Screening of urinary renal cancer metabolic biomarkers with gold nanoparticles - assisted laser desorption/ionization mass spectrometry

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Abstract

Renal cell carcinoma is a very aggressive and often fatal disease for which there are no specific biomarkers found to date. The purpose of work was to find features that differentiate urine metabolic profiles of healthy people and cancer patients. Laser desorption/ionization mass spectrometry on gold nanostructures based techniques was used for the metabolic analysis of urine of fifty patients with kidney cancer. Comparison with data from 50 healthy volunteers allowed discovering several compounds that may be considered potential renal cell carcinoma (RCC) biomarkers. Statistical analysis of data allowed discovering of \( m/z \) values that had the greatest impact on group differentiation. Database search allowed providing assignment of signals for the most promising fifteen features among them: serine, heptanol, 3-methylene-indolenine, 2-methyl-3-hydroxy-5-formylpyridine-4-carboxylate, phosphodimethylethanolamine, 4-methoxyphenylacetic acid, \( N \)-acetylglutamine, 3,5-dihydroxyphenylvaleric acid, hydroxyhexanoylglycine, valyl-leucine, leucyl-histidine, oleamide, 9,12,13-trihydroxyoctadecenoic acid, stearidonyl carnitine or squalene. Differences of metabolite profiles of human urine could be identified by gold nanoparticle-enhanced target (AuNPET) LDI MS method and used for the detection of renal cancer.

Keywords: cancer biomarker; gold nanoparticles; kidney cancer; laser desorption/ionization; mass spectrometry; renal cell carcinoma
Introduction

Worldwide, every year brings 400,000 new cases of kidney cancer and more than 175,000 deaths due to this disease.\(^1\) Renal cell carcinomas (RCCs)\(^2\) are in the 6th place among the most frequently diagnosed cases of cancer in men and the 10th place in women.\(^3,4\) RCC can develop for a long time without clinical symptoms and a significant proportion of patients (25-30\%) have metastases at the time of diagnosis.\(^5\) For these reasons, it is important to develop new methods for early detection of RCC, among them the most important are based on specific chemical compounds, called biomarkers. Diagnostic biomarkers can be proteins,\(^6\) genes\(^7\) or metabolites.\(^8\)

Due to the location of RCC inside urine tract, urine is best suited for large-scale screening to detect diagnostic markers of renal cell carcinoma. Most kidney tumors occur in the tubular epithelium and cause the secretion of specific metabolites into their lumen. This process can be used to differentiate the urinary metabolome of sick and healthy people.\(^9\) This approach has already been used several times by researchers who used various types of liquid chromatography combined with mass spectrometry.\(^10,11,12,13,14\) Unfortunately, urine is characterized by high variability among patients depending on age, gender, diet and persons activity. Moreover, biomarkers usually are present in urine at very low concentrations.\(^15\)

Commonly used for peptide and protein research matrix-assisted laser desorption/ionization (MALDI) mass spectrometry is has already been used for tumor marker research,\(^16,17\) also for RCC protein profiling.\(^18\) However, MALDI spectra contain a high chemical background below \(m/z\) 1000 due to the use of organic matrices. For small molecules, surface-assisted laser desorption/ionization (SALDI)\(^19\) solutions are generally better suited. As literature search prove, gold nanostructures are among the
most frequently used for laser MS. Some of the recent applications of gold nanostructures in LDI MS include nanostructure-embedded micro gold shells previously applied for analysis of small molecules without any significant background interferences. Colaianni et al. applied gold nanowires with good results for small peptide analysis. Nanoflowers of Au@MnO was applied for analysis of small and also large molecules of cancer cell lysates. Our group presented the advantages of gold-nanoparticle enhanced target (AuNPET) for laser desorption/ionization mass spectrometry analysis and imaging of low molecular weight compounds of different polarity, also in complex biological mixtures from plants, human kidney and prostate tissue, metabolome profiling of moulds, as well as lysine amino acid detection and quantification. Compared to commonly used MALDI MS, gold nanoparticle-based method has been proven to produce much lower chemical background, allows more precise internal calibration and is better suited for low and medium polar compounds.

The goal of this work is to demonstrate the capabilities of AuNPET-LDI-MS method for rapid metabolic profiling of fifty urine samples of patients with diagnosed RCC and statistical comparison with control group of fifty urine samples of healthy volunteers.

**Experimental**

**Participants**

Urine samples were obtained from fifty patients with diagnosed kidney cancer. Control was fifty urine samples from healthy volunteers, for which the presence of renal
tumors had been excluded by abdominal ultrasound. Specimens and clinical data from patients involved in the study were collected with written consent. Patient who agreed to participate in the study donated 10 ml of blood, 50 ml of urine according to standard medical procedure. All experiments were performed in compliance with the local laws and institutional guidelines (Rzeszów University of Technology biological material guidelines). Research protocol was approved by the local bioethics committee at the University of Rzeszów (Poland). Patient characteristics are provided in Table 1.

**Control group consisted of 50 healthy volunteers who donated 50 ml of urine.** Average age was 50.6 years old and male to female ratio was 3:2. Every volunteer underwent thorough abdominal ultrasound with the focus on genitourinary system. No bladder or kidney tumors have been diagnosed in this group.

**Materials & methods**

Chloro(trimethylphosphite)gold(I) of 97+% purity (Aldrich) was used for nanoparticle synthesis. The pyridine–borane complex (BH3:py) used was at ~8 M borane concentration (Aldrich). All solvents was of HPLC quality and were purchased from Sigma-Aldrich (Poland), except for 18 MΩ water which was produced locally. Magnetic stainless steel plate of H17 grade was made locally and used with Bruker NALDI adapter.

**Preparation of AuNPET target**

Gold nanoparticle - enhanced target was prepared as described in our recent publication. Stainless steel plate of 35x45 mm size was inserted into a large Petri dish containing acetonitrile (50 mL) and dissolved chloro(trimethylphosphite)gold(I) (25 mg). To this solution, 8M BH3:py complex in pyridine (173 µL) was added. After
48 hours of reaction, target plate was washed several times with acetonitrile, wiped with cotton wool ball and washed three times with acetonitrile and deionized water.

Sample preparation

Urine samples obtained from patients were immediately frozen and stored at -60°C. Prior to measurements, an unfreezing step was performed in room temperature, followed by 1000-times dilution with ultrapure water. Volumes of 0.5 µL of urine solutions were placed directly on target plate, air dried and inserted into MS apparatus for measurements.

LDI MS experiment

Laser desorption/ionization mass spectrometry experiments were performed using Bruker Autoflex Speed Time-of-Flight mass spectrometer equipped with a SmartBeam II laser (355 nm) in positive-ion reflectron mode. Measurement range was m/z 80-2000, suppression was turned on for m/z lower than 79. Laser impulse energy was approximately 100-190 µJ and laser repetition rate 1000 Hz. Number of laser shots was 20 000 (4x5000 shots) for each sample spot. The first accelerating voltage was held at 19 kV and the second ion source voltage at 16.7 kV. Reflector voltages used were 21 kV (the first) and 9.55 kV (the second). The data was calibrated with FlexAnalysis (version 3.3) using enhanced cubic calibration model and analyzed with mMass 5.5.0-open source program. Mass calibration was performed using internal standards (gold ions and clusters from Au⁺ to Au₅⁺).

Analysis of MS results

Database search of chemical compounds were carried out using a custom made
program. Theoretical m/z values were confirmed by using ChemCalc program available online.\textsuperscript{32} Statistical analysis of results was performed with the use of MetaboAnalyst 4.0 service.\textsuperscript{33} Data was normalized by sum, cube root transformed, default Pareto scaling was used. For creating receiver operating characteristic (ROC) curve random forests has been chosen as classification method and RandomForest built-in was selected as feature ranking method.

Results and Discussion

In order to estimate the degree of influence of method-related spectral data over sample-related data, statistical analysis with the aid of MetaboAnalyst 4.0 was performed. Principal component analysis (PCA), Partial Least Squares - Discriminant Analysis (PLS-DA), Sparse Partial Least Squares - Discriminant Analysis (sPLS-DA) and Orthogonal-Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) statistical methods implemented in MetaboAnalyst service were used. In case of domination of method-related data or signals, statistical analysis could not provide clear enough separation of studied samples.

Figure 1 contains results of statistical analysis of mass spectrometry data. It could be concluded that none of used statistical methods: PCA (Figure 1A), OPLS-DA score plot (Figure 1B), PLS-DA (Figure 1C) and sPLS-DA (Figure 1D), did not allow for complete separation of cancer patients and control group. These results are not unexpected as cancer and control samples are very similar from molecular point of view and usually complete separation in PCA is not visible. Moreover, studied cancer group
originates from patients with cancer of various stages and grades.

Based on PLS-DA, fold-change and random forest classification statistical methods, \( m/z \) values that had the greatest impact on group separation were obtained. Signals were assigned with the aid of Human Metabolome Database (HMDB)\(^{34} \) which allowed listing of fifteen potential biomarkers shown in Table 2. Random Forest classification method allowed the Out-of-bag (OOB) error to be determined at the 0.04 level, correctly classifying healthy people to the control group in 94\%, and people with diagnosed kidney cancer as patients in 98\%, based on all signals present in mass spectra.

Figure 2 presents box plots and ROC curves for each of fifteen \( m/z \) values. The largest area under the curve (AUC) was recorded for \( m/z \) 211.0990 and is 0.844, while the smallest for \( m/z \) 144.0051 is 0.561. Seven of the proposed biomarkers show up-regulation in urine samples from patients with kidney cancer, others have higher intensities in the control samples. Fourteen of the metabolites shown in Table 2 have not previously been described as a potential RCC biomarker. Only squalene was previously proposed as a tissue biomarker of the kidney cancer,\(^{35} \) however, our results suggest higher intensities among urine of cancer patients (Fig. 2O). Squalene is polyunsaturated hydrocarbon occurring naturally in living organisms. It is a metabolic precursor of sterols, including cholesterol, steroid hormones and vitamin D. It was shown that kidneys are one of the organs involved in the synthesis of squalene.\(^{36} \) Moreover, it is believed that squalene molecules may play a role in inhibiting tumor growth.\(^{37} \)

The first feature with higher mean intensity in cancer samples was \( m/z \) 155.0848 (Figure 2B) which corresponds to the potassium cation adduct of \( C_7H_{16}O \). A metabolite of this formula is heptanol, belonging to the class of organic compounds known as fatty alcohols. Next \( m/z \) value for which observed up-regulation in cancer (Fig.
2G) was 211.0715 with one of the largest area under the curve of 0.813. This value has been assigned to sodium adduct of N-acetylglutamine, a derivative of amino acid. N-acetylglutamine has already been detected in human urine by LC-MS.\textsuperscript{38} It was also found that increasing the concentration of this metabolite in urine may suggest renal tubular injury.\textsuperscript{39}  Another \textit{m/z} value with higher intensities in cancer samples was 269.1585 (Figure 3C) assigned to proton adduct of dipeptide LeuHis, a product of protein breakdown. The compound attributed to \textit{m/z} 304.2630 is oleamide for which we observed up-regulation in urine of patients with renal cell carcinoma (Fig. 3D). Oleamide is an amide of oleic acid and it occurs naturally in the body as a sleep inducing lipid whose mechanism of action is still understood.\textsuperscript{40} Studies suggest that this lipid may change its function in the urinary system causing increased calcium in bladder cancer and renal cells.\textsuperscript{41} Interestingly, the increase in oleamide levels was detected by chromatographic methods in the serum of people with colorectal cancer.\textsuperscript{42}

9,12,13-Trihydroxyoctadecenoic acid is a molecule assigned to a \textit{m/z} 353.2263 showing higher intensity in urine of RCC patients compared to controls with AUC equal to 0.741 (Figure 3E). This compound is metabolite of linoleic acid, and its increased concentration was detected in the blood of people with prostate cancer.\textsuperscript{43} A feature with up-regulation in RCC samples is \textit{m/z} 442.2921 attributed to sodium adduct of stearidonyl carnitine (Fig. 3F). Acylcarnitine to which this molecule belongs was previously found in higher concentrations than in the control group in the tissues and urine of patients with kidney cancer.\textsuperscript{13,14}

For the remaining eight metabolites (serine, 3-methylene-indolenine, 2-methyl-3-hydroxy-5-formylpyridine-4-carboxylate, phosphodimethylethanolamine, 4-methoxyphenylacetic acid, 3,5-dihydroxyphenylvaleric acid, hydroxyhexanoylglycine and valyl-leucine), down-regulation was observed for cancer samples. Serine deserves
special attention among these compounds. Serine biosynthesis also affects cellular antioxidative capacity, thus supporting tumor homeostasis and it has been shown that metabolic enzymes of serine biosynthesis are upregulated in cancer. However, a decrease in serum serine and urine and an increase in tissue are observed in human colorectal cancer, which is consistent with our results for renal cell carcinoma.

For all fifteen signals intensity table was created based on individual spectra from each sample and then receiver operating characteristic (ROC) analysis was performed in MetaboAnalyst 4.0. The results of the analysis are presented in Figure 4. Area under the curve (AUC) for the proposed biomarkers was found to be 0.915 (Figure 4A) thus they have high diagnostic accuracy to distinguish patients with kidney cancer from the healthy people from control group. Predicted class probabilities for each sample shown in Figure 4B were made on the basis of AUC. Cross-validation allowed for correct classification 44 samples to be qualified as originating from patients with kidney cancer, which gives 88% efficiency and 43 samples as derived from healthy volunteers, giving 86% correctness.

Conclusions

Laser desorption/ionization mass spectrometry with gold nanoparticle-enhanced SALDI-type target was used for rapid analysis of urine from 50 patients with diagnosed kidney cancer and 50 healthy volunteers. Methodology allowed identification of up- and downregulated fifteen compounds that could potentially serve as renal cancer biomarkers such as: serine, heptanol, 3-methylene-indolenine, 2-methyl-3-hydroxy-5-formylpyridine-4-carboxylate, phosphodimethylethanolamine,
4-methoxyphenylacetic acid, N-acetylglutamine, 3,5-dihydroxyphenylvaleric acid, hydroxyhexanoylglycine, valyl-leucine, leucyl-histidine, oleamide, 9,12,13-trihydroxyoctadecenoic acid, stearidonyl carnitine or squalene. Multivariate ROC analysis proposed biomarkers gave an area under the curve equal to 0.915, and correct classification of patients and healthy people at 87%. Statistical analysis allowed to distinguish the study group from the control.

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Table 1. Clinical characteristics of patients.

|                      | Patients |
|----------------------|----------|
| Total                | 50       |
| Age (years)          | 35-89    |
| Mean                 | 62       |
| **Sex**              |          |
| Male                 | 30       |
| Female               | 20       |
| **Stage (T)**        |          |
| T1                   | 33       |
| T2                   | 3        |
| T3                   | 10       |
| T4                   | 1        |
| undefined            | 3        |
| **Nodes (N)**        |          |
| N0                   | 46       |
| N1                   | 1        |
| undefined            | 3        |
| **Metastases (M)**   |          |
| M0                   | 42       |
| M1                   | 5        |
| undefined            | 3        |
| **Grade (Fuhrman)**  |          |
| I                    | 7        |
| II                   | 17       |
| III                  | 13       |
| IV                   | 2        |
| undefined            | 11       |
Table 2. List of ions and compounds found by statistical analysis of mass spectra.

| Metabolite                        | Ion formula         | Experimental m/z | Calculate d m/z | Δm/z [ppm] | Reg. a | AUC   | VIP   | P-Value   | log2(FC) d | Fig    |
|-----------------------------------|---------------------|------------------|-----------------|------------|--------|-------|-------|-----------|------------|---------|
| Serine                            | [C₆H₁₀NO₃+K]⁺       | 144.0051         | 144.0058        | -4.9       | ↓      | 0.561 | 1.22  | 3.9E-11   | 2.30       | 2A      |
| Heptanol                          | [C₇H₁₄O⁺K]⁺         | 155.0851         | 155.0833        | 9.7        | ↑      | 0.757 | 1.36  | 5.2E-7    | -1.97      | 2B      |
| 3-Methylene-indolene             | [C₉H₁₄N⁺K]⁺         | 168.0210         | 168.0210        | 0          | ↓      | 0.678 | 1.63  | 2.0E-14   | 4.70       | 2C      |
| 2-Methyl-3-hydroxy-5-formylpyridine-4-carboxylate | [C₈H₁₄NO₃-H]⁺     | 182.0455         | 182.0450        | -1.6       | ↓      | 0.716 | 1.72  | 9.7E-10   | 3.78       | 2D      |
| Phosphodimethylethanolamine      | [C₈H₁₄NO₃P⁺Na⁺]⁺    | 192.0382         | 192.0396        | -7.3       | ↓      | 0.579 | 1.15  | 8.5E-10   | 3.25       | 2E      |
| 4-Methoxyphenylacetic acid       | [C₇H₁₂O₃+K]⁺        | 205.0280         | 205.0262        | 8.8        | ↓      | 0.600 | 1.16  | 7.5E-9    | 2.11       | 2F      |
| N-Acetylglutamine                | [C₅H₁₀N₂O⁺Na⁺]⁺     | 211.0715         | 211.0689        | 12.3       | ↑      | 0.813 | 2.38  | 1.4E-7    | -1.30      | 2G      |
| 3,5-Dihydroxyphenylvaleric acid  | [C₁₁H₁₈O₄⁺H]⁺       | 211.0990         | 211.0965        | 11.8       | ↓      | 0.844 | 3.18  | 1.2E-13   | 9.66       | 2H      |
| Hydroxyhexanoylglycine           | [C₈H₁₄NO₃⁺Na⁺]⁺     | 212.0891         | 212.0893        | -0.9       | ↓      | 0.628 | 1.61  | 8.9E-8    | 1.32       | 2I      |
| ValLeu                           | [C₁₁H₂₂N₂O₃⁺Na⁺]⁺   | 253.1492         | 253.1523        | -12.2      | ↓      | 0.706 | 1.60  | 4.1E-10   | 1.44       | 3A      |
| LeuHis                           | [C₁₁H₂₁N₂O₃⁺H⁺]⁺    | 269.1585         | 269.1608        | -8.5       | ↑      | 0.661 | 1.34  | 3.0E-7    | -4.14      | 3B      |
| Oleamide                         | [C₁₃H₁₃NO₃⁺Na⁺]⁺    | 304.2630         | 304.2611        | 6.2        | ↑      | 0.599 | 1.24  | 1.7E-2    | -1.74      | 3C      |
| 9,12,13-Trihydroxyoctadecenoic acid | [C₁₈H₁₇O₃⁺Na⁺]⁺   | 353.2263         | 353.2298        | -9.9       | ↑      | 0.741 | 1.88  | 4.1E-3    | -1.44      | 3D      |
| Stearidonyl carnitine            | [C₂₁H₃₁NO₃⁺Na⁺]⁺    | 442.2921         | 442.2928        | -1.6       | ↑      | 0.658 | 0.96  | 7.4E-3    | -1.41      | 3E      |
| Squalene                         | [C₃₀H₄₇O⁺K]⁺        | 449.3511         | 449.3544        | -7.3       | ↑      | 0.634 | 0.87  | 1.5E-2    | -1.35      | 3F      |

a regulation of the intensity in RCC samples compared to control
b VIP value obtained on the basis of PLS-DA analysis
c P-value obtained on the basis of the t-test statistical method
d value of log2 of fold change between controls and cancer samples
Figure Captions

Figure 1. Graphical representation of statistical analysis of MS data: PCA – component 1 vs 2 (A), OPLS-DA (B), PLS-DA – component 1 vs 2 (C) and sPLS-DA component 1 vs 2 (D). Red area represents data for controls while green for cancer patients.

Figure 2. Box plots and ROC curves for m/z values: 144.0051 (A), 155.0848 (B), 168.0210 (C), 182.0445 (D), 192.0382 (E), 205.0280 (F), 211.0715 (G) and 211.0990 (H) respectively.

Figure 3. Box plots and ROC curves for m/z values: 212.0891 (A), 253.1492 (B), 269.1585 (C), 304.2630 (D), 353.2263 (E), 442.2921 (F) and 449.3511 (G) respectively.

Figure 4. Multivariate ROC curve based exploratory analysis based on 15-features intensity data processed in MetaboAnalyst service, showing area under the curve (A) and predicted class probabilities for each sample with label samples classified to the wrong groups (B).
Figure 1
Figure 2
Figure 3
Figure 4
Graphical Index