliquots of each urine sample (equivalent to 0.25 mg of creatinine) were adjusted to pH ≥ 12 after addition of 1,6-diaminohexane (200 ng) which was used as the internal standard. Briefly, a two-phase EOC reaction in aqueous phase was immediately conducted in one step by vortex mixing (10 min) with ECF (20 μL) present in dichloromethane phase (1 mL). The mixture was then saturated with sodium chloride and sequentially extracted with diethyl ether (3 mL) and ethyl acetate (2 mL). The combined extracts were evaporated down to ca. 20 μL under a gentle stream of nitrogen (40°C), with subsequent PFP derivatization by reacting (60°C for 30 min) with PFPA (20 μL) for analysis by GC–MS in SIM mode.

Statistical analysis

Gender difference of each PA in normal subjects was examined by one-way ANOVA) and calculated F value and probability ($>F$) for each PA. F-test using ratio of variances of two groups as calculated F value was examined to access the equality of two standard deviations. In addition to this, one-way MANOVA with PA levels as the dependent variables and gender as the independent variable was used to test whether gender groups appear to differ on PA levels collectively. Test statistics used for one-way MANOVA were Wilks' Lambda, Pillai's Trace, Hotelling–Lawley Trace and Roy's Maximum Root. Concentrations of nine PAs or three PAs (selected by one-way ANOVA) were used as sets of dependent variables for one-MANOVA. One-way ANOVA, F-test for equality of two standard deviations and one-way MANOVA were evaluated with stats module of R 2.6.1 (R Development Core Team, http://r-project.org).

The PA levels of each patient sample were normalized to the corresponding normal mean values. Each normalized value was then plotted as a line radiating from a common central point and the far ends of the lines were joined together to produce star patterns using MS Excel program as described elsewhere (Paik et al. 2006, 2007).

Results and discussion

Urinary polyamine levels in normal subjects

The levels of nine urinary PAs measured for normal subjects (aged 50–58 years) of eight males and eight females showed large variations among the individuals within each group (Table 1). N^1,N^{12}-diacetylspermine was not measured because it was not available as pure standard for accurate measurement of its urinary levels. As expected, the mean levels of N-acetylated PAs were higher as compared to their respective free PAs. In contrast to the young people (aged 22–37 years) examined in our previous work (Paik et al. 2006), the present aged group (50–58 years) excreted lesser amounts of PAs except for N^1-acetylspermine which showed similar values. Moreover, the level of N^1-acetylputerscine was comparable with that of N^1-acetylcadaverine unlike the younger people reported previously (Davidson et al. 1999; Khuhawar and Qureshi 2001; Inoue et al. 2005; Paik et al . 2006).

The marked gender difference was made by the three N-zacetylated PAs (Table 1). The mean levels of N^1-acetylputrescine [Pr ($>F$) = 0.021], N^1-acetylcadaverine [Pr ($>F$) = 0.039] and N^1-acetylspermidine [Pr ($>F$) = 0.047] were significantly elevated in normal female group as compared with normal male group. The result of F-test for equality of two standard deviations is also shown in Table 1 and there were significant differences in standard deviations of five PA levels by gender groups, meaning that standard deviations of PA levels of female group are significantly wider than those of male group. The gender difference was also collectively examined by one-way MANOVA (Table 2). Even though all of nine PAs did not show a significant gender difference in any of the test

Table 1 Urinary levels of aliphatic and *N*-acetylated polyamines in normal subjects

Polyamine	Concentration (nmol mg^{-1} of creatinine) Normal subject (Aged 50–58 years)						One-way ANOVA for means		*F*-Test for variance homogeneity	
	Male ($n = 8$)			Female ($n = 8$)			*F* value	*Pr* (>*F*)	*F* value	*Pr* (>*F*)
	Mean ± SD	Variance	Range	Mean ± SD	Variance	Range				
N^1-Acetylputrescine	1.05 ± 0.48	0.2287	0.53–1.80	2.35 ± 1.33	1.7813	1.04–4.76	6.772	0.021*	7.79	0.007*
N^1-Acetylcadaverine	1.07 ± 0.20	0.0417	0.85–1.35	2.64 ± 1.94	3.7684	1.00–6.63	5.193	0.039*	90.28	$<1 \times 10^{-5}$*
Putrescine	0.18 ± 0.09	0.0074	0.03–0.34	0.25 ± 0.15	0.0216	0.13–0.58	1.364	0.262	2.90	0.091
Cadaverine	0.09 ± 0.06	0.0042	0.02–0.21	0.13 ± 0.09	0.0074	0.00–0.28	1.198	0.292	1.77	0.233
N^1-Acetylspermidine	0.75 ± 0.37	0.1404	0.37–1.51	1.01 ± 1.01	1.0147	0.12–2.68	0.482	0.499	7.23	0.009*
N^8-Acetylspermidine	0.37 ± 0.37	0.1348	0.09–1.17	1.10 ± 0.88	0.7746	0.08–2.50	4.734	0.047*	5.75	0.017*
Spermidine	0.22 ± 0.16	0.0260	0.07–0.49	0.22 ± 0.17	0.0278	0.02–0.52	0.003	0.959	1.07	0.467
N^1-Acetylspermine	0.15 ± 0.18	0.0318	0.00–0.56	0.16 ± 0.32	0.1005	0.00–0.89	0.014	0.907	3.16	0.076
Spermine	0.41 ± 0.16	0.0259	0.28–0.79	0.45 ± 0.34	0.1133	0.28–1.21	0.107	0.749	4.37	0.035*

* Significantly different at the level of $\alpha = 0.05$

Table 2 Test statistics of one-way MANOVA with concentrations of polyamines used as dependent variables

Variable set	Test statistics	Value	*F*	Degrees of freedom		*Pr* (>*F*)
Nine polyamines	Wilks' lambda	0.260	1.896	9	6	0.225
	Pillai's trace	0.740	1.896	9	6	0.225
	Hotelling–Lawley trace	2.845	1.896	9	6	0.225
	Roy's maximum root	2.845	1.896	9	6	0.225
Three polyamines:	Wilks' lambda	0.479	4.346	3	12	0.027*
N^1-Acetylputrescine	Pillai's trace	0.521	4.346	3	12	0.027*
N^1-Acetylcadaverine	Hotelling–Lawley trace	1.086	4.346	3	12	0.027*
N^8-Acetylspermidine	Roy's maximum root	1.086	4.346	3	12	0.027*

* Significantly different at the level of $\alpha = 0.05$

statistics [*Pr* (>*F*) = 0.225], three PAs selected by one-way ANOVA revealed a significant gender effect by all of the four test statistics [*Pr* (>*F*) = 0.027]. Even if homogeneity of variances and covariances across the range of predictor variables of one-way MANOVA was assumed, Pillai's Trace was considered to be robust with the problems of sample sizes and homogeneity. Moreover, all of four test statistics gave the same probability level. Therefore, it was safely concluded that gender groups appear to differ on those three PA levels collectively. This required the gender of a patient to be matched with that of normal group before evaluation of therapeutic effects of acupuncture on cancer.

Urinary polyamine levels in three cancer patients under acupuncture treatment

As documented in the literature (Khuhawar and Qureshi 2001; Criss 2003; Lee et al. 2003; Thomas and Thomas 2003; Gerner and Meyskens 2004; Inoue et al. 2005), the urinary levels of three to five PA levels measured in three cancer patients before acupuncture treatment (first follow-up) were much higher (Table 3) than the respective normal values (Table 1). However, each cancer patient showed very different PA excretion patterns from each other. In the male patient (Case 1) with rectal cancer (aged 55 years), the levels of N^1-acetylputrescine, putrescine and N^8-acetylspermidine were more than two orders of magnitude higher than the respective mean values of normal male group. Similar elevation occurred only for N^1-acetylputrescine, putrescine and cadaverine in the female patient (Case 2) with lung cancer (aged 52 years) as compared with the normal mean values of female group. For the female patient (Case 3) with breast cancer (aged 59 years), the level of cadaverine was extremely higher (more than 50 times) than that for the female mean value, followed by N^1-acetylspermidine, putrescine, N^1-acetylcadaverine and N^1-acetylputrescine.

Large alterations of urinary PA levels were observed for each patient at the second and third follow-up testing stages during the long-term weekday acupuncture therapy (Table 3). In Case 1, the level of cadaverine was below its quantitation limit in all stages. In the second follow-up stage (after 2.7 month's treatment), the levels of three PAs

Table 3 Urinary levels of aliphatic and *N*-acetylated polyamines measured in three cancer patients during a long-term weekday acupuncture therapy

Polyamine	Mean concentration ± SD (nmol mg^{-1} of creatinine) (Normalized concentration)[a]								
	Case 1 (rectal cancer, male, 55 years)			Case 2 (lung cancer, female, 52 years)			Case 3 (breast cancer, female, 59 years)		
	Follow-up stage (month)[b]			Follow-up stage (month)[b]			Follow-up stage (month)[b]		
	1st (0)	2nd (2.7)	3rd (4.3)	1st (0)	2nd (1.2)	3rd (4.6)	1st (0)	2nd (2.0)	3rd (3.3)
N^1-Acetylputrescine	6.29 ± 0.72 (6.0)	3.94 ± 0.47 (3.8)	1.09 ± 0.07 (1.0)	5.61 ± 0.13 (2.4)	3.82 ± 0.24 (1.6)	2.06 ± 0.24 (0.9)	4.69 ± 0.37 (2.0)	9.68 ± 0.70 (4.1)	6.02 ± 0.63 (2.6)
N^1-Acetylcadaverine	2.05 ± 0.01 (1.9)	3.52 ± 0.05 (3.3)	1.03 ± 0.02 (1.0)	2.85 ± 0.13 (1.1)	1.49 ± 0.04 (0.6)	0.57 ± 0.02 (0.2)	8.28 ± 0.84 (3.1)	3.45 ± 0.38 (1.3)	2.59 ± 0.30 (1.0)
Putrescine	0.76 ± 0.06 (4.3)	0.23 ± 0.02 (1.3))	0.03 ± <0.01 (0.1)	0.93 ± 0.05 (3.7)	0.15 ± 0.02 (0.6)	0.34 ± 0.04 (1.4)	1.53 ± 0.09 (6.1)	1.84 ± 0.12 (7.3)	1.02 ± 0.08 (4.1)
Cadaverine	Trace (0)	Trace (0)	Trace (0)	0.66 ± 0.03 (5.1)	Trace (0)	Trace (0)	7.40 ± 0.46 (57.7)	4.56 ± 0.25 (35.5)	1.16 ± 0.02 (9.1)
N^1-Acetylspermidine	1.27 ± 0.05 (1.7)	2.92 ± 0.22 (3.9)	1.81 ± 0.10 (2.4)	0.40 ± 0.05 (0.4)	0.28 ± 0.03 (0.3)	0.32 ± 0.02 (0.3)	15.69 ± 1.33 (15.5)	0.48 ± 0.04 (0.5)	1.69 ± 0.15 (1.7)
N^8-Acetylspermidine	0.93 ± 0.03 (2.5)	0.68 ± 0.08 (1.8)	Trace (0)	0.73 ± 0.09 (0.7)	0.30 ± 0.03 (0.3)	0.20 ± 0.02 (0.2)	Trace (0)	0.56 ± 0.01 (0.5)	0.62 ± 0.05 (0.6)
Spermidine	0.17 ± 0.01 (0.8)	0.28 ± 0.01 (1.3)	0.04 ± <0.01 (0.2)	0.07 ± 0.01 (0.3)	0.01 ± <0.01 (0.0)	Trace (0)	0.02 ± <0.01 (0.1)	0.02 ± <0.01 (0.1)	0.02 ± <0.01 (0.1)
N^1-Acetylspermine	0.17 ± 0.02 (1.2)	0.11 ± 0.01 (0.8)	0.03 ± <0.01 (0.2)	0.03 ± <0.01 (0.2)	0.02 ± <0.01 (0.1)	Trace (0)	Trace (0)	Trace (0)	0.01 ± <0.01 (0.1)
Spermine	0.49 ± 0.03 (1.2)	0.76 ± 0.01 (1.9)	0.23 ± <0.01 (0.6)	0.31 ± <0.01 (0.7)	0.22 ± <0.01 (0.5)	0.11 ± <0.01 (0.2)	0.45 ± <0.01 (1.0)	0.21 ± <0.01 (0.5)	0.21 ± <0.01 (0.5)

[a] Each polyamine level in cancer patients was normalized to the corresponding normal mean value

[b] Interval (month) when polyamine levels were measured during a long-term weekday acupuncture therapy

were considerably reduced while N^1-acetylcadaverine, N^1-acetylspermidine, spermidine and spermine were more excreted. In contrast, their levels were all markedly decreased down to the normal values in the third stage (after 4.3 month's treatment) except that N^1-acetylspermidine was still higher than its initial value (1.27) and the normal level (0.75). Its isomer N^8-acetylspermidine was not detectable. In Case 2, the levels of N^1-acetylspermidine, N^8-acetylspermidine, spermidine, N^1-acetylspermine and spermine were subnormal in all follow-up stages, indicating their irrelevancy to acupuncture treatment. However, the initially higher levels of N^1-acetylputrescine and putrescine decreased with the treatment and cadaverine was not measurable from the second stage (after 1.2 month's treatment). In Case 3, the levels of N^8-acetylspermidine, spermidine and N^1-acetylspermine were subnormal in each follow-up stage and spermidine excretion did not respond to acupuncture treatment at all (Table 3). The extremely high level (15.69) of N^1-acetylspermidine was most markedly reduced below its normal value (1.01) after 2 month's treatment but backed up above the normal value in the third stage. The acupuncture treatment for 2 months elevated N^1-acetylputrescine level above its initial value and further treatment (3.3 months) reduced its level down but higher than the normal value. The high levels of N^1-acetylcadaverine, putrescine and cadaverine were considerably decreased in the third follow-up stage.

The alterations of urinary PA levels in three cancer patients recognized in the present study may explain for possible association of acupuncture therapy with PA metabolism.

Normalization and star pattern recognition

When the PA levels in each follow-up stage were normalized to the corresponding normal mean values, each normalized concentration displayed elevation of the PA levels in multiples (ranging from 0.0 to 57.7) of the respective normal mean values (Table 3). These normalized values were used as the variables to draw star graphs composed of nine rays. The differences between patients and normal group average were more clearly represented in the visual star patterns (Figs. 1, 2, 3) as compared to the numeric data (Table 3). As expected, the patient star patterns (Case 1–Case 3) were badly distorted. The nonagon shape of normal group average served well as the reference pattern for distinguishing the characteristic star shape of each cancer patient. And the abrupt alterations in the urinary PA levels from the first follow-up stage to third stage for each cancer patient during acupuncture therapy were readily perceived from the visual star graphic patterns. In Case 1 (Fig. 1), all rays of the third follow-up stage were

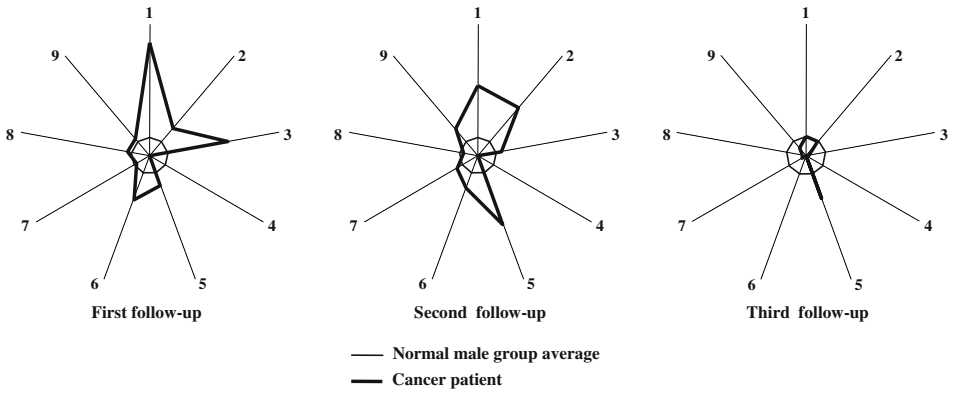


Fig. 1 Star symbol plots of first, second and third follow-up stages for the Case 1 (rectal cancer, male, 55 years) based on the mean levels of the nine polyamines as the variables after normalization to the corresponding normal male group means. *Rays* 1, N^1-Acetylputrescine; 2, N^1-Acetylcadaverine; 3, Putrescine; 4, Cadaverine; 5, N^1-Acetylspermidine; 6, N^8-Acetylspermidine; 7, Spermidine; 8, N^1-Acetylspermine; 9, Spermine

Fig. 2 Star symbol plots of first, second and third follow-up stages for the Case 2 (lung cancer, female, 52 years) based on the mean levels of the nine polyamines as the variables after normalization to the corresponding normal female group means. *Rays* the numbers correspond to those in Fig. 1

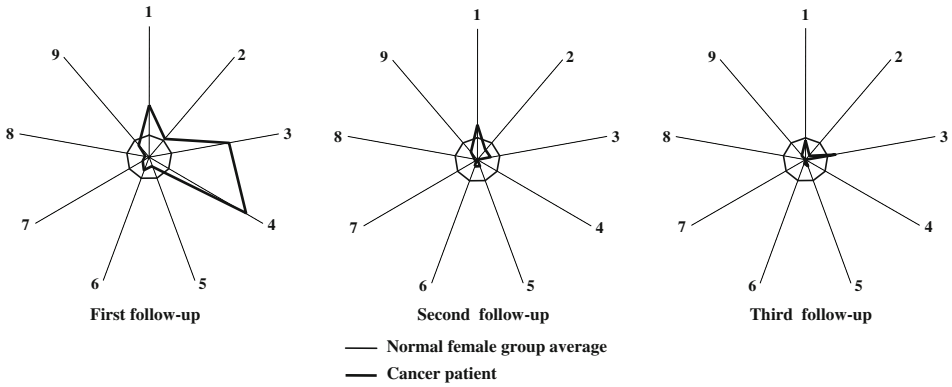
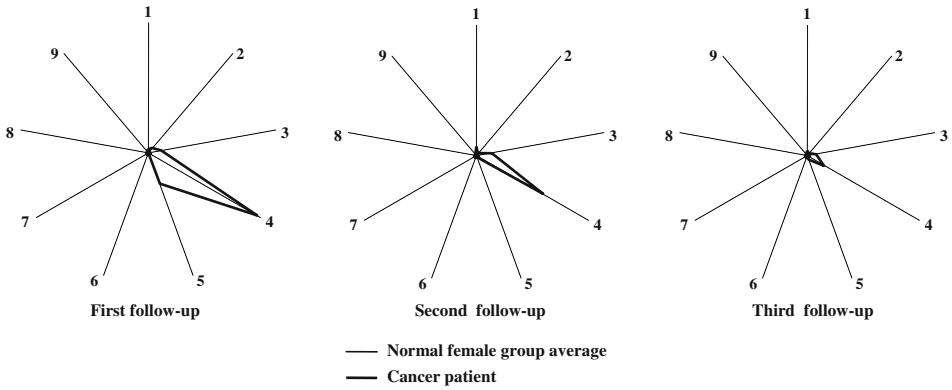


Fig. 3 Star symbol plots of first, second and third follow-up stages for the Case 3 (breast cancer, female, 59 years) based on the mean levels of the nine polyamines as the variables after normalization to the corresponding normal female group means. *Rays* the numbers correspond to those in Fig. 1



within the normal pattern except for N^1-acetylspermidine (ray no. 5). In the third follow-up stage of Case 2, all the rays were located inside the normal nonagon pattern except that putrescine (ray no. 3) slightly protruded out (Fig. 2). In Case 3 (Fig. 3), the normal nonagon was invisible due to cadaverine (ray no. 4) and putrescine (ray no. 3).

In conclusion, the present urinary PA profiling analysis combined with star graphic analysis appeared to be useful for monitoring metabolic changes induced by long-term acupuncture treatments in cancer.

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