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Preliminary Metabolomic Study of Urine Samples in Patients Affected by Renal Clear Cell Cancer by GC-MS

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Abstract: Renal cell carcinoma (RCC) represent 2-3% of all cancers. Currently, there are no invasive screening tests that could help to find diagnosis and possible follow up in clinical practice. Most renal tumors are diagnosed by abdominal ultrasound (US) or contrast-enhanced (CT) performed for other medical reasons. In this work, the metabolite profile of urine of RCC samples, has been studied by gas chromatography coupled to mass spectrometry (GC-MS) and multivariate statistical data analysis. By the same means, differences between pathological and control samples were investigated. Results of discriminant analysis were studied with the aim to find possible relevant metabolites for each class. Palmitic acid, malic acid, *allo*-inositol, oleic acid and aspartic acid were up-regulated in pathological samples while psicose was down-regulated.

Keywords: Inositol, Aspartic Acid, GC-MS, Renal Cell Cancer

1. Introduction

Among the amount of tumor types, RCC represents the 2-3% of all cancers [1], holding the highest incidence in Western countries [2]. RCC includes a broad spectrum of histopathological entities described in the 2004 World Health Organization classification [3] and modified by the International Society of Urological Pathology (ISUP) (Vancouver Classification) [4] in which the most common member is clear cell RCC (ccRCC) [5]. Several renal masses remain asymptomatic until the late disease stages. The 50% of RCCs are detected accidentally due to non-specific symptoms and for the totally absence of non-invasive tests [6, 7]. Most renal tumors are diagnosed by abdominal US or CT, regularly performed for further disease examination [7]. Currently, there are no blood or urine screening tests that can help in the diagnosis of this pathology. Numerous molecular markers, such as carbonic anhydrase IX (CaIX), vascular endothelial growth factor (VEGF), hypoxia-inducible factor (HIF), Ki67 (proliferation), p53, p21 [8], PTEN (phosphatase and tensin homolog) (cell cycle), E-cadherin, C-reactive protein (CRP), osteopontin [9], CD44 (cell adhesion) [10, 11], CXCR4 [12], and other cell cycle and proliferative markers [13, 14] have been investigated. Unfortunately, these markers were not improved as possible prognostic biomarkers. Moreover, the therapy is essentially surgical at the early stage. If RCC has spread outside of the kidneys, often into the lymph nodes, the lungs or the main vein of the kidney, then multiple therapies are used including surgery and medications. RCC is resistant to chemotherapy and radiotherapy in most cases, but it does respond well to immunotherapy with interleukin-2 or interferon-alpha, biologic, or targeted therapy. In all RCC types, prognosis worsens with stage and histopathological grade. The 5-year overall survival percentage (OS) for all types of RCC is 49%, which has improved since 2006 probably due to an increase in incidentally detected RCCs and because of the introduction of tyrosine kinase inhibitor (TKI) [15].

Metabolomics, one of the latest omics sciences, could be a useful approach to understand pathogenic mechanism in human being. In fact, this technique explores the profile of different biological matrices through the study of

metabolites. In a biological system, such as urinary samples, a metabolite up-regulation could share information concerning the physiological and functional status of such system. Metabolomics is based on the use of different hyphenated analytical techniques, such as mass spectrometry as well as nuclear magnetic resonance spectroscopy (NMR). These techniques are often associated to a multivariate statistical analysis with the aim to extract useful information from complex models [16-18] and thus understanding mechanism of action. These techniques are related to a multivariate statistical analysis useful to extract information from complex matrices [19] and to understand possible mechanism of action. In this work, we used a metabolomics approach based on GC-MS coupled with multivariate data analysis with the aim to highlight possible discriminant metabolites that could help in both diagnosis of RCC and understanding the metabolic pathways implicated in this pathology.

2. Materials and Methods

2.1. Subjects

This study was carried out on urine samples collected from patients affected by clear cell renal carcinoma~~s~~, admitted to the Urological Clinic of the University of Sassari (Sassari, Italy). This pathological pool consisted of six males and seven females with an average of age of 57 and 65 respectively, not presenting any comorbidity. Furthermore, a healthy control group comprising of six women with a mean age of 62 years and nine males with a mean age of 56 years was analyzed. As well as for ~~the~~ patients, the absence of any illnesses was verified, ~~as well as for tumors~~. Written informed consent was obtained from all participants before enrolment in the study.

2.2. Chemicals

All the chemicals used in this study were of analytical grade. Derivatized 2, 2, 3, 3-d4-succinic acid was used as internal standard, and pyridine and hexane were used as solvent (Sigma Aldrich, Milano, Italy). Methoxyamine hydrochloride, N, O-bis (trimethylsilyl)trifluoroacetamide, trimethylchlorosilane (BSTFA+TMCS), and all the analytical standards were purchased from Sigma (St. Louis, MO, USA).

2.3. Urine Samples Collection and Preparation

Urine samples (2–3 mL) were collected into a 15 mL Falcon tube. The tubes were then stored at −80°C prior analysis by GC-MS, which was carried out at the Department of Life and Environmental Sciences in Cagliari. After thawing on ice, the urine samples were centrifuged for 10 min at 14000 rpm. 150μL of surnatant from each sample was transferred into an eppendorf tube containing 1 mg of urease, sonicated for 30 min at 37°C and then centrifuged for 10 min at 13200 rpm. The resulting surnatant was separated from the precipitate and dried under a gentle nitrogen stream. Dried samples were derivatized with 50 μL of methoxyamine in pyridine solution (10 mg/mL). After 17 h, 100 μL of BSTFA+TMCS was added and after 1 h samples were resuspended with 600 μL of hexane. Trimethylsylilated 2, 2, 3, 3-d4-succinic acid at 5 mg/L was used as internal standard.

2.4. GC-MS Analysis

One microliter of derivatized sample was injected splitless into a 6850 gas chromatograph coupled with a 5973 Network mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped with a 30 m × 0.25 mm ID, fused silica capillary column, which was chemically bonded with 0.25 µm DB5-MS stationary phase (J&W scientific, Folsom, CA, USA). The injector temperature was 200°C. The gas flow rate through the column was 1 mL/min. The column initial temperature was kept at 50°C for 10 min. The temperature was then increased up to 300°C at 10°C/min and held at 300°C for 10 min. The transfer line and the ion source temperatures were at 280°C and 180°C, respectively. Ions were generated at 70 eV with electron ionization and were recorded at 1.6 scan/sec over the mass range m/z 50-550. GC-MS data analysis was conducted by integrating each resolved chromatogram peak and normalizing the area for the corrected total area of the chromatogram. Peaks were examined for their mass spectra and their identification was performed using the NIST08 library after deconvolution with AMDIS.

2.5. Multivariate Statistical Data Analysis

Multivariate statistical data analysis of GC-MS ~~data~~ analysis was performed using the SIMCA software (version 13.0, Umetrics, Umea, Sweden). Untargeted principal components analysis (PCA) was applied to visualize tendency of samples to cluster based on similarities and dissimilarities in their metabolite profile and to detect the presence of outliers that may be due to errors in sample preparation or instrumentation parameters. Outliers can be detected by different tests, as implemented in SIMCA software. For sample classification and for the search of biomarkers that differentiate the predefined classes, the Orthogonal Partial Least Square-Discriminant Analysis (OPLS-DA) was performed, ~~this is~~ a supervised classification method that requires information about class membership of samples. In OPLS-DA, class separation is maximized in the predictive component (*x*-axis) and its orthogonal component (*y*-axis) express intra-class variability. The goodness of models was evaluated by the parameters: R^2Y (goodness of classification) and Q^2Y (goodness of classification in cross-validation). For what concerns prediction power, a permutation test was used. The study of discriminant metabolites was performed using the S-plot model, which combines covariance and correlation loading profiles [19].

3. Results and Discussion

A total of 28 urine samples were analyzed by GC-MS. Chromatograms and spectra analysis yield 71 metabolites from GC-MS (Table 1).

Table 1. *GC-MS characteristics of metabolites.*

Compound	Retention time (min)	EI-MS, *m/z* (amu) (% relative ion abundances)
glycolic acid	16,228	147-73-177
alanine	16,771	116-73-147
glycine	17,183	73-147-102
oxalic acid	17,405	73-147-190
3-hidroxybutiric acid	17,884	147-73-233
U1	18,224	73-221-248
isovaleric acid	18,733	73-147-131
Valine	18,815	73-147-144
canavanine	18,901	73-156-201
glycine	20,235	174-73-248
butanedioic acid	20,369	73-147-247
gliceric acid	20,552	73-147-187-292
erithronic acid	20,73	73-117-292
U2	20,859	73-117-147-292
serine	20,998	73-204-218
U3	21,208	73-147-117
threonine	21,332	73-218-291
2,4-butanoic acid	21,695	73-103-147-219
butanoic acid 3,4-dioh	21,967	73-147-233-189
homoserine	22,206	73-218-128
U4	22,245	73-147-350
aminomalonic acid	22,473	73-147-218
malic acid	22,695	73-147-233
l-aspartic acid	23,107	73-232-218
pyroglutamic acid	23,171	73-156-147
U5	23,23	73-129-247
2,3,4-trihidroxy butiric acid	23,348	73-147-292
creatinine	23,575	115-73-329
U6	23,779	147-129-247
U7	24,317	73-147-231
tartaric acid	24,472	73-147-292
U8	24,602	73-147-245
U9	24,714	73-217-147
U10	24,802	73-147-245
ribose	24,923	73-217-103
U11	25,24	73-175-217
U12	25,319	136-166-210
xylitol	25,398	73-217-147
U13	25,708	73-217-204
cis-aconitate	25,781	73-147-229
U14	25,889	73-357-292
ribonic acid	26,066	73-147-292
citric acid	26,528	73-147-273
allo-inositol	26,617	73-217-260
U15	26,776	221-117-292
psicose	26,822	73-245-147
fructose	26,98	73-217-307
galactose	27,105	73-205-319
tagatose	27,129	73-217-307-103
glucose	27,183	73-204-147
glucose	27,229	73-319-205
mannose	27,289	217-305-321
mannitol	27,627	73-205-319
U16	27,695	73-147-160-333
U17	28,286	73-147-292
gluconic acid	28,33	73-147-333
scyllo-inositol	28,595	73-318-305
palmitic acid	28,886	313-75-317
myo-inositol	29,216	73-305-217
quiric acid	29,723	73-345-255
oleic acid	30,472	73-117-129-339
2-o-glycerol-a-d-galactopyranoside	31,165	73-204-217
U18	31,400	73-285-222
digalacturonic acid	32,144	73-147-375

Compound	Retention time (min)	EI-MS, *m/z* (amu) (% relative ion abundances)
U19	32,389	73-285-147
U20	32,507	73-375-147-254
sucrose	33,764	361-73-217
lactose	34,142	73-204-361
maltose	34,56	73-361-217
U21	34,642	204-73-217
galactinol	34,796	73-204-217

To investigate correlations between compositional and analytical data, and to observe samples distribution in the multivariate space, an unsupervised PCA analysis was performed. The first two principal components were able to describe the 35% of the total information (data not shown). No outliers were observed. With the aim to find those metabolites that mainly differentiate urines from patients affected by renal cell carcinoma and control samples, a supervised pair-wise OPLS-DA were applied. Validation parameters of the discriminant analysis were: 0.80 for R^2Y, and 0.54 for Q^2 (Figure 1).

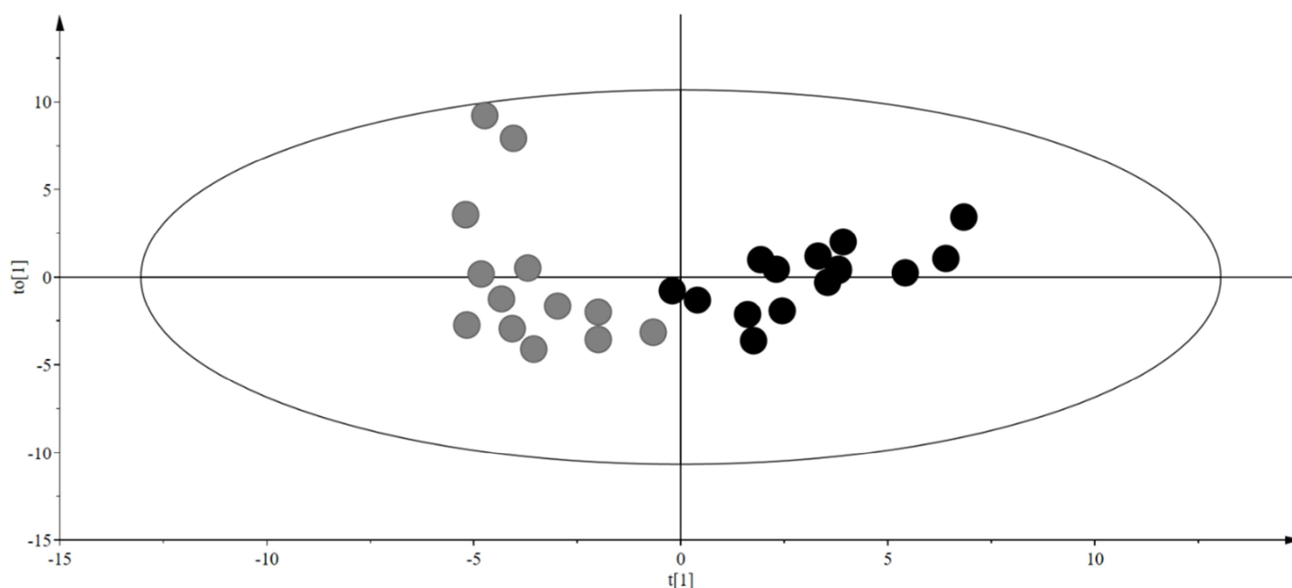

Figure 1. *OPLS-DA scores plot of RCCs samples vs control samples. Validation parameters of the discriminant analysis were: 0.80 for R^2Y, and 0.54 for Q^2. Permutation test showed a Q^2 value of -0.31.*

Permutation test showed a Q^2 value of -0.31. To identify statistically significant metabolites, an S-plot was performed. The resultant most discriminant metabolites for the pathological class were: palmitic acid, malic acid, *allo*-inositol, oleic acid, aspartic acid. The high levels of two chain fatty acids, such as palmitic acid and oleic acid, in pathological samples highlight possible alterations in the lipid metabolism. In fact, recently Hakimi el al. [20] showed an increase of fatty acid biosynthesis and a decrease in oxidative phosphorylation at the beginning of the pathogenesis and a reversal of these patterns during tumor progression. Moreover, Schieda et al. found intracellular lipid in a minority of papillary renal cell cancers [21]. Furthermore, it is still clear that lipid peroxidation, which is increased in obese and hypertensive subjects, is the mechanism responsible for their increased risk of renal cell carcinoma [22]. Furthermore, the up-regulation of malic acid in the pathological samples suggests an alteration of the citric acid cycle. In fact, different syndromes of renal cancer were recently described by Launonen et al. [23]. These syndromes evidenced a co-segregation of cutaneous leiomyomas on type II papillary renal cell carcinoma in two familiar lines that showed modification in the chromosome 1. This gene caused the deficiency of fumarate hydratase which is responsible of the conversion of fumarate into malate through the citric acid cycle. The depletion of fumarate hydratase causes an increase of the glycolytic metabolism and an up-regulation of hypoxia-inducible factors transcripts that helps tumors in their survival and proliferation [24]. Furthermore, the overall inositol phosphate metabolism pathway was significantly associated with risk of lung cancer ($P = 2.00 \times 10^{-4}$), esophageal squamous cell carcinoma ($P = 5.70 \times 10^{-3}$), gastric cancer ($P = 3.03 \times 10^{-2}$) and renal cell carcinoma ($P = 1.26 \times 10^{-2}$) [25].

4. Conclusion

In this work, the metabolite profile of urine patients affected by RCC has been investigated and compared with control samples. Gas-chromatography coupled to mass spectrometry and multivariate statistical data analysis were useful to highlight discriminant metabolites that could be worthwhile to understand the possible metabolic pathways implicated in this pathology. Considering these results and

taking into account the need of further experiments, this study can be considered the first step towards the prediction of RCC disease.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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