Metabolomic profiling identifies novel biomarkers and mechanisms in human bladder cancer treated with submucosal injection of gemcitabine

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Abstract. Bladder cancer (BCa) is a common urinary tract malignancy with frequent recurrences after initial resection. Submucosal injection of gemcitabine prior to transurethral resection of bladder tumor (TURBT) may prevent recurrence of urothelial cancer. However, the underlying mechanism remains unknown. In the present study, ultra-performance liquid chromatography Q-Exactive mass spectrometry was used to profile tissue metabolites from 12 BCa patients. The 48 samples included pre- and post-gemcitabine treatment BCa tissues, as well as adjacent normal tissues. Principal component analysis (PCA) revealed that the metabolic profiles of pre-gemcitabine BCa tissues differed significantly from those of pre-gemcitabine normal tissues. A total of 34 significantly altered metabolites were further analyzed. Pathway analysis using MetaboAnalyst identified three metabolic pathways closely associated with BCa, including glutathione, purine and thiamine metabolism, while gluta-thione metabolism was also identified by the enrichment analysis using MetaboAnalyst. In search of the possible targets of gemcitabine, metabolite profiles were compared between the pre-gemcitabine normal and post-gemcitabine BCa tissues. Among the 34 metabolites associated with BCa, the levels of bilirubin and retinal recovered in BCa tissues treated with gemcitabine. When comparing normal bladder tissues with and without gemcitabine treatment, among the 34 metabolites associated with BCa, it was observed that histamine change may be associated with the prevention of relapse, whereas thiamine change may be involved in possible side effects. Therefore, by employing a hypothesis-free tissue-based metabolomics study, the present study investigated the metabolic signatures of BCa and found that bilirubin and retinal may be involved in the mechanism underlying the biomolecular action of submucosal injection of gemcitabine in urothelial BCa.

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Abbreviations: TURBT, transurethral resection of bladder tumor; PCA, principal component analysis; ACN, acetonitrile; KEGG, Kyoto Encyclopedia of Genes and Genomes; HMDB, human metabolome database; SMPDB, Small Molecule Pathway Database; HPLC/MS, high-performance liquid chromatography/ mass spectrometry; UPLC-MS, ultra-performance LC-MS; UPLC-TOF-MS, UPLC time-of-flight MS; UPLC-HRMS, UPLC high-resolution MS; BCa, bladder cancer

Key words: tissue metabolomics, submucosal injection, gemcitabine, biomarkers, bladder cancer

Introduction

Bladder cancer (BCa) ranks ninth among the most common solid tumors worldwide (1). Approximately 75% of newly diagnosed BCa cases are non-muscle invasive, and the majority are histologically low-grade cancer (2). Routine surveillance to monitor BCa recurrence includes cystoscopic examination due to a high risk of recurrence after initial resection. The repeat transurethral resection of bladder tumor (TURBT) remains the first-line treatment for BCa recurrence (3). However, all these invasive procedures result in a high cost of care, and are often associated with significant morbidity. Therefore, more effective interventions to prevent BCa recurrence are urgently needed.

Submucosal injection of antitumor drugs (pirarubicin) after standard TURBT was proven to be an effective approach to reducing superficial tumor recurrence (4). Gemcitabine is a pivotal chemotherapeutic agent widely used for BCa due to its low toxicity in general (5) and as an intravesical instillation (3). Data from our experimental and clinical studies also demonstrated that submucosal injection of gemcitabine prior to TURBT significantly reduced BCa recurrence (6). However, the underlying mechanisms are largely unknown.

Metabolomics is a newly emerging technology, which enables the identification of endogenous compounds and potentially novel mechanisms associated with disease processes (7). Metabolomics has been used to profile metabolites in various biological samples, such as serum (8), urine (9-15) and tissue (16,17), which are the results of the metabolic response of living systems to drug toxicity or disease (10). Potential biomarkers identified from metabolomic profiling studies on BCa may be of diagnostic value and act as indicators of cancer recurrence (18). Currently, a number of analytical platforms, such as high-performance liquid chromatography/mass spectrometry (HPLC/MS) (10), ultra-performance LC-MS (UPLC-MS) (12), and UPLC time-of-flight MS (UPLC-TOF-MS) (15), have been employed to study the metabolomics of BCa by using urine samples. However, metabolomic studies on BCa tissues is relatively scarce (16,17). Notably, different metabolomic platforms with their unique analytical approaches provide complementary insights into metabolome changes (9,11-15). Therefore, there is a need to study the tissue-based metabolic signatures of BCa using a new metabolomics platform.

Metabolomics has also been applied to cancer treatment and drug target discovery. Eidelman *et al* reported using metabolomics to screen the potential therapeutic pathways in prostate cancer (PCa) (19). Metabolomics has also been proven to be a promising approach to developing reliable therapeutic targets for PCa treatment (20). The present study employed liquid chromatography (LC)-Q-Exactive MS-based metabolomic technology to study the metabolic changes in BCa tissues before and after treatment with gemcitabine. Identification of the key metabolites may reveal new metabolic changes associated with BCa and uncover the changes that mediate the effect of gemcitabine in the treatment of BCa.

Materials and methods

Clinical samples. A total of 12 patients (9 men and 3 women; age range, 55-85 years) who had undergone TURBT at the Affiliated Huai'an No. 1 People's Hospital of Nanjing Medical University were recruited between December 2016 and September 2017. Bladder tissue samples were collected from the same patient immediately prior to and 30 min after submucosal injection of gemcitabine (50 mg, dissolved in 20 ml normal saline). The bladder tissues included BCa as well as adjacent non-cancerous bladder tissues. Therefore, a total of 48 samples were collected in four groups: Pre-gemcitabine normal, pre-gemcitabine BCa, post-gemcitabine normal, and post-gemcitabine BCa. Histopathological diagnosis was conducted by two independent pathologists according to the classification criteria of the World Health Organization/International Society of Urological Pathology (21). Written informed consent was obtained from each participant prior to recruitment. The Ethics Committee of The Affiliated Huai'an No. 1 People's Hospital of Nanjing Medical University reviewed and approved the study protocol (serial no. YL-P-2013-21-01). The sample size of the present study was similar to that of a previous study on PCa tissues, which produced valuable findings (22). In addition, the experimental design of self-control and complete collection of samples in our study may improve statistical power by avoiding confounding factors.

Tissue preparation for metabolomic analysis. Following harvesting, all tissues were snap-frozen in liquid nitrogen, and kept in a -80°C freezer until further analysis. The sample preparation was conducted as described previously (23). Briefly, the tissues were fragmented, ultrasonicated for 5 min (power: 60%, pulses: 6/4) in distilled water, and then 150 μl homogenate and 450 μl methanol (Merck KGaA) were mixed in a 1.5-ml Eppendorf tube for protein precipitation. The mixture was centrifuged at 16,000 x g for 15 min at 4°C, and the supernatant was collected and dried in a vacuum centrifugal concentrator. The dry residue was reconstituted in ultra-pure water and used for metabolomic analysis.

Metabolomic analysis. The metabolomic analysis was conducted as previously reported (23). LC-HRMS analysis was performed on an UPLC Ultimate 3000 system (Dionex), coupled with a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Inc.). The instrument operated at a 70,000 resolution with a full-scan acquisition ranging from 70 to 1,500 m/z. The chromatographic separation of metabolites associated with the metabolomic profiling used a multistep gradient containing ultra-pure water (mobile phase A) and acetonitrile (ACN; mobile phase B), both acidified with 0.1% formic acid. The gradient operated at a flow rate of 0.4 ml/min over a 15-min period. The metabolites were identified based on the accurate mass and the retention time compared with the commercial standards. The metabolite standards were purchased from Sigma-Aldrich; Merck KGaA, Damas-beta Co., Ltd., Aladdin Reagent Company and Adamas Reagent Co., Ltd.

Statistical analysis. Data collected from the mass spectrometer were processed for pattern recognition analysis (principal component analysis, PCA). Normalized MS data were exported to SIMCA-P+ software (V14.0, Umetrics AB) to perform PCA where grouping trends could be observed. The difference in metabolites between two groups was compared by paired t-test. According to previous reports (24,25), the

correlation between metabolite changes and cancer stage was analyzed based on the comparison between two groups using paired t-test based on the cancer stage classification. A P-value of <0.05 was considered as the threshold for statistically significant differences.

To further characterize the metabolic changes and the metabolic pathways involved, the differentiated metabolites were first annotated with Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) and Human Metabolome Database (HMDB, http://www.hmdb.ca/) (date of access for databases: April 19, 2018). Data were then processed and analyzed using MetaboAnalyst 4.0 (http://www.metaboanalyst.ca/MetaboAnalyst/) by R software (v3.4.3, GitHub). Two modules of MetaboAnalyst were used, namely pathway analysis and enrichment analysis, which are based on the KEGG database and Small Molecule Pathway Database (SMPDB, http://smpdb.ca/), respectively (26). The metabolic network of the differential metabolites and altered metabolic pathways in KEGG general metabolic pathway was visualized by iPath 3.0 (http://pathways.embl.de/).

Results

Clinical characteristics of 12 subjects. The mean age of the subjects, including 9 men and 3 women, was 67 years (range, 55-85 years). In all 12 patients, the diagnosis of urothelial carcinoma was confirmed by histopathological examination. A total of 2 patients had T_a, 1 patient had T_1, and 9 patients had T_2 disease. Two patients were diagnosed with high-grade T_2 tumors with squamous metaplasia. The clinical characteristics of the participants are shown in Table I, and are considered to be representative according to the general population of BCa in China (2,27).

PCA. A total of 165 metabolites were detected. PCA was performed to process the metabolite data based on a mean center-scaling model, which is an unsupervised projection method employed to visually display the intrinsic similarities and differences in the dataset. As shown in Fig. 1, PCA (pre-gemcitabine normal vs. pre-gemcitabine BCa tissues) revealed a well-differentiated and clustered pattern in score plots, indicating the significant metabolome changes between these two groups.

Altered metabolites and cancer-associated metabolic pathways in BCa. UPLC-Q-Exactive analysis identified 34 differentially expressed metabolites annotated in the KEGG and HMDB databases in pre-gemcitabine BCa tissues compared with pre-gemcitabine adjacent normal tissues (Table II).

The 34 BCa-associated metabolites were then submitted to MetaboAnalyst for analysis. Table II lists all metabolites found to be altered in BCa. Table III and Fig. 2A show the metabolic pathways connected with these 34 metabolites, among which three pathways, namely glutathione, purine and thiamine metabolism, were significantly associated with BCa. Furthermore, in order to expand our understanding of metabolic pathways related to BCa, the module of enrichment analysis of MetaboAnalyst was used, which verified that glutathione metabolism was significantly associated with BCa

(Table IV and Fig. 2B). The metabolic network of the differential metabolites and altered metabolic pathways in the KEGG general metabolic pathway map is shown in Fig. 2C.

Candidate targets of submucosal injection of gemcitabine. Based on targeted metabolomic analysis, we next analyzed these 34 differential metabolite changes in post-gemcitabine BCa tissues, and compared their levels with those in pre-gemcitabine normal tissues. A total of 32 metabolites maintained significant changes with identical trends in the comparison between pre-gemcitabine normal vs. pre-gemcitabine BCa tissues, indicating that the findings for differential metabolites in BCa are reliable. Importantly, the significant decrease in the levels of two metabolites associated with BCa recovered to insignificant levels following submucosal injection of gemcitabine (Table II and Fig. 3A, B and E-F). These were bilirubin and retinal, which may be the candidate targets of gemcitabine for the prevention of recurrence of urothelial BCa. We further analyzed the changes in the two metabolites in association with cancer stage. In Ta/T1 stage disease, when comparing pre-gemcitabine normal vs. pre-gemcitabine BCa tissues, bilirubin was decreased significantly in the BCa tissues (P=4.15E-2) (Table SI and Fig. 3C), while this decrease recovered to an insignificant level following submucosal injection of gemcitabine (P=5.40E-1) (Table SI and Fig. 3D); however, at this stage, the decrease of retinal in the tumor was still not statistically significant (P=4.76E-1) (Table SI). In T2 stage disease, when comparing pre-gemcitabine normal vs. pre-gemcitabine BCa tissues, retinal was decreased significantly in the BCa tissues (P=4.98E-2) (Table SI and Fig. 3G), while its change became statistically insignificant following submucosal injection of gemcitabine (P=3.22E-1) (Table SI and Fig. 3H); however, at this stage, the decrease of retinal in tumor was not statistically significant (P=1.68E-1) (Table SI). These results indicate that bilirubin and retinal changes were correlated with cancer stage, and gemcitabine may exert its effect through metabolic pathways associated with cancer stage.

Effects of submucosal injection of gemcitabine on normal tissues. To identify the effects of submucosal injection of gemcitabine on normal bladder tissues, the metabolomes of normal tissues were compared pre- and post-gemcitabine treatment. A total of 10 metabolites were found to be significantly altered in the normal tissues following submucosal injection of gemcitabine, whereas only 2 metabolites were associated with BCa (Table V). Histamine was significantly decreased in BCa, and its level was significantly increased in normal tissues after submucosal injection of gemcitabine (Fig. 3I and J). However, thiamine was significantly decreased in BCa, and its level was significantly decreased in normal tissues after submucosal injection of gemcitabine (Fig. 3M and N). We further analyzed the changes in the two metabolite in relation to cancer stage. In Ta/T1 stage disease, when comparing pre-gemcitabine normal vs. pre-gemcitabine BCa tissues, the changes in histamine (P=2.60E-1) and thiamine (P=8.39E-2) in the tumor were insignificant (Table SI). However, in T2 stage disease, when comparing pre-gemcitabine normal vs. pre-gemcitabine BCa tissues, the decrease in histamine (P=4.61E-3) (Table SI and Fig. 3K) and thiamine (P=4.13E-4) (Table SI and Fig. 3O) in

Table I. Clinical characteristics of 12 BCa patients.

				Histopathology	
Number	Sex	Age (years)	Tumor type	Stage	Grade
01	Male	55	MIUC	T2	High
02	Male	56	MIUC	T2	High
03	Male	59	MIUC	T2	High
04	Female	82	MIUC	T1	High
05	Male	67	MIUC	T2	High
06	Male	76	MIUC	T2b	Squamous metaplasia
07	Male	76	MIUC	T2	High
08	Female	60	MIUC	T2	High
09	Male	67	NMIUC	Ta	High
10	Male	58	NMIUC	Ta	High
11	Male	85	MIUC	T2a	High
12	Female	61	MIUC	T2	Squamous metaplasia

MIUC, muscle-invasive urothelial carcinoma; NMIUC, non-muscle-invasive urothelial carcinoma. BCa, bladder cancer.
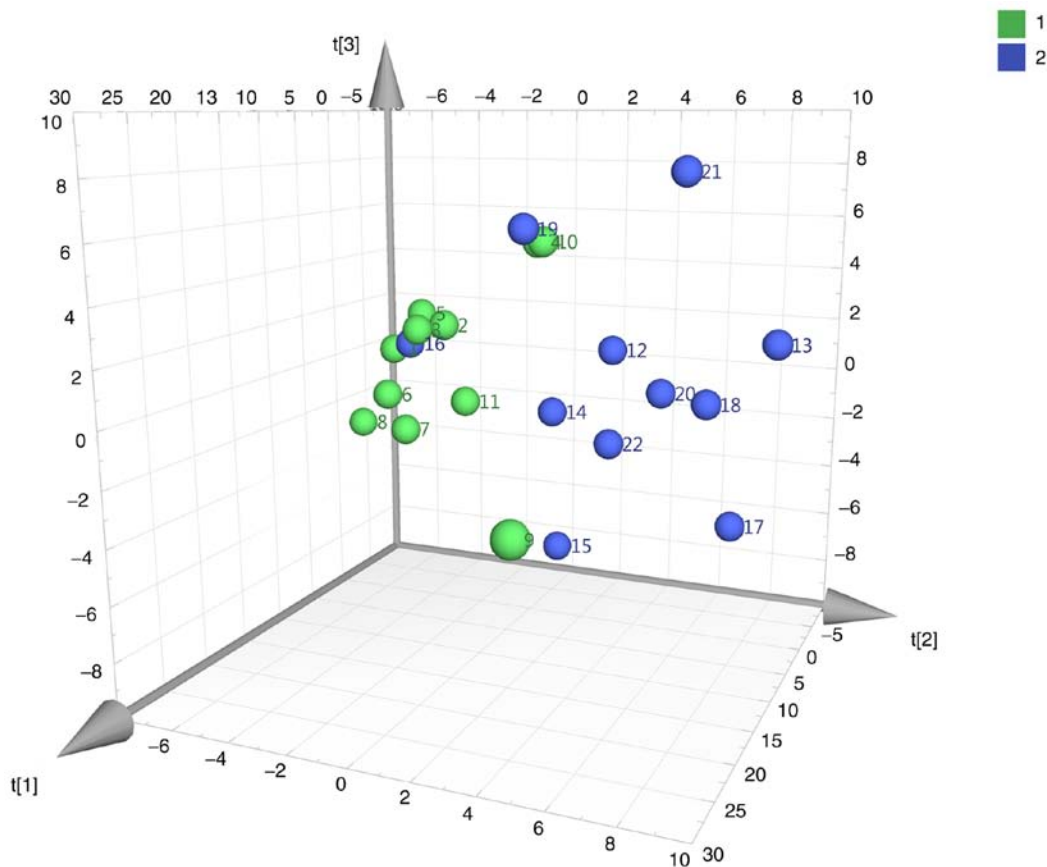

Figure 1. PCA score plots derived from pre-gemcitabine normal (green) and pre-gemcitabine BCa (blue) tissues. Comparison of pre-gemcitabine normal (green) and pre-gemcitabine BCa (blue) tissues. PCA, principal component analysis; BCa, bladder cancer.

the BCa tissues was significant. In T2 stage disease, the histamine level was significantly increased in normal tissues after submucosal injection of gemcitabine (P=3.55E-3) (Table SI and Fig. 3L), while thiamine was significantly decreased (P=4.11E-2) (Table SI and Fig. 3P). These results indicate that these metabolite changes were correlated with cancer stage, and gemcitabine may exert its effects mainly through these metabolic pathways in T2 stage disease.

Table II. List of the altered metabolites identified in BCa and their changes in the comparison between BCa tissues with or without gemcitabine pretreatment and pre-gemcitabine normal tissues.

Metabolites	KEGG	HMDB	Pre-gemcitabine BCa vs. normal tissues[a]		Post-gemcitabine BCa vs. pre-gemcitabine normal tissues[a]	
			Fold change[a]	P-value	Fold change[a]	P-value
Deoxycytidine	C00881	HMDB0000014	4.03	1.22E-02	10.10	4.15E-05
5'-Methylthioadenosine	C00170	HMDB0001173	15.16	2.38E-04	12.22	4.16E-04
3'-AMP	C01367	HMDB0003540	4.95	3.02E-02	6.36	3.33E-02
Androstenedione	C00280	HMDB0000053	0.16	4.09E-02	0.10	5.76E-03
Bilirubin	**C00486**	**HMDB0000054**	**0.4**	**3.97E-02**	**1.26**	**2.04E-01**
Cholic acid	C00695	HMDB0000619	0.29	3.14E-02	0.24	2.00E-03
Cytidine	C00475	HMDB0000089	3.83	2.43E-03	3.05	6.77E-03
5-Hydroxylysine	C16741	HMDB0000450	0.64	4.40E-02	0.48	7.49E-03
Deoxyinosine	C05512	HMDB0000071	2.44	3.87E-02	4.12	1.70E-02
Glucosamine 6-phosphate	C00352	HMDB0001254	6.04	6.26E-03	6.87	3.02E-05
Glyceraldehyde	C02154	HMDB0001051	0.29	5.86E-04	0.32	1.65E-03
Sphingosine	C00319	HMDB0000252	2.03	3.30E-02	3.34	2.18E-05
Glycerophosphocholine	C00670	HMDB0000086	56.88	1.93E-03	51.03	3.44E-03
Glycine	C00037	HMDB0000123	0.55	2.22E-02	0.47	4.68E-03
Guanidine	C17349	HMDB0001842	0.47	5.25E-03	0.01	3.79E-04
Hexadecanedioic acid	C19615	HMDB0000672	0.27	1.34E-02	0.23	3.44E-03
Histamine	C00388	HMDB0000870	0.58	2.49E-02	0.35	7.49E-03
Hypotaurine	C00519	HMDB0000965	0.07	4.19E-04	0.08	5.33E-04
Inosinic acid	C00130	HMDB0000175	92.72	1.73E-05	150.57	1.43E-03
L-Carnitine	C00318	HMDB0000062	2.24	2.39E-03	2.70	3.97E-03
L-Cystine	C00491	HMDB0000192	0.49	2.70E-02	0.13	1.17E-03
L-Phenylalanine	C00079	HMDB0000159	0.51	2.80E-02	0.31	4.70E-03
N-Acetylneuraminic acid	C19910	HMDB0000230	2.68	2.13E-02	2.90	3.38E-02
Oxidized glutathione	C00127	HMDB0003337	13.97	4.36E-03	16.05	2.77E-03
L-Palmitoylcarnitine	C02990	HMDB0000222	3.59	1.88E-02	5.09	2.77E-04
Pantothenol	C05944	HMDB0004231	0.44	3.12E-02	0.18	8.44E-05
Pyroglutamic acid	C01879	HMDB0000267	0.29	1.85E-03	0.16	5.30E-04
Quinic acid	C06746	HMDB0003072	0.09	4.05E-02	0.25	4.76E-02
Retinal	**C00376**	**HMDB0001358**	**0.13**	**4.98E-02**	**0.43**	**3.81E-01**
Rhamnose	C00507	HMDB0000849	0.22	2.90E-03	0.17	2.40E-04
Deoxycholic acid glycine conjugate	C05464	HMDB0000631	0.19	6.50E-03	0.15	4.52E-04
Sorbitol	C00794	HMDB0000247	0.29	9.19E-03	0.13	1.44E-03
Tetradecanedioic acid	C11002	HMDB0000872	0.44	4.46E-02	0.36	4.47E-02
Thiamine	C00378	HMDB0000235	0.34	6.95E-05	0.18	2.47E-05

[a]In the fold change calculation, the metabolite in pre-gemcitabine normal tissues served as the denominator. Bold print indicates statistical significance. BCa, bladder cancer; KEGG, Kyoto Encyclopedia of Genes and Genomes; HMDB, human metabolome database.

Discussion

In the present study, UPLC-Q-Exactive-based metabolomic analysis was utilized to profile metabolites in BCa tissues. The major findings may be summarized as follows: i) A total of 34 key metabolites associated with BCa were identified (Table II); ii) three metabolic pathways, namely glutathione, purine and thiamine metabolism, were altered in BCa, and glutathione metabolism was the consistently altered pathway in enrichment and pathway analyses (Tables III and IV, and Fig. 2A and B); iii) among the 34 cancer-associated metabolites, the levels of bilirubin and retinal recovered after gemcitabine injection, suggesting that these two are likely the targets of gemcitabine treatment (Table II, Fig. 3A-D and E-H); iv) the effects of gemcitabine on normal bladder tissues were also investigated, and it was deduced that histamine may have the

Table III. Pathway analysis of metabolite changes in BCa[a].

KEGG pathway	Total	Hits	P-value
Glutathione metabolism	38	3	1.55E-02
Purine metabolism	92	4	3.86E-02
Thiamine metabolism	24	2	4.40E-02
Nitrogen metabolism	39	2	1.04E-01
Primary bile acid biosynthesis	47	2	1.41E-01
Fructose and mannose metabolism	48	2	1.46E-01
Cysteine and methionine metabolism	56	2	1.86E-01
Cyanoamino acid metabolism	16	1	2.04E-01
Pyrimidine metabolism	60	2	2.07E-01
Taurine and hypotaurine metabolism	20	1	2.48E-01
Retinol metabolism	22	1	2.70E-01
Ether lipid metabolism	23	1	2.80E-01
Aminoacyl-tRNA biosynthesis	75	2	2.86E-01
Alanine, aspartate and glutamate metabolism	24	1	2.90E-01
Sphingolipid metabolism	25	1	3.01E-01
Pantothenate and CoA biosynthesis	27	1	3.20E-01
Phenylalanine, tyrosine and tryptophan biosynthesis	27	1	3.20E-01
Methane metabolism	34	1	3.86E-01
Glycerophospholipid metabolism	39	1	4.28E-01
Porphyrin and chlorophyll metabolism	104	2	4.36E-01
Galactose metabolism	41	1	4.45E-01
Histidine metabolism	44	1	4.68E-01
Phenylalanine metabolism	45	1	4.76E-01
Lysine degradation	47	1	4.91E-01
Glycine, serine and threonine metabolism	48	1	4.98E-01
Fatty acid metabolism	50	1	5.13E-01
Amino sugar and nucleotide sugar metabolism	88	1	7.21E-01
Steroid hormone biosynthesis	99	1	7.63E-01

[a]The analysis was conducted by the module of pathway analysis of MetaboAnalyst 4.0. BCa, bladder cancer; KEGG, Kyoto Encyclopedia of Genes and Genomes.

ability to protect against disease recurrence, whereas thiamine may be involved in the side effects of treatment (Table V, Fig. 3I-L and M-P), which requires further confirmation in future studies.

The identification of the three metabolic pathways significantly altered in BCa may be pathophysiologically important. Glycine is an important amino acid that participates in all three metabolic pathways. A decrease in glycine was reported as a biomarker in BCa (14). As a precursor of purine synthesis, reduced glycine in BCa indicates that a critical metabolic process associated with cell proliferation is altered in BCa (14). Notably, glutathione metabolism is the consistently altered pathway in enrichment and pathway analysis. Glutathione is the most abundant low-molecular-weight peptide present in eukaryotic cells (28). Glutathione is a primary cellular antioxidant that effectively scavenges free radicals and other reactive oxygen species (29) and, therefore, plays an important role in protecting cells from oxidative injury (30). Glutathione is also involved in cellular detoxification, and is required in several aspects of the immune response (31). Ke *et al* reported

altered oxidized glutathione in BCa; they discovered four single-nucleotide polymorphisms in the glutathione synthetase gene, and these changes were associated with BCa recurrence after TUR and Bacillus Calmette Guerin treatment (31). Our findings, together with others, suggest that oxidative stress in BCa cells is at least partially due to the disrupted glutathione metabolism.

By using LC-Q-Exactive MS, this study is, to the best of our knowledge, the first to investigate the metabolite changes in BCa treated with gemcitabine followed by TURBT. Among the 34 cancer-associated metabolites, the levels of bilirubin and retinal recovered after gemcitabine treatment, indicating that they may be the potential targets of gemcitabine for reducing BCa recurrence. Bilirubin, a degradation product of free heme groups, protected LLC-PK1 cells against cisplatin-induced death (32). A large population-based study demonstrated that patients with primary biliary cirrhosis (PBC) have a nine-fold increased risk of developing urinary BCa, and BCa and PBC share a number of etiological factors (33). Uridine 5'-diphospho-glucuronosyltransferases (UGTs) are enzymes
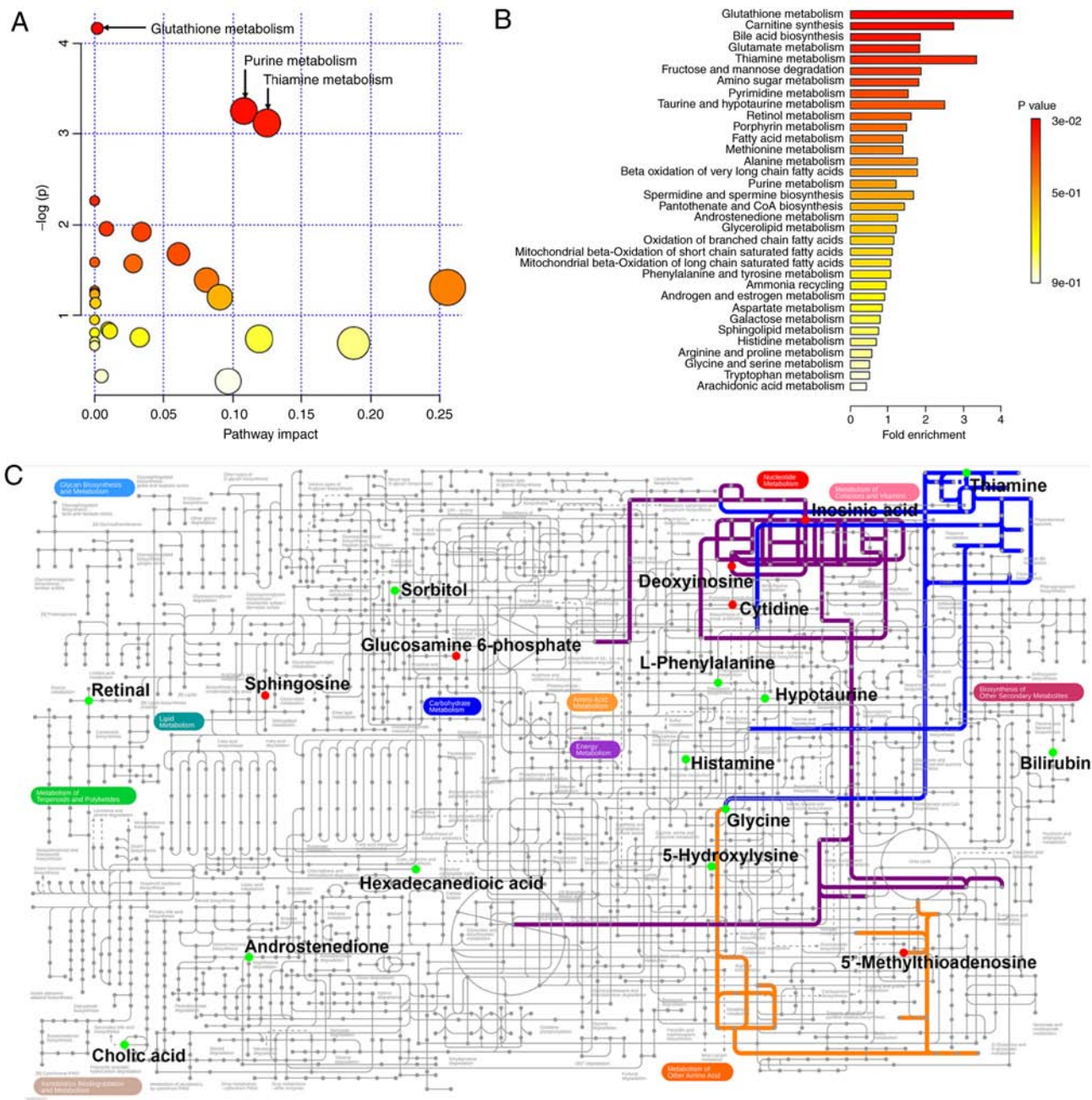
Figure 2. Results of the metabolic connection analysis of the changed metabolomic data in BCa. (A) Pathway analysis based on the KEGG database. (B) Enrichment analysis based on SMPDB. (C) Metabolic network of the differential metabolites and altered metabolic pathways in KEGG general metabolic pathway map. Red dots represent the increased metabolites in BCa; green dots represent the specifically decreased metabolites in BCa; orange line, glutathione metabolism; purple line, purine metabolism; blue line, thiamine metabolism. The metabolites and pathways not indicated in the general pathway map are not shown. The original general metabolic pathway map is available at https://pathways.embl.de/ipath3.cgi. KEGG, Kyoto Encyclopedia of Genes and Genomes; SMPDB, Small Molecule Pathway Database; BCa, bladder cancer.

that participate in several biological processes involving bilirubin conjugation. UGTs catabolize carcinogens and, therefore, protect bladder cells from the harmful effects of toxic chemicals accumulated in the bladder. Targeting UGT1A may serve as a novel therapeutic intervention against uroepithelial carcinomas (34). Retinal (retinaldehyde; Rald) is derived from retinol (vitamin A) oxidized by alcohol dehydrogenases (35). Retinal plays an essential role in molecular signaling in vision, and serves mainly as a retinoic acid (an active form of vitamin A) precursor outside the eye (36). The serum levels of vitamin A were found to be decreased in patients with

BCa (37). A high dietary intake of vitamin A reduces the incidence of BCa (38). Ziouzenkova *et al* reported retinal as a distinct biological regulator involved in suppressing adipogenesis, diet-induced obesity and insulin resistance (39). The potential effect of bilirubin and retinal on the clinical outcome of patients with BCa identified in the present study is worthy of further investigation in the future.

The effects of gemcitabine on the metabolism of adjacent normal tissues were also examined. Most gemcitabine-induced metabolites do not overlap with those identified in BCa, indicating that gemcitabine did not exert notable adverse effects on

Table IV. Enrichment analysis of metabolite changes in BCa[a].

Pathway from SMPDB	Total	Hits	P-value
Glutathione metabolism	21	3	2.96E-02
Carnitine synthesis	22	2	1.64E-01
Bile acid biosynthesis	65	4	1.64E-01
Glutamate metabolism	49	3	2.19E-01
Thiamine metabolism	9	1	2.63E-01
Fructose and mannose degradation	32	2	2.88E-01
Amino Sugar metabolism	33	2	3.00E-01
Pyrimidine metabolism	59	3	3.11E-01
Taurine and hypotaurine metabolism	12	1	3.35E-01
Retinol metabolism	37	2	3.50E-01
Porphyrin metabolism	40	2	3.87E-01
Fatty acid metabolism	43	2	4.23E-01
Methionine metabolism	43	2	4.23E-01
Alanine metabolism	17	1	4.39E-01
Beta oxidation of very long-chain fatty acids	17	1	4.39E-01
Purine metabolism	74	3	4.51E-01
Spermidine and spermine biosynthesis	18	1	4.58E-01
Pantothenate and CoA biosynthesis	21	1	5.11E-01
Androstenedione metabolism	24	1	5.59E-01
Glycerolipid metabolism	25	1	5.74E-01
Oxidation of branched chain fatty acids	26	1	5.89E-01
Mitochondrial beta-oxidation of short-chain saturated fatty acids	27	1	6.03E-01
Mitochondrial beta-oxidation of long-chain saturated fatty acids	28	1	6.16E-01
Phenylalanine and tyrosine metabolism	28	1	6.16E-01
Ammonia recycling	32	1	6.66E-01
Androgen and estrogen metabolism	33	1	6.78E-01
Aspartate metabolism	35	1	6.99E-01
Galactose metabolism	38	1	7.29E-01
Sphingolipid metabolism	40	1	7.48E-01
Histidine metabolism	43	1	7.73E-01
Arginine and proline metabolism	53	1	8.41E-01
Glycine and serine metabolism	59	1	8.72E-01
Tryptophan metabolism	60	1	8.76E-01
Arachidonic acid metabolism	69	1	9.10E-01

[a]The analysis was conducted by the module of enrichment analysis of MetaboAnalyst 4.0. BCa, bladder cancer. SMPDB, Small Molecule Pathway Database.

normal bladder tissues. It was observed that histamine change may be associated with the prevention of relapse. Histamine is derived from the decarboxylation of histidine by histidine decarboxylase in mammals (8). Histamine is primarily released in inflammatory processes by mast cells (8), which are closely associated with BCa (40). Histamine H1 receptor (HRH1) expression was identified in BCa and found to be associated with the prognosis (41). It was observed that thiamine change may be involved in treatment-related side effects. Thiamine, or vitamin B-1, is a water-soluble vitamin (42). An early report by Pamukcu *et al* demonstrated that the incidence of urinary bladder carcinomas in rats fed bracken fern and additionally s.c. injected once weekly with 2 mg of thiamine hydrochloride was significantly higher compared with that in rats fed bracken fern but receiving no thiamine supplements, as thiamine may interfere with the absorption, distribution, metabolism, or excretion of the bracken fern (43). However, a case-control study from New Hampshire investigated the effect of minerals and vitamins on the risk of BCa, and found that a higher total intake of thiamine was inversely correlated with BCa risk in older participants (44).

There is currently a lack of effective biomarkers for BCa diagnosis and prognosis. Metabolomic profiles from tissue have the potential to be used, along with other current diagnostics, to help guide the clinical management of patients with BCa. The changed metabolites identified in the present

Table V. Changes in normal tissues after gemcitabine treatment.

Metabolites	Pre-gemcitabine BCa vs. normal tissues[a]		Post-gemcitabine vs. pre-gemcitabine normal tissues[a]	
	Fold-change[a]	P-value	Fold-change[a]	P-value
3-Methyladenine	1.54	3.78E-01	0.17	2.33E-02
Ascorbic acid	22.26	1.85E-01	0.28	5.46E-03
Creatinine	0.48	3.44E-01	0.13	1.16E-02
D-Glyceraldehyde 3-phosphate	0.72	3.33E-01	0.15	2.16E-02
Histamine	0.58	2.49E-02	2.26	3.73E-04
N-Acetylglutamic acid	0.52	8.58E-02	0.57	4.95E-02
N-Acetylglutamine	0.68	5.45E-01	0.11	4.25E-02
Thiamine	0.34	6.95E-05	0.65	2.11E-02
Tryptamine	0.10	7.66E-02	0.11	3.23E-02
Uridine	1.41	6.74E-02	1.51	3.65E-02

[a]In the fold change calculation, the metabolite in pre-gemcitabine normal tissues served as the denominator. Bold print indicates statistically significant differences. BCa, bladder cancer.
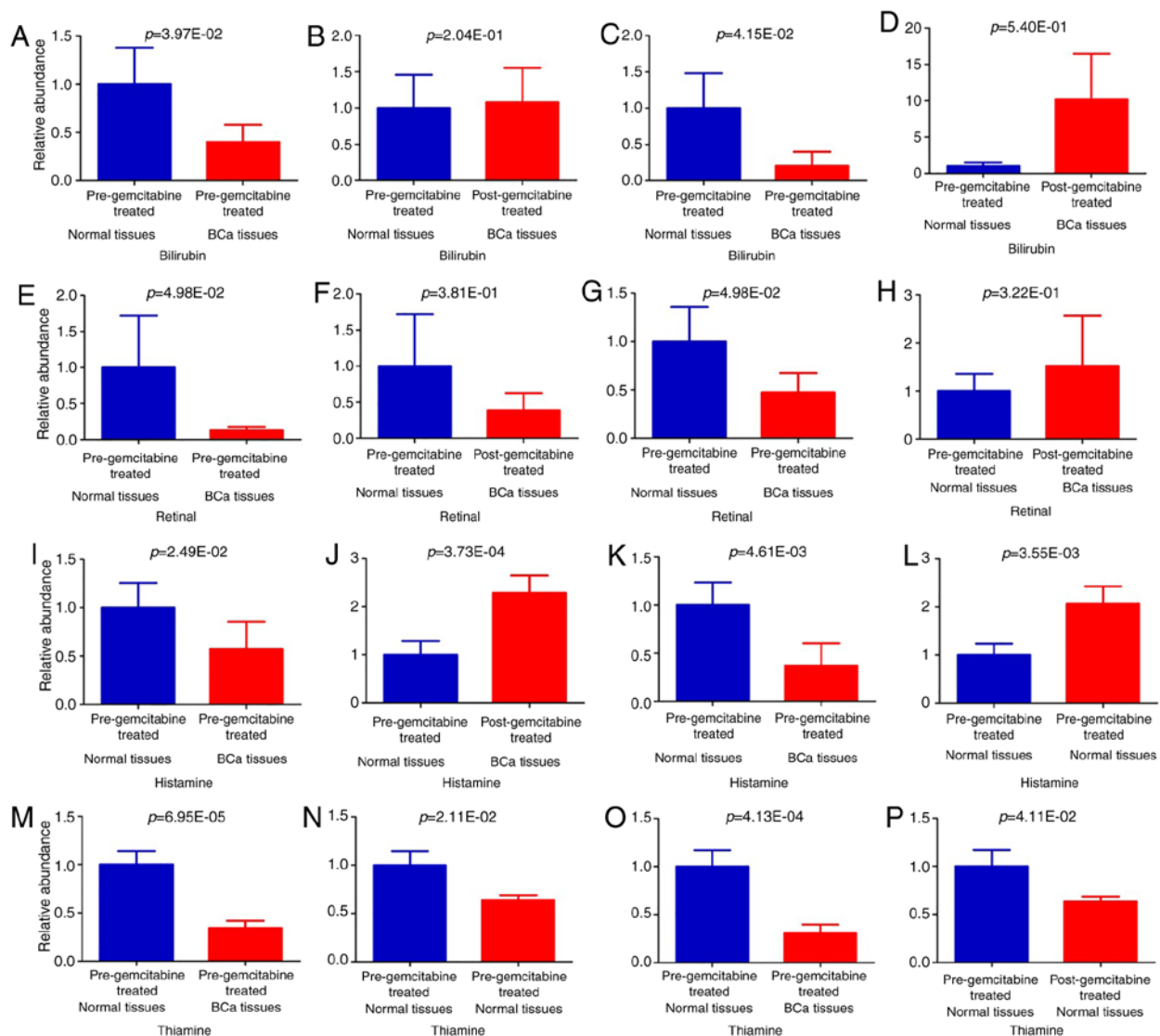
Figure 3. Relative levels of potential candidate targets of submucosal injection of gemcitabine (A, B, E, F, I, J, M and N). All samples; (C and D) Ta/T1 stage; (G, H, K, L, O and P) T2 stage. Values are presented as mean ± standard error of the mean. The P-values of paired t-test are indicated.

study, such as glycine, may be used as potential biomarkers for BCa. In addition, those metabolites discovered in BCa with gemcitabine treatment may reveal new metabolic pathways that mediate the anti-recurrence effect of gemcitabine, which currently remain elusive.

In summary, the present study employed UPLC-HRMS-based metabolomic analysis to investigate metabolite changes in bladder tissues from BCa and BCa treated with gemcitabine. The findings may provide new insights into metabolic changes in BCa and the biomolecular basis of submucosal injection of gemcitabine for BCa. In addition, the study demonstrated that the UPLC-HRMS-based metabolomic analysis provides comprehensive metabolite profiling data that may pave the way to a novel approach to BCa research.

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Availability of data and materials

The datasets generated and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

CY, XS, KW, YG, RS and MC designed and performed the experiments. TL and MC analyzed and interpreted the raw data. SZ, XJ, BZ and HW collected the bladder tissue samples. ZG and SS performed the histopathological examination of BCa tissues. JL and JT preserved the samples and collected the basic information of the participants. CY, TL and MC wrote the manuscript. XW, HJ, XN, XW, MC and GF critically revised the manuscript for important intellectual content. MC and GF supervised the project. All authors have read and approved the final version of the manuscript for publication.

Ethics approval and consent to participate

The Ethics Committee of The Affiliated Huai'an No. 1 People's Hospital of Nanjing Medical University reviewed and approved the study protocol (serial no. YL-P-2013-21-01).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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