Bladder Cancer Biomarker Screening based on Non-targeted Urine Metabolomics

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Abstract

Background: Bladder cancer is one of the most common malignancies of the urinary system, and its screening relies heavily on invasive cystoscopy, which increases the risk of urethral injury and infection. This study aims to use non-targeted metabolomics approaches to screen the metabolites that are expressed significantly differently from the urine of bladder cancer patients and the cancer-free controls.

Methods and Results: This study performed non-targeted metabolomic analysis and metabolite identification on the urine of bladder cancer patients (n=57) and the cancer-free controls (n=38) using a liquid chromatography-mass spectrometry analyzer. The results showed that there were significant differences in the expression of 27 metabolites between bladder cancer patients and the cancer-free controls.

Conclusions: In the multivariate statistical analysis of this study, the urinary metabolic profile data of bladder cancer patients are analyzed, and the receiver operating characteristic curve analysis shows that it is possible to perform non-invasive clinical diagnoses of bladder cancer through these candidate biomarkers.

Introduction

Bladder cancer is the tenth most common cancer in the world and one of the deadliest cancers [1]. Currently, screening for bladder cancer still relies heavily on invasive cystoscopy [2], which can directly show tumor lesions in the bladder, and can perform biopsies for further pathological examination. However, this uncomfortable procedure can lead to urethral injury, urinary tract infection, and hematuria [3]. On the other hand, urine cytology is a non-invasive method to detect cancer cells in urine. Although it has a high specificity (86%-99%), it has a low sensitivity, which is between 48% and 70% [4]. In addition, CT and MRI are mainly used for staging and evaluation of distant metastasis in bladder cancer patients [2].

Urine stored in the bladder not only comes into direct contact with a bladder tumor, but may also be affected by certain chemicals secreted by the tumor. Therefore, the non-invasive and easy collection of urine makes it an ideal sample for early screening of bladder cancer [5]. Through multivariate statistical analysis and metabolite identification via a bioinformatics platform, this study aims to use non-targeted metabolomics approaches to screen the metabolites that are expressed significantly differently from the urine of bladder cancer patients and the cancer-free controls.

Materials And Methods

4.1 Patient and sample collection

The samples in this study are urine samples from bladder cancer patients (n = 57) and cancer-free controls (n = 38) that were collected from patients in Zhongshan Hospital affiliated with Xiamen University in 2021. Table 1 shows statistical data on the clinical characteristics of the patients. Urine samples for bladder cancer patients were collected from patients who had been pathologically diagnosed but not receiving treatment. According to the pathological diagnosis, they were classified into muscle-invasive bladder cancer (MIBC) and non-muscle invasive bladder cancer (NMIBC), as well as high and low urothelial carcinoma. The study design was carried out in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Zhongshan Hospital Affiliated to Xiamen University (XMZSYY-AF-SC-12-03). All subjects had given informed consent to be included in this study before participation.

Table.1

Clinical characteristics of BC patients and cancer-free controls

Group	Number of Samples (F/M)	Age Range (years)	Mean Age $\pm$ SD (years)
Cancer-Free	38(13/25)	61	61 $\pm$ 4.1
BC Patients	57(11/46)	65.8	65.8 $\pm$ 3.5
Pathological type			
MIBC	20(3/17)	69.3	69.3 $\pm$ 5.0
NMIBC	37(8/29)	63.9	63.9 $\pm$ 4.6
Urothelial carcinoma grade			
High	28(3/25)	67.7	67.7 $\pm$ 4.2
Low	29(8/21)	64.0	64.0 $\pm$ 5.6

4.2 UPLC-MS analysis and metabolite identification

The Thermo Scientific UPLC system (DIONEX, Ultimate 3000, USA) and the Q-Exactive Mass Spectrometer (Thermo Scientific, USA) were used for non-targeted urine metabolome detection. The test procedure for UPLC-MS analysis is consistent with the previous studies [6] and is explained in detail in Supplementary Material B.

Compound Discoverer 2.1 (Thermo Scientific, USA) software was used to extract metabolic data from the original LC-MS data, and the result data matrix was returned with sample name, the mass-to-charge ratio (m/z), peak intensity, retention time, and expression level. In addition, the Human Metabolome Database (www.hmdb.ca) and ChemSpider database (<http://www.chemspider.com>) were consulted to assist in the identification and interpretation of the urinary metabolic biomarkers. The final data matrix is aggregated based on the data collected in the positive and negative ion mode, with exact compound names and corresponding HMDB ID.

4.3 Multivariate statistical analysis

Two different statistical analysis methods were combined to screen different metabolites between the two groups: (1) Principal Component Analysis (PCA) and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) were applied to the data matrix in SIMCA 14.1 software, and the Variable Importance in Projection (VIP) score of the OPLS-DA model was applied to screen the metabolites that can best distinguish the two groups. (2) The p-value of each variable in the data matrix was calculated in MetaboAnalyst 5.0 (www.MetaboAnalyst.ca) using the unpaired Wilcoxon rank-sum test, and the false discovery rate (FDR) [7] was calculated to correct the p-value of multiple comparisons [8]. Variables with VIP > 1.5 and FDR < 0.05 were considered as metabolites with statistically significant differences between the groups. Then, receiver operating characteristic curve analysis (ROC) was used to evaluate the ability of this group of metabolites to diagnose bladder cancer. Finally, the Pearson correlation coefficient r value between age, BMI, gender, and this statistically significant metabolites group was calculated by MetaboAnalyst5.0 to evaluate the potential impact of these possible confounding factors

Results

The PCA score plot was drawn by the principal component analysis (PCA) of the quality control samples (QC = 16) of the urine samples collected in this experiment (Fig. S1), which were closely clustered, indicating that the experimental data has high consistency in detection.

5.1 Analysis of metabolite differences between bladder cancer patients and the cancer-free controls

In this study, a multivariate statistical analysis of metabolites between bladder cancer patients (n = 57) and cancer-free controls (n = 38) was performed. Figure 1a is an OPLS-DA model score plot constructed based on the urinary metabolite level of bladder cancer patients and cancer-free controls (95 objects × 677 variables). The PCA score plot shows a significant difference between the bladder cancer patients and the cancer-free controls, and the permutation test result (Fig. S2) shows that the classification model does not appear to be over-fitting.

Table 2 summarizes the statistical indicators of these significantly different urinary metabolites, of which 27 statistically significantly different variables (VIP > 1.5 and FDR < 0.05) were identified as the differential metabolites between bladder cancer patients and cancer-free controls.

Table.2

A list of 27 metabolites found changed in the urine of bladder cancer patients (n=57) compared with cancer-free controls (n=38).

Metabolite Name	VIP	p-value FDR	Log ₂ (FC)	AUC	Down or Up	HMDB ID	Metabolite sites
Mesobilirubinogen	2.79	0.0001	-1.91	0.764	DOWN	HMDB0001898	Organoheterocyclic compounds
Isoleucyl-Phenylalanine	2.77	0.0003	5.26	0.737	UP	HMDB0028914	Organic acids
2-Hydroxycaproic acid	2.63	0.0015	-2.22	0.703	DOWN	HMDB0001624	Fatty Acyls
L-Urobilin	2.54	0.0001	-2.82	0.762	DOWN	HMDB0004159	Organoheterocyclic compounds
Choline	2.34	0.0000	3.22	0.772	UP	HMDB0000097	Organic nitrogen compounds
1-Methylhistidine	2.29	0.0001	1.14	0.759	UP	HMDB0000001	Organic acids
N-Undecanoylglycine	2.24	0.0000	1.43	0.823	UP	HMDB0013286	Organic acids
6-Hydroxyhexanoic acid	2.16	0.0040	-0.93	0.681	DOWN	HMDB0012843	Fatty Acyls
Linoleyl carnitine	2.14	0.0000	3.35	0.777	UP	HMDB0006469	Fatty Acyls
Cytidine	1.99	0.0137	0.40	0.655	DOWN	HMDB0000089	Carbohydrates
Adenosine monophosphate	1.98	0.0034	3.42	0.685	UP	HMDB0000045	Nucleic acids
Dihydrotestosterone	1.94	0.0000	-1.25	0.844	DOWN	HMDB0002961	Sterol Lipids
L-Histidine	1.89	0.0008	-0.87	0.717	DOWN	HMDB0000177	Organic acids
Flavin Mononucleotide	1.89	0.0040	-2.59	0.681	DOWN	HMDB0001520	Nucleic acids
Valyl-Serine	1.85	0.0000	-0.95	0.768	DOWN	HMDB0029136	Organic acids
4-Acetamidobutanoic acid	1.85	0.0008	0.61	0.717	DOWN	HMDB0003681	Organic acids
Niacinamide	1.80	0.0034	5.52	0.685	UP	HMDB0001406	Organoheterocyclic compounds
(5R)-5-Hydroxyhexanoic acid	1.72	0.0035	-0.92	0.684	DOWN	HMDB0000409	Fatty Acyls
Adenosine	1.71	0.0011	-0.56	0.711	DOWN	HMDB0000050	Nucleic acids
L-Targinine	1.70	0.0001	-1.24	0.759	DOWN	HMDB0029416	Organic acids
Phenylalanyl-Asparagine	1.65	0.0025	2.58	0.692	UP	HMDB0028990	Organic acids
1,11-Undeannedicarboxylic acid	1.63	0.0180	-1.44	0.648	DOWN	HMDB0002327	Fatty Acyls
Ubiquinone-2	1.58	0.0028	0.99	0.689	DOWN	HMDB0006709	Lipids and lipid-like

							molecules
Butenylcarnitine	1.57	0.0293	-0.37	0.635	DOWN	HMDB0013126	Lipids and lipid-like molecules
Sebacic acid	1.56	0.0154	0.64	0.652	DOWN	HMDB0000792	Organic acids
Alanyl-Tyrosine	1.56	0.0295	0.29	0.635	DOWN	HMDB0028699	Organic acids
Taurine	1.54	0.0155	1.40	0.651	UP	HMDB0000251	Organic acids

VIP: Variable Importance in Projection; FDR: false discovery rate

5.2 The influence of NMIBC and MIBC on urinary metabolites

Based on non-targeted urine metabolomics data, MIBC/NMIBC and low/high OPLS-DA models were established. MIBC and NMIBC (57 observations × 677 variables) showed some differences in the score plot of the OPLS-DA model, but when the metabolites were further analyzed for differences, after FDR correction, no metabolites displayed statistical differences (Fig. S3). The OPLS-DA model constructed based on the low/high grouping of urothelial carcinoma grade also did not show statistically significant differences in metabolites (Fig. S4).

5.3 Evaluation of differential metabolites to distinguish bladder cancer and cancer-free controls

The OPLS-DA model was constructed based on the 27 different metabolite sets obtained from the above analysis, and a ROC curve (Fig. 1b) was drawn through marker analysis to evaluate its performance in the classification of bladder cancer, where the AUC value was 0.951, the sensitivity was 91.2%, the specificity was 86.8%, and the accuracy was 89.5%. The AUC value of each differential metabolite is shown in Table 2. For the OPLS-DA model containing 27 metabolites, the AUC value was 0.943, the sensitivity was 81.6%, the specificity was 94.6%, and the accuracy was 88%, which were used to distinguish NMIBC from cancer-free controls (Fig. 1c). When distinguishing MIBC from cancer-free controls, the AUC value was 0.944, the sensitivity was 90%, the specificity was 89.7%, and the accuracy was 72% (Fig. 1d).

5.4 Potential influence of age, gender, and BMI on statistically different metabolite levels

The Pearson correlation coefficients of differential metabolites with age, gender, and BMI were calculated by MetaboAnalyst 5.0 (Fig. S5), and the results showed that the correlation between age, gender, and BMI and the 27 differential metabolites were small ($|r| \leq 0.3$). Thus, it can be concluded that age, gender, and BMI in this study did not show a significant correlation with the changes in metabolites detected between bladder cancer patients and the cancer-free controls [3].

5.5. Enrichment analysis and KEGG pathway analysis of differential metabolites

The enrichment analysis revealed that the differential metabolites are mainly contained in organic acids, fatty acyl groups, organic heterocyclic compounds, sterol lipids, nucleic acids, organic nitrogen compounds, lipids and lipid-like molecules, and carbohydrate metabolites (Fig. 3a). In the KEGG pathway analysis, this group of differential metabolites was concentrated in the pathways of histidine metabolism, steroid hormone biosynthesis, glycerophospholipid metabolism, glycine, serine and threonine metabolism, purine metabolism, β -alanine metabolism, amino-tRNA biosynthesis, riboflavin metabolism, niacin and nicotinamide metabolism, taurine and sub taurine metabolism, primary bile acid biosynthesis, arginine and proline metabolism, and pyrimidine metabolism in metabolic pathways (Fig. 3b).

Discussion

Our study selected a total of 27 differential metabolites ($VIP > 1.5$ and $FDR < 0.05$) from the urine of bladder cancer patients and cancer-free controls through non-targeted metabolomic analysis. The ROC curve analysis showed that when distinguishing bladder cancer patients from cancer-free controls, the AUC value of this group of metabolites was 0.851, the sensitivity was 70%, the specificity was 89%, and the accuracy was 80%. Compared with FDA-approved urine biomarkers, this group of significantly different urinary metabolites showed better overall sensitivity and specificity than NMP-22 (69% and 77%, respectively) and BTA Track (65% and 74%, respectively) [9].

The ability to grade bladder cancer staging will effectively reduce unnecessary cystoscopy and overtreatment of bladder cancer [10]. However, in this study, there was not a statistically significant difference in metabolites between NMIBC and MIBC. Sriastava's NMR study also did not find significant changes in the concentration of metabolites between bladder cancer groups [11]. However, subsequent studies by Issaq et al. [12], X. Cheng [13], Alberice [14], X. Liu [15], and J. Peng [16] have shown that urinary metabolomic markers for bladder cancer indeed are able to distinguish the tumor stages. The study by Dinges et al. suggested that metabolomics may show the potential to differentiate cancer stages when studied in larger cohorts [17].

This group of differential metabolites may provide new insights into dysregulated urinary metabolism in bladder cancer patients. Significant changes have occurred in ten organic acids in the urine of patients with bladder cancer, including 1-methylhistidine, L-histidine, taurine, 4-acetyl aminobutyric acid, N-undecanoyl glycine, isoleucine-phenylalanine, phenylalanine-aspartic acid, valine-serine, L-arginine, and sebacic acid. The significant increase in the taurine in the urine of bladder cancer patients is consistent with the study by Wittmann et al. [10], that is, the taurine in the urine of bladder cancer patients is higher than that of the healthy control group. The biological background of taurine shows it to be a known free radical scavenger that can prevent cell damage and is expected to be a marker for cancer screening [17]. Whereas leucine-phenylalanine, phenylalanine-aspartic acid, and valine-serine are collectively referred to as dipeptides, which are incomplete breakdown products of proteolytic metabolism, most of which are only transient intermediates that enter specific amino acid degradation pathways after further protein hydrolysis. Changes in amino acids and dipeptides in urine may reflect shedding into the urine due to high levels of cell division, cell death, and protein degradation. A literature search did not find any reports of dipeptides associated with bladder cancer and other cancers. The content of 1-methylhistidine in bladder cancer is increased, while the content of L-histidine is decreased. The research of Alberice and T. Zhang showed that the histidine and its methylated derivatives in the urine of patients with bladder cancer and ovarian cancer changed significantly [5, 18]. The decrease in the level of L-arginine in the urine of bladder cancer patients may be related to the catabolic disease state of cancer, resulting in increased arginine utilization, which will lead to arginine consumption [19]. 4-Acetyl aminobutyric acid is a product of the urea cycle and amino metabolism, while N-undecanoyl glycine is a secondary metabolite of fatty acids; and metabolomics studies searched in the literature have not shown that they are associated with tumors.

The six fatty acyl groups in the urine of patients with bladder cancer are changed, including 6-hydroxyhexanoic acid, (5R)-5-hydroxyhexanoic acid, 2-hydroxyhexanoic acid, 1,11-undecanedicarboxylic acid, linoleoyl carnitine, and butenyl carnitine. The general function of acylcarnitine is to transport acyl groups (organic acids and fatty acids) from the cytoplasm to the mitochondria so that they can be broken down to produce energy. This process is known as β -oxidation. Considering that the urine under normal physiological conditions does not contain lipids, Paskenta's study showed increased urinary acetyl carnitine and adipic acid in patients with bladder cancer, suggesting disturbances in fatty acid transport and changes in energy metabolic processes [20, 21]. Increased urinary adenosine monophosphate content and decreased ubiquinone-2 and flavin mononucleotide (FMN) in the electron chain of cellular oxygen respiration in bladder cancer patients are also considered to be associated with disturbed energy metabolism.

Mesobilirubinogen and L-urobilin are reduced in bladder cancer patients. Their metabolism is derived from the degradation of heme to produce urobilinogen, but whether their metabolic changes are related to tumors and other wasting diseases requires further research. Nicotinamide, which is a heterocyclic aromatic compound, is relatively increased in bladder cancer, and it is related to metabolic disorders of the NAD⁺ signaling pathway (cancer) [22].

In this study, compared with the controls, bladder cancer patients have lower levels of dihydrotestosterone in urine. Although there are no reports of androgens metabolism changes in the literature, the research by Miyamoto et al. pointed to suggest that androgens and their receptors play a contributory role in the development of bladder cancer [23]. The increase in urinary choline levels in bladder cancer patients is consistent with the study results by Kouznetsova et al., in which patients with advanced bladder cancer have significant multiplicative changes in urinary choline [24].

The change of urinary adenosine in bladder cancer patients is consistent with Jadidi-Niaragh's study. His research pointed out that adenosine is an important factor in the tumor microenvironment that inhibits the anti-tumor response of cancer cells and immune cells. Inhibiting the production of adenosine can significantly prevent tumor growth in vivo, and targeted inhibition of adenosine receptors may be a promising therapeutic approach in tumor treatment.

Overall, our study shows the potential of a statistically significantly different set of urinary metabolites in the early detection of bladder cancer, providing new insights into changes in urinary metabolism in patients with bladder cancer. This includes changes in lipid metabolism and protein catabolism in bladder cancer patients, as well as changes in the metabolism of adenosine monophosphate (AMP), ubiquinone-2, and flavin mononucleotide (FMN) in the urine in the energy metabolism pathway. This group of differential metabolites of taurine, nicotinamide, histidine, adenosine, dihydrotestosterone, choline, and arginine has also been reported to be metabolically altered in previous tumor-related studies. However, the potential influences of diet, environment, lifestyle, medications, and other disease conditions still cannot be ruled out. Comprehensively considering multiple confounding variables and testing the effectiveness of the proposed markers are important steps in the development of markers for clinical use, which need to be verified in larger cohort studies in the future.

The advantage of using metabolomics is that it provides a functional measurement of the physiological state of an organism. Our study found that the difference between the urinary metabolites of bladder cancer patients and the cancer-free controls is statistically significant, which may provide clinically useful biomarkers for identifying metabolic changes and provide new insights into the occurrence and development of bladder cancer. In the future, more mature and standardized urinary metabolomics tests will be able to effectively supplement existing screening tools. A positive result for altered urinary metabolism prompts more detailed tests to detect and identify specific cancers or precancerous conditions.

Declarations

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Author contribution

JL,BS and CZ conceptualized and designed the study. JL, BS and SL performed the experiments. JL, BS, CZ and SL analyzed the results. The manuscript was drafted by JL, BS, CZ and SL and edited PB. All authors have read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest

Authors declare no conflict of interest. All authors have read and approved the manuscript for submission to Molecular Biology Reports.

Ethical approval

The study design was carried out in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Zhongshan Hospital Affiliated to Xiamen University (XMZSYY-AF-SC-12-03).

Consent to participate

Written informed consent was obtained from individual participants included in the study.

Consent to publish

Patients signed informed consent regarding publishing their data.

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Figures

Figure 1

OPLS-DA score plot of the urinary metabolites profile of (a) bladder cancer patients (green circles, n=57) and the cancer-free controls (blue circles, n=38), and the ROC curve obtained for the set of 27 discriminant metabolites; (b) bladder cancer patients (n = 57) and the cancer-free controls (n = 38); (c) NMIBC patients (n = 37) and the cancer-free controls (n = 38); (d) MIBC patients (n = 20) and the cancer-free controls (n = 38). Abbreviations: AUC: Area Under Curve, Sens: sensitivity, Spe: specificity, Accu: accuracy

Figure 2

Overview of the enriched metabolite sets (a) and KEGG pathway analysis; (b) obtained from the metabolites profile of 27 urinary biomarkers in bladder cancer patients (n=57) and the cancer-free controls (n=38).

Supplementary Files

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