Study on changes of polyamine levels in mice with the development of U14 cervical cancer

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Received 5 April 2012; accepted 19 July 2012
Available online 27 July 2012

Abstract This study was performed to investigate the possible involvement of polyamines in the development of cervical cancer. The objective of the present study was therefore to find the specific polyamine indicators, which could be used as useful markers for the early determination of cervical cancer. A simple method for the simultaneous determination of plasma concentrations of five polyamines in normal and U14 model mice was developed by using HPLC-MS. The samples were derivatized by benzoyl chloride. The derived polyamines were separated on a C18 column by a gradient elution with methanol–water, and then detected with HPLC-MS. The results showed that all polyamine levels in the U14 model mice were higher than those in normal mice. The cadaverine, putrescine and 1, 3-diaminopropane levels were significantly higher in U14 model mice plasma than those in normal mice plasma, especially the putrescine and 1, 3-diaminopropane (\(P<0.01\)). The cadaverine, putrescine and 1, 3-diaminopropane levels were significantly higher in U14 mice model urine than those in normal mice urine, especially the cadaverine and 1, 3-diaminopropane (\(P<0.01\)). Putrescine, cadaverine and 1, 3-diaminopropane might be the indicators of the cervical cancer.

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1. Introduction

Globally, cervical cancer is the second most common cause of cancer death in women, with 510,000 estimated new cases and 288,000 deaths annually [1]. Human Papillomavirus (HPV) is a necessary cause for invasive cervical cancer worldwide. HPV DNA is found in 99.7% of all cervical cancer [2]. However, despite this association, HPV alone is not sufficient to induce
the disease. HPV virus can spread directly through skin contact and the incubation period of it was even more than 10 years without any symptoms. Most women who are HPV positive show no evidence of cervical abnormalities [3] and only a small portion of women with HPV develop cervical cancer [4]. Therefore, finding more effective indicators to diagnose the cervical cancer is necessary. Cervical cancer is preventable and can be cured. The purpose of cervical cancer screening is the early detection, diagnosis and treatment of cancer precursor lesions and cancer. Accordingly, searching out the indicators of cervical cancer has become an important task for cancer diagnosis and anti-tumor drug monitoring.

Polyamines are endogenous substances and the products of the cell metabolism. Their metabolites putrescine (PUT), 1, 3-diaminopropane (DAP), cadaverine (CAD), spermidine (SPD) and spermine (SPM) are the most important in the regulation of cell growth, differentiation, and synthesis of proteins and nucleic acids [5–7]. Many papers and reviews on the relationship between polyamines and cancer have been substantiated [8–13]. However, most of the literatures only studied PUT, SPD and SPM. In addition, the changes of five different polyamine metabolite levels with the development of cervical cancer remain undefined. Which polyamine metabolites can be used to diagnose the cervical cancer is not clear.

In the present study, the U14 mice models were established. Plasma samples were collected at day 2, 4, 8 and 12 after inoculation. Urine samples were collected at day 5 and 11 after inoculation. Another normal mice group was used as control. An HPLC-MS method was applied to determine the concentrations of five polyamine metabolites in these mice simultaneously. The relationship between polyamines and cervical cancer was studied. The aim of this study was to search out the specific polyamine indicators of the cervical cancer, which could be used as useful markers for the early determination of cervical cancer.

2. Materials and methods

2.1. Chemicals, reagents and materials

DAP, PUT, SPM, 1, 6-diaminohexane (DAH, used as an internal standard), CAD hydrochloride, SPD hydrochloride and benzoyl chloride were obtained from Sigma–Aldrich (St.Louis, MO, USA). Methanol and acetonitrile of HPLC grade were purchased from Fisher Chemicals (Fair Lawn, NJ, USA). All the other reagents were of analytic grade. Distilled and deionized water was used throughout the experiment.

2.2. Animals and cell lines

Female Kunming mice with body weight ranging from 18 to 22 g were provided by the Experimental Animal Center of Shenyang Pharmaceutical University (no. 0225336). Animals were bred under conditions (22±2 °C, RH 50±20%) with a natural light–dark cycle for 3 days before the experiment started. All procedures involving animals were in accordance with the Regulations of Experimental Animal Administration Issued by the State Committee of Science and Technology of China. U14 cervical carcinoma cells were gifted by School of Pharmacology, Shenyang Pharmaceutical University.

2.3. Animal experiments

U14 cervical carcinoma cells were diluted with normal saline (NS) at a density of 1 × 10⁶ cells/mL. Kunming mice were subcutaneously implanted with 1 × 10⁷ cells/mice on the right armpit as model group (n=8). The interval between the ascites taking and the last mice inoculation was less than 2 h. Another normal mice group (n=8) was used as control. Plasma samples were collected at day 2, 4, 8 and 12 after inoculation. Urine samples were collected at day 5 and 11 after inoculation. Then the plasma samples were kept at −20 °C. Urine samples were centrifuged at 13,000 rpm for 3 min to aid settling of coarse material. The supernatant liquid was transferred to polypropylene tube and kept at −20 °C. Two mice were combined into a sample (n=4) due to the difficult sample collection from mice except the last plasma collection at day 12 (n=8). Twelve days after inoculation, all the mice were sacrificed and tumors were removed and weighted individually.

2.4. Preparation of standard and calibration solutions

Stock solutions for DAP, PUT, CAD, SPD, SPM and DAH were prepared in methanol at 1 mg/mL. Working standard solutions were prepared by serial dilution with methanol. All solutions were stored at 4 °C before analyzed.

Calibration samples at PUT, DAP, CAD, SPD and SPM concentration of 2.5, 12.5, 25, 125, 250, 500, 1000 ng/mL were prepared by spiking appropriate amounts of the standard solution in blank mice plasma.

Calibration samples at DAP, CAD, SPD and SPM concentration of 10, 50, 100, 500, 1000, 2000, 4000 ng/mL, PUT concentration of 20, 100, 200, 1000, 2000, 4000, 8000 ng/mL, were prepared by spiking appropriate amounts of the standard solution in blank mice urine.

2.5. Preparation of plasma and urine samples

Plasma samples (200 µL) were spiked with 50 µL of internal standard and 120 µL of 10% perchloric acid, then mixed thoroughly for 3 min and centrifugated at 15,000 rpm for 5 min. The supernatant liquid was transferred to polypropylene tube and submitted to the derivatization procedure.

Urine samples (50 µL) were spiked with 50 µL of internal standard and 30 µL of 10% perchloric acid, then mixed thoroughly for 3 min and centrifugated at 15,000 rpm for 5 min. The supernatant liquid was transferred to polypropylene tube and submitted to the derivatization procedure.

2.6. Derivatization procedure

2.6.1. Plasma sample derivatization procedure
Polymamines were derivatized according to the method described by our research group [14]. To the perchloric supernatants, 620 µL of 2 M NaOH solution, and 250 µL of 4% benzoyl chloride in acetonitrile were added sequentially. After standing for 20 min under ultrasonication at 30 °C, excess benzoyl was removed by mixing with the equal volume of saturated sodium chloride solution. Polyamine derivatives were extracted into diethyl ether, followed by evaporating to dryness under nitrogen. The residue was reconstituted in 100 µL methanol, and 5 µL of which was injected for HPLC-MS analysis.
2.6.2. Urine sample derivatization procedure
To the perchloric supernatants, 530 µL of 2 M NaOH solution was added. The remaining processes were the same with the plasma sample derivatization procedure. The residue was reconstituted in 100 µL methanol, and 2 µL of which was injected for HPLC-MS analysis.

2.7. Instrumentation and chromatographic conditions
HPLC-ESI-MS analytical procedures were performed on an ACQUITY UPLC system coupled to a Quattro Micro API mass spectrometer (Waters Milford, MA, USA). MassLynx V4.1 software (Waters) was applied to system operation and mass spectrometer (Waters Milford, MA, USA). MassLynx HPLC-ESI-MS analytical procedures were performed on an in injected for HPLC-MS analysis. The ESI source was set in positive ionization mode. Target quadrupole tandem mass spectrometer equipped with electrospray ionization (ESI) interface was used for analytical detection. The ESI source was set in positive ionization mode. Target ions were monitored [M+Na]⁺ at m/z 305.3 for DAP and cone at 45 V, [M+Na]⁺ at m/z 319.4 for PUT and cone at 48 V, [M+Na]⁺ at m/z 333.4 for CAD and cone at 50 V, [M+Na]⁺ at m/z 347.4 for DAH (I.S.) and cone at 51 V, [M+Na]⁺ at m/z 480.6 for SPD and cone at 52 V, [M+Na]⁺ at m/z 641.8 for SPM and cone at 56 V using the selected ion record (SIR) mode. The capillary voltage was set at 3500 V, extractor voltage at 2 V, SPM and cone at 56 V using the selected ion record (SIR) mode. The capillary voltage was set at 3500 V, extractor voltage at 2 V, SPM and cone at 56 V using the selected ion record (SIR) mode.

2.8. Validation of the method
2.8.1. Validation of the method of plasma sample
To evaluate linearity, seven calibration solutions were prepared and assayed together with spiked amount of IS (125 ng/mL) in triplicate by derivative procedure as described above. The lower limit of quantification (LLOQ) was assessed by determining the concentration of polyamine at 2.5 ng/mL. For intra-day and inter-day assays, the samples were prepared individually in triplicate at low (10 ng/mL), medium (125 ng/mL) and high (400 ng/mL) concentration in methanol. Likewise, the recovery was determined at three concentrations by comparing the recovered polyamine concentrations with the nominal concentrations. In order to evaluate the matrix effect on the ionization of analytes, i.e., the potential ion suppression or enhancement due to the matrix components, three concentration levels of polyamines were tested. The matrix effect was evaluated by comparing the peak area ratio of post-spiked derivative polyamines standards in the plasma derivatives and IS to that of the neat standard solutions. The stability of polyamines in mice plasma was studied under a range of times (0, 2, 4, 8, 12, and 24 h) after derivatization. During the analysis, the samples were stored at 4°C. The stability of plasma samples was also studied under a range of times (0, 2 and 4 h) at ambient temperature. The stability of polyamines in mice plasma was studied for three freeze–thaw cycles. In each freeze–thaw cycle, the plasma samples were frozen at −20°C for 24 h and thawed at room temperature.

2.8.2. Validation of the method of urine sample
To evaluate linearity, seven calibration solutions were prepared and assayed together with spiked amount of I.S. (500 ng/mL) in triplicate by derivative procedure as described above. The LLOQ was assessed by determining the concentration of polyamine at 10 ng/mL. For intra-day and inter-day assays, the samples were prepared individually in triplicate at low (40 ng/mL, PUT: 80 ng/mL) and high (1600 ng/mL, PUT: 3200 ng/mL) concentration in methanol. The remaining processes were the same with the plasma sample processes.

2.9. Data analysis
All data were presented as mean±SD. Significances were analyzed by the t-test for two independent means. p<0.05 was considered significant and p<0.01 was considered highly significant. The SPSS (version 16.0) statistical software was applied.

| Table 1 | Validation data for the analysis of polyamines in plasma using the proposed method (n=3). |
|---------|-----------------------------------------------------------------------------------------|
| Polymine | Calibration range (ng/mL) | Linearity (r) | Calibration equation | Plasma recovery (%) |
| PUT     | 2.5–1000.0 | 0.9907 | \( y = 4.302 \times 10^{-3}x + 5.039 \times 10^{-2} \) | QC1 | QC2 | QC3 | Mean |
| DAP     | 2.5–1000.0 | 0.9939 | \( y = 7.349 \times 10^{-2}x + 2.163 \times 10^{-2} \) | 83.3 | 92.5 | 81.5 | 85.8 |
| CAD     | 2.5–1000.0 | 0.9975 | \( y = 6.414 \times 10^{-3}x + 3.857 \times 10^{-2} \) | 76.1 | 93.6 | 90.5 | 86.7 |
| SPD     | 2.5–1000.0 | 0.9977 | \( y = 1.294 \times 10^{-2}x + 1.080 \times 10^{-1} \) | 83.6 | 84.6 | 84.9 | 84.3 |
| SPM     | 2.5–1000.0 | 0.9975 | \( y = 3.187 \times 10^{-2}x + 7.314 \times 10^{-2} \) | 75.6 | 93.3 | 96.1 | 88.4 |

| Polymine | Intra-day precision (RSD, %) | Inter-day precision (RSD, %) |
| QC1 | QC2 | QC3 | QC1 | QC2 | QC3 |
| PUT | 8.9 | 7.3 | 5.7 | 11.6 | 9.3 | 9.3 |
| DAP | 5.8 | 3.9 | 5.3 | 14.2 | 5.1 | 11.2 |
| CAD | 11.1 | 11.1 | 4.0 | 12.4 | 5.7 | 7.3 |
| SPD | 7.5 | 2.7 | 6.9 | 13.7 | 12.0 | 12.3 |
| SPM | 13.2 | 5.5 | 4.0 | 14.0 | 12.9 | 10.0 |
3. Results

3.1. Tumor growth

After the tumor cells inoculated, all the Kunming mice were inoculated successfully, tumorigenicity was 100%. The mean tumor weight of the U14 model group was 1.39 ± 0.44 g at day 12.

3.2. Method validation

The results of method validation are summarized in Tables 1 and 2 for the plasma sample and Tables 3 and 4 for the urine sample. The intra-day and inter-day precisions were within 14.0% and 15.0%, respectively. Furthermore, the recoveries ranged from 75.60% to 96.1% for mice plasma and 77.40% to 95.5% for mice urine. In terms of matrix effect, all the ratios defined in Section 2.8 were between 85.2% and 113.8%, which meant no significant matrix effect in this method. Polyamines are endogenous substances existing in the blank samples. Therefore, all values were corrected for polyamine concentration of the blank mice samples.

3.3. Determination of polyamines in plasma and urine samples

3.3.1. Determination of polyamines in plasma samples

The typical mass chromatograms of polyamines in U14 bearing mice plasma are shown in Fig. 1B. The derivative

| Table 2 | Result of stability test of polyamines in plasma (n=3). |
|---------|-----------------------------------------------------------|
| Polyamines | Post-preparative stability (RSD, %) | Plasma sample at ambient temperature stability (RSD, %) | Freeze-thaw stability (RSD, %) |
| PUT | 8.8 | 1.9 | 10.8 |
| DAP | 3.2 | 4.1 | 5.1 |
| CAD | 6.7 | 6.9 | 6.5 |
| SPD | 7.9 | 5.2 | 4.0 |
| SPM | 14.7 | 1.6 | 8.4 |

| Table 3 | Validation data for the analysis of polyamines in urine using the proposed method (n=3). |
|---------|-------------------------------------------------------------------------------|
| Polyamines | Calibration range (ng/mL) | Linearity(r) | Calibration equation | Plasma recovery (%) |
| PUT | 20–8000 | 0.9979 | $y = 2.019 \times 10^{-2}x - 2.336 \times 10^{-1}$ | QC1 QC2 QC3 Mean |
| DAP | 10–4000 | 0.9940 | $y = 4.473 \times 10^{-3}x - 1.369 \times 10^{-2}$ | 77.4 83.9 84.2 85.8 |
| CAD | 10–4000 | 0.9928 | $y = 4.745 \times 10^{-3}x - 4.788 \times 10^{-2}$ | 82.1 89.9 81.7 86.7 |
| SPD | 10–4000 | 0.9940 | $y = 6.412 \times 10^{-3}x - 3.163 \times 10^{-1}$ | 82.4 84.7 95.5 84.3 |
| SPM | 10–4000 | 0.9959 | $y = 1.676 \times 10^{-2}x - 1.059 \times 10^{-2}$ | 87.5 81.8 89.5 88.4 |

| Polyamines | Post-preparative stability (RSD, %) | Inter-day precision (RSD, %) |
| PUT | 11.7 | QC1 QC2 QC3 |
| DAP | 10.0 | QC1 QC2 QC3 |
| CAD | 4.6 | QC1 QC2 QC3 |
| SPD | 5.1 | QC1 QC2 QC3 |
| SPM | 6.4 | QC1 QC2 QC3 |

| Table 4 | Result of stability test of polyamines in urine (n=3). |
|---------|----------------------------------------------------------|
| Polyamines | Post-preparative stability (RSD, %) | Urine sample at ambient temperature stability (RSD, %) | Freeze-thaw stability (RSD, %) |
| PUT | 6.0 | 3.8 | 7.9 |
| DAP | 7.3 | 5.5 | 2.8 |
| CAD | 5.3 | 2.1 | 7.9 |
| SPD | 11.2 | 1.4 | 6.6 |
| SPM | 10.0 | 3.8 | 10.5 |
Polyamines were identified from the comparison of MS characteristic of standard polyamines. The mean levels of each polyamine (nM) are shown in Table 5. Polyamine levels in the U14 group were compared with those in the normal group. All of polyamines of model mice were higher than those of normals. Highly marked increases were found in the levels of PUT and DAP \((p<0.01)\). CAD in the model group was significantly higher than that in the normal group \((p<0.05)\). However, experimental results showed that the levels of SPD and SPM were no significant difference between model and normal groups. To classify the model and the normal mice, PUT, DAP and CAD were analyzed using the SPSS (version 16.0). The between-groups linkage method was adopted and squared Euclidean distance was chosen to

Fig. 1 Total ion chromatograms (TIC) obtained from standard polyamines (A), plasma sample (B) and urine sample (C) by HPLC-ESI-MS. (1) PUT, (2) DAP, (3) CAD, (4) DHA (I.S.), (5) SPD, and (6) SPM.
Table 5  Amounts of polyamines (nM) in plasma from mice.

| Time (day)          | Group    | Polyamines (nM) (Mean ± SD) | Total polyamines |
|---------------------|----------|-----------------------------|-----------------|
|                     | PUT      | DAP            | CAD       | SPD       | SPM       |                  |
| Second days (n=4)   | Normal   | 1.27±0.19      | 0.17±0.04  | 0.17±0.06 | 2.10±0.67 | 0.32±0.05 | 4.03             |
|                     | U14      | 1.87±0.61      | 0.17±0.03  | 0.19±0.02 | 1.71±0.52 | 0.39±0.04 | 4.33             |
| Differences         |          | +0.60          | 0.00      | -0.02     | -0.39     | +0.07     | +0.30            |
| Fourth days (n=4)   | Normal   | 1.52±0.15      | 0.16±0.02  | 0.14±0.01 | 3.80±0.38 | 0.43±0.04 | 6.05             |
|                     | U14      | 1.59±0.13      | 0.20±0.04  | 0.46±0.63 | 2.51±0.48 | 0.43±0.05 | 5.20             |
| Differences         |          | +0.07          | +0.04     | +0.32     | -1.29     | 0.00      | -0.85            |
| Eighth days (n=4)   | Normal   | 1.35±0.24      | 0.18±0.03  | 0.14±0.02 | 2.85±0.67 | 0.36±0.07 | 4.88             |
|                     | U14      | 1.96±0.73      | 0.27±0.04  | 0.18±0.04 | 2.98±0.51 | 0.38±0.05 | 5.78             |
| Differences         |          | +0.61          | +0.09     | +0.04     | +0.13     | +0.02     | +0.90            |
| Twelfth days (n=8)  | Normal   | 1.09±0.08      | 0.14±0.03  | 0.17±0.06 | 2.87±0.49 | 0.38±0.09 | 4.64             |
|                     | U14      | 2.00±0.43**    | 0.21±0.07**| 0.26±0.07*| 3.47±0.99 | 0.44±0.18 | 6.39             |
| Differences         |          | +0.91          | +0.07     | +0.09     | +0.60     | +0.06     | +1.75            |

*Compared with normal group p<0.05.
**Compared with normal group p<0.01.

![Fig. 2 Result of the cluster analysis from mice plasma samples, the Between-groups linkage method was adopted and squared Euclidean distance was chosen as measurement. Sixteen samples were clustered into two groups, i.e. Group I and Group II. Bar=5 rescaled distance.](image)

3.3.2. Determination of polyamines in urine samples

The typical mass chromatograms of polyamines in U14 bearing mice urine are shown in Fig. 1C. The derivative polyamines were identified from the comparison of MS characteristic of standard polyamines. All values were corrected for the concentration of creatinine in the same urine. Creatinine was all excreted in the urine without any absorption. The excretion of creatinine was relatively constant without effects by the amount of urine. As shown in Table 6, the mean levels of each polyamine (nM of creatinine) of U14 models were higher than those of normals. At day 5, the levels of DAP and CAD were significant difference between model and normal groups (p<0.05). At day 11, highly marked increases were found in the levels of DAP and CAD (p<0.01). PUT and SPD in the model group were significantly higher than those in normals (p<0.05). PUT, DAP, CAD, SPD and SPM were analyzed using the SPSS (version 16.0). The results are shown in Fig. 3. Urine samples were gathered into two groups, the samples of normals were classified in Group I and U14 model mice were classified in Group II.

4. Discussion

Nowadays, in order to get valuable data for rapidly qualitative and quantitative assessment of anti-tumor activity, many different tumor models have been established to test the activity of anti-tumor drugs in vivo [15]. These transplantation tumors grow rapidly in vivo with short experimental cycle. The tumor models can be established successfully in 7 to 10 day [16]. Therefore, the last mice samples were collected at day 12.

PUT, DAP, CAD, SPD and SPM are the metabolic products of ornithine and lysine. As shown in Fig. 4, PUT is the precursor of SPD and SPM, and formed by direct decarboxylation of L-ornithine by Ornithine decarboxylase (ODC) [17]. DAP is catalyzed by S-adenosylmethionine decarboxylase (SAMDC). CAD is synthesized by a direct decarboxylation of lysine by lysine decarboxylase. Our results showed that the levels of PUT, DAP and CAD were significantly higher in U14 mice than those in normals. It could be concluded the high activity of ODC, SAMDC and lysine decarboxylase in the cervical cancer. The inhibitors of ODC, SAMDC and lysine decarboxylase may be designed to develop anticancer drugs for cervical cancer.

Compared to cervical cancer, the Hepatoma-22 (H22) bearing mice models were established in our previous research. PUT and CAD were significantly higher in H22 mice than those in normals. However, beside PUT and CAD, DAP was significantly increased (p<0.01) in cervical cancer mice. Therefore, it might be concluded that DAP was the specific polyamine indicators of the cervical cancer, which could be one means to classify the cervical cancer and the hepatoma. Our results showed that SPD decreased in the early stage of the tumor growth, but then increased rapidly. It could suggest...
that polyamines have a different metabolic pattern in the cervical cancer mice. This discrepancy may be due to the transient suppression of spermidine synthase activity in the early stage of cervical cancer. With the cervical cancer development, the activity of spermidine synthase was gradually increased. Furthermore, the increase value of SPD in urine samples at day 11 was 19.9 times than that at day 5. The increase value of SPD in plasma samples at day 12 was 4.6 times than that at day 8. What’s more, many literatures reported that SPD was increased significantly in cervical cancer patients [18,19]. Therefore, we speculated that SPD levels could be increased significantly with the development of cervical cancer. As shown in Table 5, in the early stage of cervical cancer, DAP and CAD were increased more rapidly than the others. It was suggested that the activity of SAMDC and lysine decarboxylase was activated firstly after inoculation. With the development of cervical cancer, PUT, SPD and SPM were increased, especially PUT and SPD. It could be inferred that the activity of ODC and spermidine synthase was activated at this period. Therefore, SAMDC and lysine decarboxylase might be the targets of the anti-cancer drugs in the early stage of cervical cancer, while ODC and spermidine synthase in the medi-terminal stage of cervical cancer. Further studies are required to determine whether enzyme levels are constitutively linked. Our data showed that polyamines which were increased significantly in plasma were consistent with those obtained in urine. In clinic, the urine samples were easier to collect than the plasma samples. Therefore, monitoring polyamine levels in urine might play an important role in diagnosing the cervical cancer.

5. Conclusion

DAP was the specific polyamine indicators of the cervical cancer, which could be one means to classify the cervical cancer and the hepatoma. Our findings confirmed that polyamines were useful biochemical markers of cervical cancer. PUT, CAD and DAP might be used to diagnose the cervical cancer.

Acknowledgments

This study was financially supported by the National Science Foundation (81102784/H2803) and the Liaoning Science Foundation (20102211).

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