Metabolic Pathway Signatures Associated with Urinary Metabolite Biomarkers Differentiate Bladder Cancer Patients from Healthy Controls

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ABSTRACT

Metabolic Pathway Signatures Associated with Urinary Metabolite Biomarkers Differentiate Bladder Cancer Patients from Healthy Controls

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Purpose: Our previous high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry study identified bladder cancer (BCA)-specific urine metabolites, including carnitine, acylcarnitines, and melatonin. The objective of the current study was to determine which metabolic pathways are perturbed in BCA based on our previously identified urinary metabolome.

Methods: A total of 135 primary BCA samples and 26 control tissue samples from healthy volunteers were analyzed. The association between specific urinary metabolites and their related encoding genes was analyzed.

Results: Significant alterations in the carnitine-acylcarnitine and tryptophan metabolic pathways were detected in urine specimens from BCA patients compared to those of healthy controls. The expression of eight genes involved in the carnitine-acylcarnitine metabolic pathway (*CPT1A*, *CPT1B*, *CPT1C*, *CPT2*, *SLC25A20*, and *CRAT)* or tryptophan metabolism *(TPH1* and *IDO1*) was assessed by RT-PCR in our BCA cohort (n = 135). *CPT1B*, *CPT1C*, *SLC25A20*, *CRAT*, *TPH1*, and *IOD1* were significantly downregulated in tumor tissues compared to normal bladder tissues ($p < 0.05$ all) of patients with non-muscle invasive bladder cancer, whereas *CPT1B*, *CPT1C*, *CRAT*, and *TPH1* were downregulated in those with muscle invasive bladder

cancer ($p < 0.05$), with no changes in *IDO1* expression.

Conclusions: Alterations in the expression of genes associated with the carnitine-acylcarnitine and tryptophan metabolic pathways, which were the most perturbed pathways in BCA, were determined.

Key words : Bladder cancer; urine metabolites, gene expression; qRT-PCR; diagnostic marker

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I. INTRODUCTION

Among men, bladder cancer (BCA) is the seventh most common cancer worldwide and the fourth most common in developed countries.[1] Although cystoscopy and cytology are the standard diagnostic tools for BCA, cystoscopy has low sensitivity for high-grade superficial tumors (i.e., carcinoma in situ, CIS) and cytology has poor sensitivity for low-grade tumors.[2,3] Therefore, there is a growing interest in the development of new, noninvasive, and accurate methods for the detection of BCA.

Metabolic dysfunction has been implicated in a wide variety of human diseases including BCA.[4] Recent advances on technological and data analysis have enabled the characterization of metabolites in biofluids. Metabolomics has been applied increasingly in the field of toxicology and biomarker research,[5,6] in particular for cancer diagnosis.[7,8] Growth and division of tumor cells are associated with an increase in the activity of a variety of metabolic pathways. Genes linked to altered cancer metabolism contribute to produce and secrete the cancer-specific metabolites into biofluids.[9] Recent studies reported that metabolites uniquely detected in urine samples from patients with BCA, not those in the matched non-cancer controls, could be a useful biomarker for BCA diagnosis and prognosis.[4,10-12] Because the bladder

serves as a temporary urine reservoir, analysis of urine metabolites could provide potential candidates of sensitive and specific biomarkers of BCA.[4,10-12]

In our previous study, we used a comprehensive mass spectrometry-based metabolomics approach to identify potential diagnostic biomarkers of BCA using 138 patients with BCA and 121 control subjects.[13] This previous metabolomic analysis revealed twelve metabolites as potential biomarkers for the detection of BCA. They were carnitine, ovalerylcarnitine, glutarylcarnitine, octenoylcarnitine, decanoylcarnitine, succinate, pyruvate, oxoglutarate, phosphoenolpyruvate, trimethyllysine, melatonin, and acetyl-CoA. Carnitine has two key functions: (a) the transport of long-chain fatty acids (FAs) into the mitochondria, where they are oxidized to produce energy, and (b) the removal of toxic compounds to prevent their accumulation.[14] The carnitine-acylcarnitine metabolic pathway involves several enzymes, including carnitine palmitoyltransferase type 1 (CPT1) and carnitine acetyltransferase (CRAT).[15] CPT1, which has several isoforms including CPT1A, CPT1B, and CPT1C, is a mitochondrial enzyme that plays a role in FA import into mitochondria for β-oxidation. Loss of CPT1 leads to the impaired FA oxidation and contributes to the cancer-associated cachexia, which is characterized by progressive weight loss resulting from a reduction in adipose tissue and skeletal muscle. CRAT catalyzes the reversible conversion of acetyl-CoA into acetyl-carnitine.[15]

Our urinary metabolomics analysis showed the decreased urinary levels of melatonin in BCA patients compared to controls.[13] Melatonin, a derivative of tryptophan, enters to cells via glucose transporters and has a broad range of biological functions, including regulation of circadian rhythms and detoxification of free radicals.[16] Because of its antioxidant properties, melatonin is considered as a potential agent for the prevention and/or treatment of cancer.[16]

Due to the limited metabolome databases, there have been few studies analyzing the metabolomics databases in the context of the related genes/proteins and biological pathways/networks. The potential association between specific metabolites and their related tissue gene/protein expression in BCA remains unclear. An association between BCA-related metabolites and altered biochemical processes fundamental to BCA development and progression was proposed in a previous study, however, the findings were based on the limited experimental data of phase I/II metabolic enzymes (CYP1A1 and CYP1B1).[4] In the present study, we assessed the relationship between the BCA-associated urinary metabolites and gene expression associated with relevant biological pathways using bladder tissues derived from BCA patients and controls.

II. MATERIALS AND METHODS

1. Ethics statement

The study protocol was approved by the Ethics Committee of Chungbuk National University, and written informed consent was obtained from each subject. The Institutional Review Board of Chungbuk National University approved the protocols for the collection and analysis of samples (IRB approval number GR 2010-12-010).

2. Patients and tissue samples

A total of 135 primary BCA samples and 26 control tissue samples from healthy volunteers were analyzed. 120 of 135 BCA samples were overlapped between our previous studies[13] and the current one. The study included primary tumor samples from patients with histologically-verified transitional cell carcinoma treated at our institute. All tumors were macrodissected, typically within 15 minutes of surgical resection. Each BCA specimen was verified by pathological analysis of tissue samples from fresh frozen sections of transurethral resection or radical cystectomy specimens. Specimens were frozen in liquid nitrogen and stored at -80°C until use. Tumors were staged according to the 2002 TNM classification,[17] while they were graded according to the 1973 WHO grading system.[18]

3. Extraction of RNA and synthesis of cDNA

RNA was isolated from BCA and control tissue using 1 ml of TRIzol (Invitrogen, Carlsbad, CA) and homogenized in a 5 ml glass tube. The homogenate was transferred to a 1.5 ml tube and mixed with 200 µl of chloroform. After 5 min of incubation at 4°C, the homogenate was centrifuged for 13 min at 13,000 × *g* at 4°C. The upper aqueous phase was transferred to a clean tube, mixed with 500 µl of isopropanol, incubated for 60 min at 4°C, and centrifuged for 8 min at 13,000 × *g* at 4°C. The upper aqueous phase was discarded and the lower phase was mixed with 500 µl of 75% ethanol, and then centrifuged for 5 min at 13,000 × *g* at 4°C. After discarding the upper aqueous layer, the pellet was dried at room temperature, dissolved in diethylpyrocarbonate (DEPC)-treated water, and stored at -80°C. The quality and integrity of the RNA were verified by agarose gel electrophoresis and ethidium bromide staining, followed by visual inspection under ultraviolet light. The cDNA was prepared from 1 µg of total RNA using a First-Strand cDNA Synthesis Kit (Amersham Biosciences Europe GmbH, Freiburg, Germany) according to the manufacturer's protocol.

4. Real-time PCR

The expression of the selected eight genes was quantified by real-time PCR amplification using a Rotor-Gene 6000 instrument (Corbett Research, Mortlake, Australia). Real-time PCR assays were performed in micro-reaction tubes (Corbett Research, Mortlake, Australia) using SYBR Premix EX Taq (TAKARA BIO INC., Otsu, Japan). The primers used for gene amplification are shown in Table 1. The PCR reaction was set up as follows: 1 µl of sample cDNA, 5 µl of 2× SYBR Premix EX Taq buffer, and 0.5 µl of each 5'- and 3'- primer (10 pmol/µl), in a final volume of 10 µl. Products were purified with a QIAquick Extraction kit (QIAGEN, Hilden, Germany) and quantified using a spectrophotometer (Perkin Elmer MBA2000,

Fremont, CA). Fragments were sequenced with an automated laser fluorescence sequencer (ABI PRISM 3100 Genetic Analyzer, Foster City, WI). For real-time PCR, 10-fold serial dilutions of a known concentration of the product (100–0.1 pg/μl) were used to establish the standard curve. Real-time PCR was performed under the following conditions: 1 cycle of 20 seconds at 96°C, followed by 40 cycles of 2 sec at 96°C for denaturation, 15 sec at 60°C for annealing, and 15 sec at 72°C for extension. The melting program was performed at 72–95°C with a heating rate of 1°C per 45 sec. Spectral data were captured and analyzed using Rotor-Gene Real-Time Analysis Software 6.0 Build 14 (Corbett Research, Mortlake, Australia). In this study, a standard plasmid was included in real-time PCR to produce a standard curve using copy number (with copy numbers of 10^5, 10^4, 10^3, 10^2, and 10) and threshold cycle (Ct) values. To construct standard plasmids, 360bps of each gene including the PCR amplified target region were synthesized and ligated into pUC57 plasmid DNA (GenScript, Piscataway, NJ, USA). The synthesized target regions of each gene were confirmed by capillary sequencing. The Ct values of each gene from the real-time PCR run were plotted on the standard curve to calculate copy number. All samples were analyzed in triplicate. *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* was used as an endogenous RNA reference gene and gene expression was normalized to the expression of *GAPDH*.[19] The primers used for gene amplification are shown in Table 1.

Table 1. Sequences of the primers used in this study

Gene	Primer
CPT1A	S: 5'-CCAAGATCGACCCCTCGTTA-3' `
	AS: 5'-AGAGCAGCACTTTCAGGGAGTA-3'
CPT1B	S: 5'-CATCCAGCTTGCCTGGTTC-3'
	AS: 5'-CACAAACACGAGGCAAACAG-3'
CPT1C	S: 5'-CATCCAGCTTGCCTGGTTC-3'
	AS: 5'-CACAAACACGAGGCAAACAG-3'
CPT2	S: 5'-TTGAAGACACCATTAGGAGATACC-3'
	AS: 5'-CCAGCTGCTCATGCAGTTCTTTT-3'
CRAT	S: 5'-CAGGCCAGATGCTGCATG-3'
	AS: 5'-TAGTCCAGAAGGGTGACAATAGGG-3'
SLC25A20	S: 5'-ACCGAGTTTGCCTGGACA-3'
	AS: 5'-ACCAAACCCAAAGAAGCACAC-3'
TPH1	S: 5'-TTTCATCTGCTGAAGTCTCATACC-3'
	AS: 5'-AAACCAAGGAACAGTTT-3'
IDO1	S: 5'-TGCTAAACATCTGCCTGATCTCAT-3'
	AS: 5'-CCCCACACATATGCCATGGT-3'

CPT, carnitine palmitoyltransferase; CRAT, Carnitine O-acetyltransferase; SLC25A20, solute carrier family 25 (carnitine/acylcarnitine translocase (CACT)), member 20; TPH, tryptophan hydroxylase; IDO, Indoleamine 2,3-dioxygenase.

4. Data and statistical analysis

In this study, two pathways, the carnitine-acylcarnitine metabolic pathway and tryptophan metabolism pathway, were selected for further analysis. The metabolic pathways associated with the selected urinary metabolites were identified using KEGG pathways (http://www.genome.jp/kegg). The association between specific urinary metabolites and their related encoding genes was analyzed (Table 2). Differences between two groups were analyzed using the Mann-Whitney U test. All statistical analyses were performed using SPSS® 21.0 software (SPSS Inc., Chicago, IL, USA), with $P < 0.05$ considered statistically significant.

Table 2. Selected urinary metabolites in bladder cancer patients and their related pathways, enzymes, and encoding genes

Urinary metabolites	Up/ down	Associated pathways	Associated enzymes	Encoding genes
Carnitine	up	Carnitine-acylcarnitine pathway	Carnitine Palmitoyltransferase	*CPT1A, CPT1B, CPT1C, CPT2*
			Carnitine/acylcarnitine translocase	*SLC25A20*
Acetylcarnitine	-		Carnitine O-acetyltransferase	*CRAT*
Melatonin	down	Tryptophan metabolism pathway	Tryptophan Hydroxylase	*TPH1*
			Indoleamine 2,3-dioxygenase	*IDO1*

CPT, carnitine palmitoyltransferase; SLC25A20, solute carrier family 25 (carnitine/acylcarnitine translocase (CACT)), member 20; CRAT, Carnitine O-acetyltransferase; TPH, tryptophan hydroxylase; IDO, Indoleamine 2,3-dioxygenase.

III. RESULTS

1. Baseline characteristics

The mean age of the 135 BCA patients was 65.9 ± 12.62 years. Eighty-two (60.7%) patients had non-muscle invasive bladder cancer (NMIBC) and 53 (39.3%) had muscle invasive bladder cancer (MIBC). The baseline characteristics of the patients are shown in Table 3.

Table 3. Baseline characteristics of primary bladder cancer patients

Variables	Incidence or mean value (%)
Age (years)	65.9 ± 12.62
Gender	
Male	112 (83.0%)
Female	23 (17.0%)
Grade	
G1	30 (22.2%)
G2	58 (43.0%)
G3	47 (34.8%)
Stage	
NMIBC	
Ta	47 (34.8%)
T1	35 (25.9%)
MIBC	
T2	23 (17.0%)
T3	11 (8.1%)
T4	19 (14.1%)

NMIBC, non-muscle invasive bladder cancer; MIBC, muscle invasive bladder cancer

2. Comparison of gene expression levels between NMIBC patients and controls

Our previous urinary metabolomics-based analysis[13] showed that patients with BCA had the elevated levels of urinary carnitine and several acylcarnitines, such as isovalerylcarnitine and octenoylcarnitine, and the decreased levels of urinary melatonin, glutarylcarnitine, and decanolycarnitine, compared to the control group. Bioinformatics analysis suggested that the levels of these metabolites may be altered mainly in two metabolic pathways, the carnitine-acylcarnitine metabolic pathway and the tryptophan metabolism. In order to quantify the expression levels of key genes in these two pathways we performed qRT-PCR analysis using samples from 135 BCA patients (82 NMIBC and 53 MIBC) and 26 controls. Several key enzymes of the carnitine-acylcarnitine metabolic pathway or tryptophan pathway were selected for further validation (Table 2).

CPT catalyzes the transfer of the acyl group of a FA to carnitine to allow its movement from the cytosol to mitochondria for oxidation.[20] Carnitine-acylcarnitine translocase (CACT, gene symbol: SLC25A29) plays a role in transporting both carnitine-FA complexes and carnitine across the inner mitochondrial membrane for beta-oxidation.[21] CRAT catalyzes the reaction of acetyl-CoA plus carnitine to CoA plus acetylcarnitine.[22] Tryptophan hydroxylase (TPH) is involved in the synthesis of the neurotransmitters serotonin and melatonin in tryptophan metabolism.[23] Indoleamine 2,3-deoxygenase (IDO) is involved in the degradation of tryptophan in the tryptophan metabolic pathway.[24] In the carnitine-acylcarnitine metabolic pathway, the mRNA expression levels of *CPT1B* and *CPT1C*, *SLC25A20*, and *CRAT*, but not *CPT1A* or *CPT2*, were significantly lower in NIMBC patients than in controls ($p < 0.05$ each) (Table 3) (Figure 1). In the tryptophan metabolic pathway, the mRNA expression of *TPH1* and *IDO1* was significantly lower in NIMBC patients than in controls ($p < 0.05$).

3. Comparison of gene expression levels between MIBC patients and controls

Among the carnitine-acylcarnitine metabolic pathway-associated genes, the expression levels of *CPT1B*, *CPT1C*, and *CRAT* were significantly lower in MIBC tissues than those of controls ($p < 0.05$) (Table 4) (Figure 1). No significant differences in the expression of *CPT2* and *SLC25A20* were observed between MIBC patients and controls. *CPT1A* expression was significantly higher in MIBC patients than in controls ($p = 0.008$). Among tryptophan metabolism-associated genes, only *TPH1*, but not *IDO1*, was significantly downregulated in MIBC compared to controls ($p = 0.001$).

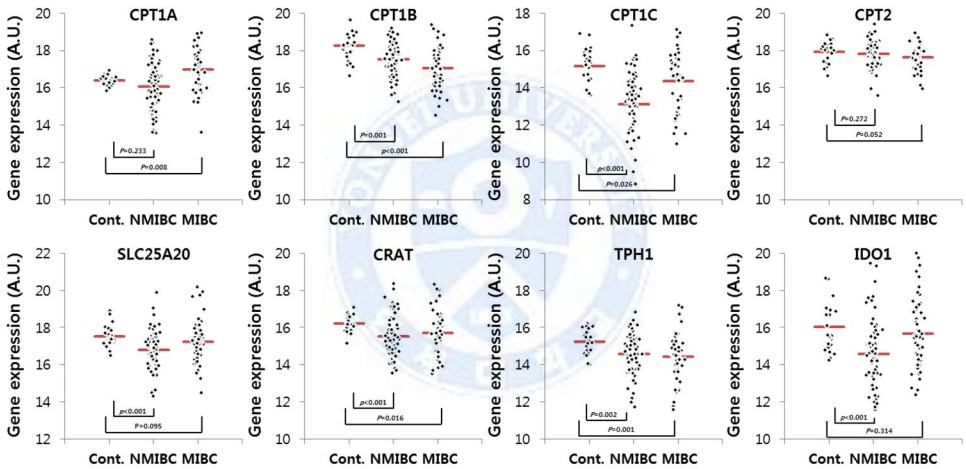

Figure 1. Jitter plots showing mRNA levels in normal controls and patients with NMIBC or MIBC. RT-PCR analysis of the mRNA expression of CPT1A, CPT1B, CPT1C, CPT2, SLC25A20, CRAT, TPH1, and IDO1 was performed as describe in the Materials and Methods section. P values were calculated using the Mann-Whitney U test.

Table 4. Comparison of tissue mRNA expression levels in control vs. NMIBC or MIBC

	Control	NMIBC		MIBC	
Gene	mRNA expression, x 10^5 copies/μg Median (IQR)	mRNA expression, x 10^5 copies/μg Median (IQR)	P-value (control vs.)	mRNA expression, x 10^5 copies/μg Median (IQR)	P-value (control vs.)
CPT1A	133.16 (108.30-153.62)	81.60 (42.30-211.09)	0.233	301.59 (110.27-560.94)	0.008
CPT1B	869.68 (409.04-1530.84)	410.13 (227.18-734.81)	0.001	249.00 (127.34-505.11)	<0.001
CPT1C	40.68 (17.27-79.82)	6.46 (2.89-15.06)	<0.001	25.14 (6.21-43.09)	0.026
CPT2	707.51 (372.11-	579.02 (427.90-	0.272	519.46 (303.93-	0.052

	867.09)	829.59)		727.81)	
SLC25A20	332.79	209.25	<0.001	312.97	0.095
	(288.58-	(132.17-		(170.26-	
	562.43)	307.49)		527.46)	
CRAT	112.16	44.09	<0.001	67.14	0.016
	(71.56-	(25.89-		(40.62-	
	154.01)	90.50)		108.69)	
TPH1	44.15	26.33	0.002	21.58	0.001
	(26.64-	(13.17-		(11.42-	
	84.18)	39.24)		37.15)	
IDO1	54.74	21.36	<0.001	55.16	0.314
	(31.30-	(6.39-		(19.71-	
	228.58)	51.36)		249.63)	

IQR, interquartile range; CPT, carnitine palmitoyltransferase; SLC25A20, solute carrier family 25 (carnitine/acylcarnitine translocase (CACT)), member 20; CRAT, Carnitine O-acetyltransferase; TPH, tryptophan hydroxylase; IDO, Indoleamine 2,3-dioxygenase.

4. Correlation analysis between urinary metabolites and tissue mRNA expression in 120 bladder cancer patients.

Octenoylcarnitine, urinary metabolites, was positively correlated with CPT1A and SLC25A20 (r=0.250, p=0.006 and r=0.255, p=0.005, respectively), while decanolylcarnitine was negatively correlated with CPT2 (r=-0.226, p=0.013). Other candidate urinary metabolites were not able to show the significant correlation with tissue RNA expression in bladder cancer patients (Table 5).

Table 5. Correlations between urinary metabolites and tissue mRNA expression in 120 bladder cancer patients

	Carnitine		Isovalerylcarnitine		Glutarylcarnitine	
	r	p-value	r	p-value	r	p-value
CPT1A	-0.133	0.149	0.087	0.342	0.044	0.635
CPT1B	-0.097	0.298	0.084	0.365	-0.013	0.888
CPT1C	-0.062	0.515	0.067	0.481	0.041	0.665
CPT2	-0.106	0.249	-0.010	0.913	-0.005	0.955
SLC25A20	0.049	0.597	0.088	0.335	-0.027	0.766
CRAT	0.102	0.269	0.045	0.626	-0.043	0.645
TPH1	-	-	-	-	-	-
IDO1	-	-	-	-	-	-

	Octenoylcarnitine		Decanoylcarnitine		Melatonin	
	r	p-value	r	p-value	r	p-value
CPT1A	0.250	0.006	-0.172	0.06	-	-
CPT1B	-0.132	0.155	-0.099	0.284	-	-
CPT1C	0.025	0.788	-0.105	0.264	-	-
CPT2	0.021	0.816	-0.226	0.013	-	-
SLC25A20	0.255	0.005	-0.090	0.324	-	-
CRAT	0.134	0.147	-0.097	0.295	-	-
TPH1	-	-	-	-	-0.092	0.321
IDO1	-	-	-	-	0.042	0.649

r=correlation (r); CPT, carnitine palmitoyltransferase; SLC25A20, solute carrier family 25 (carnitine/acylcarnitine translocase (CACT)), member 20; CRAT, Carnitine O-acetyltransferase; TPH, tryptophan hydroxylase; IDO, Indoleamine 2,3-dioxygenase.

IV. DISCUSSION

Our previous study identified twelve urinary metabolites, including carnitine, and their derivatives and melatonin, as potential diagnostic biomarkers for BCA.[13] The following bioinformatics analysis showed that these metabolites were enriched in the carnitine-acylcarnitine or tryptophan metabolic pathways. Given that metabolic pathways and associated genes/proteins/metabolites are altered in BCA, we next tested whether the expression levels of key genes involved in the carnitine-acylcarnitine metabolic pathway and tryptophan metabolism are also perturbed in bladder tissues obtained from patients with BCA.

Few studies have analyzed carnitine or carnitine metabolism-associated genes in BCA. Huang *et al.* performed a liquid chromatography mass spectrometry-based metabolomics analysis and showed that the level of urinary carnitine C9:1 was significantly decreased in patients with BCA, particularly in those with early BCA.[11] However, the contribution and regulatory mechanism of the carnitine metabolic pathway to BCA remains unclear.

Urinary levels of tryptophan and tryptophan metabolites have been reported increased in BCA.[10,12] Alberice *et al.* showed that the levels of tryptophan and its related metabolite N-acetyltryptophan were significantly increased in the urine of patients with early BCA.[10] Pasikanti *et al.* showed elevated concentrations of tryptophan metabolites in the urine of BCA patients.[12] Despite accumulating evidence supporting the importance of tryptophan and its urinary metabolites in bladder carcinogenesis,[25] the role of THP1, a key enzyme in tryptophan metabolism that catalyzes the conversion of tryptophan to serotonin and the depletion of tryptophan, in BCA has not been investigated to date. Our present data indicate that TPH1 levels are significantly decreased in patients with NMIBC and MIBC compared to controls. A tryptophan metabolite, melatonin, was also decreased in the urine of patients with

BCA.

Metabolic profiling includes high resolution spectroscopy, mainly nuclear magnetic resonance (NMR), and mass spectrometry with liquid chromatography (LC) or gas-chromatography (GC). Because the final products of the metabolism of DNA, RNA, and protein are metabolites, the levels of metabolites may be a more accurate reflection of changes in metabolic activity than the levels of DNA, RNA, and protein. Therefore, metabolites could be important diagnostic markers because their levels may vary without significant changes in DNA, RNA, and protein levels. Metabolomics studies of BCA have been performed using urine, serum, and cancer tissues.[4,10-12,26,27] Urinary biomarkers for the detection of BCA are attractive because of their direct contact to cancer and the noninvasive nature of urine collection. Therefore, there has been growing interest in urinary metabolomics for BCA detection. However, validating urinary metabolomics profiles in independent cohorts or using different methods, and determining their association with biological properties and specific disorders have been challenges that need to be resolved.

Urinary metabolomics study is very convenient and non-invasive diagnostic tools. Generally, urinary metabolites could be affected by diet, environment, and individual factor. However, OPLS-DA analysis could eliminate the confounding variables such as diet and environment between normal controls and cancer patients.[28] Therefore, selected metabolites by OPLS-DA could be a useful marker in cancer patients according to the contribution for cancer versus normal controls.

In this study, we proposed the possibility of the association between urinary metabolites and tissue mRNA expression in bladder cancer in the figure 2. In the β-

oxidation, carnitine and their carnitine derivatives, excreted to the urine from bladder cancer tissue, because CPT1, CPT2, CACT mRNA expression associated with Acyl-carnitine and carnitine metabolism were decreased. Also, the decreased TPH1 led to the inhibition of metabolizing the tryptophan pathway to target metabolite, melatonin. In NMIBC, decreased IDO1 also might lead to the inhibition of metabolites, N-formyl-kynureinine but, might lead to activation of another pathway to metabolites, indoleacetate and anthranilate to final metabolite, acetyl-CoA in tryptophan pathway. Although the decreased TPH1 level might be associated with the reduced metabolite such as melatonin in tryptophan metabolism. A metabolite in the tryptophan metabolic pathway, Acetyl CoA, is the last metabolite in the fatty acid metabolism, the glycolysis metabolism and the tryptophan metabolism. We found the increase of Acetyl-CoA level in our study, which might be caused by glycolysis metabolism and another tryptophan pathway. IDO1 encode the enzyme Indoleamine 2,3-dioxygenase in human. This enzyme is produced by macrophage and other immunoregulatory cells.[29] Elevation of IDO1 levels in bladder tumor cells might be associated with immune escape of tumor cells. In particular, in early tumorigenesis periods like NMIBC, the human immune systems maybe downregulate IDO1 and lead the protection of cancer cells from immune response.

Collectively, the present data generated by the use of metabolomics technologies may broaden our understanding of BCA. However, further data interpretation requires a rich integrated database of metabolome, transcriptome, and proteome, and a systematic bioinformatics approach to ensure that the biological function of metabolite candidates.

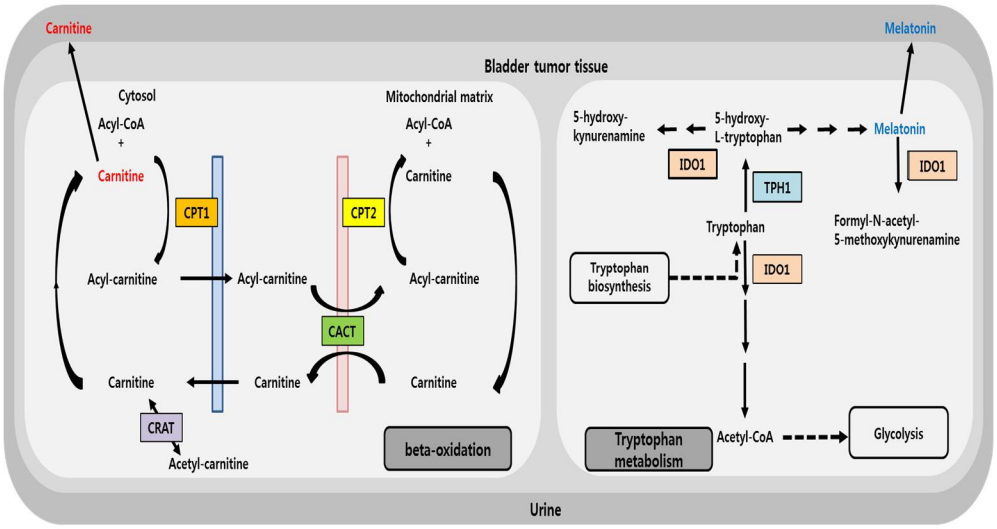

Figure 2. Schematic pathway of bladder cancer-associated metabolic signatures in BCA patients. CPT: carnitine palmitoyltransferase, CACT: carnitine/acylcarnitine translocase, CRAT: carnitine O-acetyltransferase, TPH: tryptophan hydroxylase, IDO: indoleamine 2,3-dioxygenase.

V. CONCLUSION

We showed that the expression of genes involved in the carnitine-acylcarnitine and tryptophan pathways is associated with alterations in the levels of urinary metabolites in BCA patients. The present experimental results may advance our understanding of the association between urinary metabolites and their related tissue gene expression in BCA.

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ABSTRACT(IN KOREAN)

방광암 환자를 정상대조군으로부터 구분해주는 소변내 대사체 바이오마커와 연관된 대사경로의 특징

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목적: 방광암은 세계에서 7번째로 많은 암이다. 방광내시경과 요세포검사가 방광암의 진단에 일반적인 방법이지만, 일부에서는 민감도가 낮은 단점으로 새롭고, 비침습적이면서도 정확한 방광암의 진단법에 대한 관심이 증가하고 있다. 최근의 기술의 발전으로 생체내 액체에서 대사체의 특성을 관찰하는 연구가 가능해졌고, 이미 독성학이나 암진단과 같은 바이오마커 연구에 적용이 되고 있다. 최근들어 방광암 환자의 소변에서 대사체들이 방광암의 진단이나 예후예측에 유용한 바이오마커가 될 수 있다는 연구들이 발표가 되고 있으며, 이는 방광이 일시적인 소변의 저장고로 작용하기 때문에, 더욱 더 방광암 특이적인 바이오마커로써의 가능성을 제공해 줄 수 있다고 여겨진다.

이전의 high-performance liquid chromatography mass spectrometry 방법을 이용하여 방광암의 소변내 대사체 중 carnitine, acylcarnitine, melatonin등이 방광암에 특이적인 대사체임을 보고하였다. 본 연구의 목적은 이전에 보고한 소변내 대사체를 중심으로 방광암 내의 대사경로의 특징에 대하여 알아보고자 하였다.

재료 및 방법: 총 135명의 방광암과 26명의 정상 방광조직을 이용하여 RT-PCR분석을 시행하였다. 본 연구에서는 carnitine, acylcarnitine, melatonine등의 대사체와 연관있는 경로로 carnitine-acylcarnitine 대사경로 및 tryptophan 대사경로를 선택하였고, KEGG경로를 이용하여 연관있는 유전자를 선택하였다. 소변내 방광암 특이 대사체와 대사체와 연관있는 유전자의 상관관계를 분석하였다.

결과: Carnitine-acylcarnitine 대사경로에서는 CPT1A, CPT1B, CPT1C, CPT2, SLC25A20, CRAT의 유전자의 발현양을, 또한 tryptophan 대사경로에서는 TPH1과 IDO1의 유전자 발현양을 조사하여 총 8개의 유전자 발현양을 조사하였다. 방광암 환자와 정상대조군의 소변샘플에서 carnitine-acylcarnitine과 tryptophan 대사경로의 의미있는 유전자 변화가 관찰되었다. CPT1B, CPT1C, SLC25A20, CRAT, TPH1, IDO1은 정상방광과 비교하여 비근육침윤방광암 조직에서 유의하게 발현양이 감소되었으며 ($p < 0.05$ all), 반면, CPT1B, CPT1C, CRAT, TPH1은 근육침윤방광암 조직에서 유의하게 발현양이 감소되었으나 ($p < 0.05$), IDO 발현양의 변화는 없었다.

120명의 방광암 환자의 소변내 대사체와 조직 유전자 발현양의 상관관계를 확인하였을 때, Octenoylcarnitine은 CPT1A와 SLC25A20은 양의 상관관계를 보였고 ($r=0.250$, $p=0.006$ and $r=0.255$, $p=0.005$, respectively), 반면, decanoylcarnitine은 CPT2와 음의 상관관계를 보였다 ($r=-0.226$, $p=0.013$). 다른 소변내 대사체는 방광암 조직 유전자 발현양과 상관관계를 보이지 않았다.

결론: 방광암 환자에서 carnitine-acylcarnitine과 tryptophan 대사경로

에 연관되어 있는 유전자 발현양과 소변내 대사체의 변화와 연관이 있는 것으로 나타났다. 본 연구를 통해 방광암에서 소변내 대사체와 그것과 연관되어있는 조직내 유전자 발현양과의 관련성을 이해하는데 도움이 될 것으로 생각된다.

핵심되는 말: 방광암, 소변 대사체, 유전자 발현, qRT-PCR, 진단 마커