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Urinary Metabolite Profiling Combined with Computational Analysis Predicts Interstitial Cystitis-Associated Candidate Biomarkers

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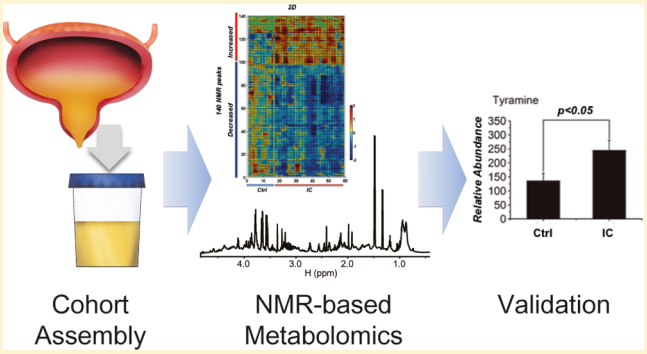
ABSTRACT: Interstitial cystitis/painful bladder syndrome (IC) is a chronic syndrome of unknown etiology that presents with bladder pain, urinary frequency, and urgency. The lack of specific biomarkers and a poor understanding of underlying molecular mechanisms present challenges for disease diagnosis and therapy. The goals of this study were to identify noninvasive biomarker candidates for IC from urine specimens and to potentially gain new insight into disease mechanisms using a nuclear magnetic resonance (NMR)-based global metabolomics analysis of urine from female IC patients and controls. Principal component analysis (PCA) suggested that the urinary metabolome of IC and controls was clearly different, with 140 NMR peaks significantly altered in IC patients (FDR < 0.05) compared to that in controls. On the basis of strong correlation scores, fifteen metabolite peaks were nominated as the strongest signature of IC. Among those signals that were higher in the IC group, three peaks were annotated as tyramine, the pain-related neuromodulator. Two peaks were annotated as 2-oxoglutarate. Levels of tyramine and 2-oxoglutarate were significantly elevated in urine specimens of IC subjects. An independent analysis using mass spectrometry also showed significantly increased levels of tyramine and 2-oxoglutarate in IC patients compared to controls. Functional studies showed that 2-oxoglutarate, but not tyramine, retarded growth of normal bladder epithelial cells. These preliminary findings suggest that analysis of urine metabolites has promise in biomarker development in the context of IC.



KEYWORDS: *Metabolomics, NMR, metabolites, interstitial cystitis, bladder, biomarker*

INTRODUCTION

Interstitial cystitis/painful bladder syndrome/bladder pain syndrome (IC) is a chronic visceral pain syndrome of unknown etiology that presents with a constellation of symptoms, including bladder pain, urinary frequency and urgency, and small voided volumes, in the absence of other identifiable etiologies.[1−4] IC is a common condition affecting approximately 1 out of 77 people, which translates into three to eight million women and one to four million men in the United States alone. Of those affected, approximately 80% of patients are female. Due to lack of consistent and effective treatments, the chronic pain from IC reduces quality of life and generates a great public health burden. IC results in more than $100 million/year in both direct healthcare expenses and indirect costs due to reduced productivity and work performance.

Diagnostic tests for IC include urine cytology, potassium sensitivity tests (considered outdated), cystoscopy with and without hydrodistention and/or bladder biopsy, and biofluid-based assays.[5−8] However, cytology is nondiagnostic, and cystoscopic appearance is often normal in IC patients. Classic ulcerations of the bladder lining (Hunner's ulcers) are found in only 5−10% of patients with IC symptoms, and bladder biopsy is also often nondiagnostic. Although hydrodistension in patients with IC demonstrates punctuate bleeding (also called glomerulations), these findings also occur with hydrodistention of normal bladders. On the contrary, assays of urine components are noninvasive and can be easily repeated. Urine is also much less complex than serum but nevertheless can contain disease biomarkers.[9]

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Metabolic profiling using nuclear magnetic resonance (NMR) spectroscopy can provide global chemical fingerprints of the physiology and metabolism of cells and can identify physiological and pathological states of biological samples. NMR profiles of metabolites can be interpreted through computational methods using multivariate statistical analysis. Metabolomics approaches have been used to identify biomarkers of disease using urine, plasma, saliva, fecal extract, and sputum.[10−12] These sources represent noninvasive methods for disease profiling and are thus much preferred to invasive methods.

In this study, we attempted to identify IC-associated metabolites by NMR using urine specimens from IC patients and control subjects. Our findings provide preliminary evidence that metabolomics analysis of urine can potentially segregate IC patients from control subjects.

MATERIALS AND METHODS

Ethics Statement

This study was approved by the Ethics Committee of Inha University Hospital in South Korea. Written informed consent was obtained from all subjects. The Institutional Review Board of Inha University Hospital approved collection, curation, and analysis of all samples.

Reagents

Cell culture medium and heat-inactivated and dialyzed fetal bovine serum were purchased from Invitrogen (Carlsbad, CA). Crystal violet solution was obtained from Promega (Madison, WI). All reagents for sample preparation for NMR and liquid chromatography−mass spectrometry (LC−MS) analysis as well as tyramine and 2-oxoglutarate were obtained from Sigma-Aldrich.

Subjects and Urine Specimen Collection

Patients and healthy control subjects were recruited from an outpatient urology clinic at Inha University Hospital. Workup included symptom assessment, cystoscopic evaluation, physical examination, urodynamics, and/or urine culture. Patients with a history of other diseases (such as any types of cancer, inflammation, or diabetes, etc.) were excluded. All subjects were of Asian female descent living in South Korea. To avoid possible contamination with vaginal or urethral cells, first morning urine specimens were obtained using clean catch methods in a sterile environment. The deidentified specimens were centrifuged to remove cell debris, and supernatants were processed into individual aliquots of 1 mL/tube before storage at −80 °C until further analysis.

^{1}H NMR Analysis of Urine

^{1}H NMR spectroscopy-based metabolomics analysis was performed to search for soluble metabolites that segregate with the diagnosis of IC. The NMR facility at the Korea Basic Science Institute was used for this study. Sample preparation for ^{1}H NMR was done as follows: aliquots of urine specimens (500 μL/each) were resuspended in 50 μL of D_2O containing sodium-3-trimethylsily-[2,2,3,3-2*H*4]-1-propionate (TSP, 0.025%, w/v) in a 5 mm NMR tube. An NMR spectrometer (Bruker Biospin, Avance 500) operating at a proton NMR frequency of 500.13 MHz and equipped with a triple-resonance cryogenic probe was used for these experiments. All one-dimensional spectra of the urine samples were measured.

Data Processing

The NMR data in urine samples from IC patients and healthy subjects were preprocessed as previously described.[13] We excluded one subject from the IC patient group and three subjects from the control group because their spectra were outliers based on PCA analysis. Fourier transformation and phase and baseline correction of the time domain data were manually performed. The resulting frequency domain data were binned at a 0.002 ppm interval. The signals were normalized against total integration values and 0.025% TSP. The region corresponding to water (4.6−5.0 ppm) was removed from all spectra. Data pretreatment including baseline correction, chromatogram alignment, time-window setting, hierarchical multivariate curve resolution, H-MCR, and normalization were performed in MATLAB (version 7.3) using custom scripts. Identification of detected NMR spectra was performed using VNMRS500 (Varian Inc.). The metabolites were identified by a database search, based on spectra and chromatographic retention index, using Chenomx (spectral database; Edmonton, Alberta, Canada) by fitting the experimental spectra to those in the database.

Identification of Metabolic Marker Candidates

To identify potential metabolites as marker candidates that can discriminate IC patients from healthy subjects, we applied a two-step approach. First, a nonparametric Wilcoxon rank sum test was performed to extract significant features from normalized NMR data. Second, the resultant NMR peak profiles, which contain profiles of 140 variables, were then imported into MetaboAnalyst, version 2.0.[14] Mean-centering with Pareto scaling was performed prior to multivariate statistical analysis. Partial least-squares discriminant analysis (PLS-DA), important variable selection with sum of absolute regression coefficient, and model evaluation with permutation strategy were carried out according to a published protocol.[15]

Liquid Chromatography−Mass Spectrometry (LC−MS or Alternatively HPLC−MS)

For LC−MS analysis, the supernatant of centrifuged urine samples was directly injected with an injection volume of 5 μL. HPLC was performed on an Agilent 1100 series liquid chromatography (Agilent, CO). The chromatographic separation was performed on a Zic-Philic Polymeric Beads Peek Column (150 × 2.1 mm, 5 μm, Merck kGaA, Darmstadt, Germany) at 35 °C. Mobile phases A and B were DW with 10 mM ammonium carbonate (pH 9.0) and acetonitrile, respectively. The mobile phase was delivered at a flow-rate of 0.15 mL/min. The linear gradient was as follows: 80% B at 0 min, 35% B at 10 min, 5% B at 12 min, 5% B at 25 min, 80% B at 25.1 min, and 80% B at 35 min. An API 2000 mass spectrometer controlled by the Analyst 1.6 software (AB/SCIEX, Framingham, MA) and equipped with an electrospray ionization (ESI) source was used in positive ion mode for detecting tyramine and in negative ion mode for detecting 2-oxoglutarate. For mass detection, multiple reaction monitoring (MRM) was performed with the m/z value of parent and fragment ions. The MRM transitions were 138 > 121 (tyramine) in positive ion mode and 145 > 101 (2-oxoglutarate) in negative ion mode. Two samples were excluded from the LC−MS analysis because of their abnormal detection levels.

Cell Culture and Proliferation Assay

Immortalized normal human bladder epithelial cells, TRT-HU1, were maintained as described previously.[16] TRT-HU1 cells were

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seeded in 24-well culture plates at a density of 1×10^2 cells per well in standard growth medium. For the next 3 days, the cells were treated with varying doses of tyramine or 2-oxoglutarate. Crystal violet staining analysis was performed for determination of cell proliferation.[18]

RESULTS

Characteristics of the Study Subjects

The Inha Institutional Review Board approved collection and analysis of all samples (IUH-IRB no. 10-0751). All patients and healthy control subjects were recruited for this study from an outpatient urology clinic at Inha University Hospital (South Korea). A clinical diagnosis of IC was made by two independent urologists (T.L. and S.P.) according to NIDDK criteria (e.g., frequency, urgency, bladder pain, discomfort, and the presence of glomerulations during cystoscopic hydrodistention) before any treatment or medication was given. In total, we enrolled 64 female subjects (43 IC patients and 21 normal subjects) with a mean age of around 51. Population-based, age-matched controls were recruited from one clinic using the same standard operating procedures (SOPs) during the same research period (2010−2013). The clinical and pathological features of the subjects are described in Table 1.

Table 1. Clinical and Pathological Features of Patients with IC and Control Subjects

variables	no. of patients (%)	no. of controls (%)
no.	43	21
mean age ± SD	50.7 ± 10.7	51.4 ± 13.7
Gender		
male	0	0
female	43	21
Grade (IPSS Symptom Score)		
severe (>20)	16 (37.2)	0 (0)
modest (9−19)	17 (39.5)	6 (28.6)
mild (0−8)	10 (23.3)	15 (71.4)
Symptoms		
frequency	31 (72.1)	2 (9.5)
urgency	28 (65.1)	2 (9.5)
discomfort	9 (20.9)	0 (0)
pain	17 (39.5)	0 (0)

^{1}H NMR Spectra of Urine Specimens from IC Patients and Controls

Because analysis of urine metabolites is a promising, noninvasive approach to study bladder disease, as shown with bladder cancer,[17] we investigated the metabolite profile of the individual urine samples using ^{1}H NMR spectroscopy. An NMR spectrometer equipped with a triple-resonance cryogenic probe was used for the analysis. NMR-based metabolomics requires relatively simple sample preparation and provides structural information on metabolites. Our analysis and data requisition resulted in a total of 4501 metabolites detected. The spectra featured visually identifiable differences in the signal ranges of 6.5−7.5, 3.5−4.0, and 2.0−2.5 ppm, suggesting metabolic differences between IC patients and controls (Figure 1).

To compensate for possible outliers within samples, principal component analysis (PCA) was performed on the NMR spectral data of the urine samples from patients and controls. The Perato scaling method and division of the mean-centered data by the square root of the standard deviation were used (Figure 2A). The scores plot for partial least-squares (PLS) components showed differentiation of the IC samples from controls with good separation and dispersion (Figure 2B). We further attempted to assess how accurately our predictive model fit using the leave-one-out cross-validation method (also called rotation estimation) as well as randomized permutation. The observed statistic of this analysis using MetaboAnalyst software[14] was significant (p = 0.012), suggesting that these signatures may significantly differentiate patients from healthy controls (Figure 2C).

Identification of NMR Peaks Increased in IC Specimens

Given the above result, we tried to identify NMR signals responsible for the difference. We sought to capture the most significantly and differentially detected NMR peaks and found that there was a significant difference in the NMR peak distribution between IC and control specimens. On the basis of multivariate statistical analysis, a total of 140 NMR peaks were significantly different between IC and controls (FDR < 0.05) (Figure 3). We then focused on the NMR peaks that most heavily contributed to the separation with respect to high correlation and signal-to-noise ratio values. We selected the top 15 NMR peaks based on the partial least-squares discriminant analysis (PLS-DA) model using MetaboAnalyst software.[14] NMR signals at 3.2485, 4.3505, 3.243, 2.9606, 2.2924, 3.2504, 3.0157, 3.0212, 2.9625, 4.4422, 0.7017, 4.3523, 4.3432, 9.2718, and 3.0102 ppm are among the major factors separating the groups with high correlation and intensity of signal (Figure 4A). These key candidate metabolites contribute to the separation of patients and controls with a coefficient 0.7 or more (Figure 4A). Given that a coefficient 0.53 or above is considered to be statistically significant (with a correlation coefficient of a risk of 5% or less), levels of these top 15 NMR peaks are considered to be strongly correlated to the IC group. Although the intensities of four NMR peaks at 4.3505, 4.4422, 4.3523, and 4.3432 ppm were significantly decreased in the IC group, the intensities of the other 11 peaks were increased in this group (Figure 4B). These findings suggest that these top 15 NMR peaks would be useful for further annotation and analysis.

Identification of Differentially Expressed Metabolites in Urine of IC Patients

Independent quantification of the metabolites that were upregulated in patients showed that 11 NMR peaks (e.g., 3.2485 and 3.243 ppm) were significantly upregulated in IC patients (p < 0.05). Annotation of the NMR peaks was performed using MeltDB, Chenomx, and MetaboloAnalyst software. We were able to annotate several of the discriminant peaks, including the most significant peak at 3.2485 ppm, which was identified as tyramine, a neuro-transmodulator related to pain.[18] Other NMR peaks, such as 3.243 and 2.924 ppm, were also annotated to tyramine, and peaks at 3.0157 and 3.0212 ppm were annotated to 2-oxoglutarate (Figure 5A). Figure 5B shows the structures and relative abundance of tyramine and 2-oxoglutarate. Urinary concentrations of 3.2485, 2.924, and 3.243 ppm (annotated as tyramine) and 3.0157 and 3.0212 ppm (annotated as 2-oxoglutarate) were increased in the IC patient group compared to that in controls (Figure 5C). An additional LC−MS analysis was able to confirm the NMR-based data and showed that levels of tyramine and 2-oxoglutarate were significantly increased in urine specimens from IC patients compared to that in controls (approximately 2-fold, p < 0.05) (Figure 5D,E).

The findings above suggest that bladder cells may sense higher level of metabolites, resulting in biological changes. We then

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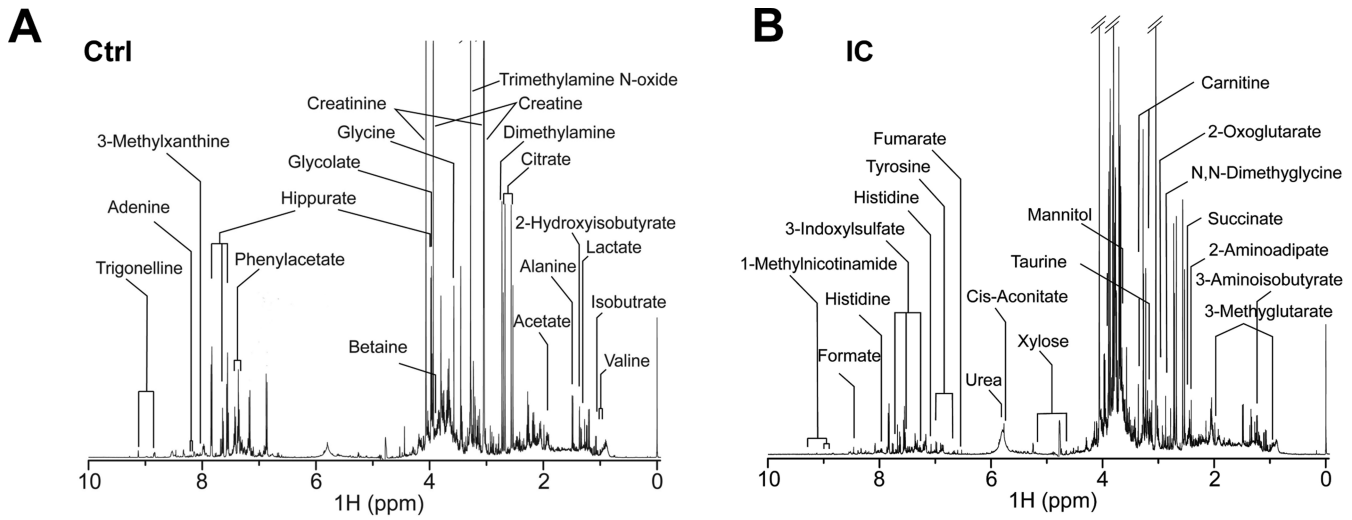

Figure 1. ^{1}H NMR spectra of urine samples derived from controls (Ctrl, A) and IC subjects (IC, B). Metabolite peaks were assigned as described in Materials and Methods.

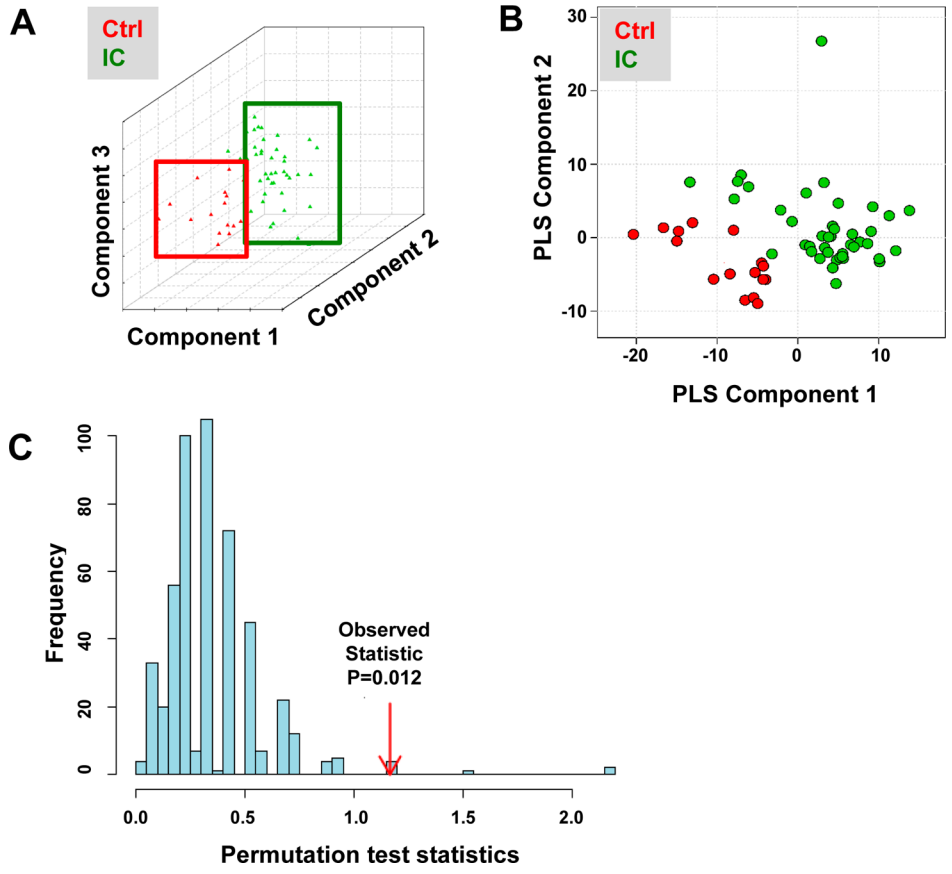

Figure 2. Differentiation of IC patients and healthy control groups using multivariate statistical analysis. (A) Principal component analysis (PCA) showed a clear separation of NMR peaks between patients and matched control subjects. (B) Partial least-squares discriminant analysis (PLS-DA) score plot of the IC and control groups. Red, control samples; green, IC patient samples. The model was established using three principal components. PLS-DA analysis differentiated IC patients from controls. (C) For model evaluation, the class prediction results based on cross-model validation predictions of the original labeling compared to the permuted data assessed using the separation distance. Histogram shows distribution of separation distance based on permutated data. Red arrow indicates observed statistic (p = 0.012).

tested the effects of tyramine and 2-oxoglutarate on cell proliferation in the hTERT-immortalized urothelial cell line TRT-HU1 (Figure 5F). To do this, TRT-HU1 cells were treated with varying concentrations of tyramine or 2-oxoglutarate. 2-Oxoglutarate, also known as α-ketoglutarate, is a key intermediate in the Krebs cycle, which plays a role in amino acid synthesis, nitrogen transport, and oxidation reactions. TRT-HU1 bladder cell proliferation was suppressed in the presence of 2-oxoglutarate in a dose-dependent manner. This observation is consistent with previous observations by other groups. Previous

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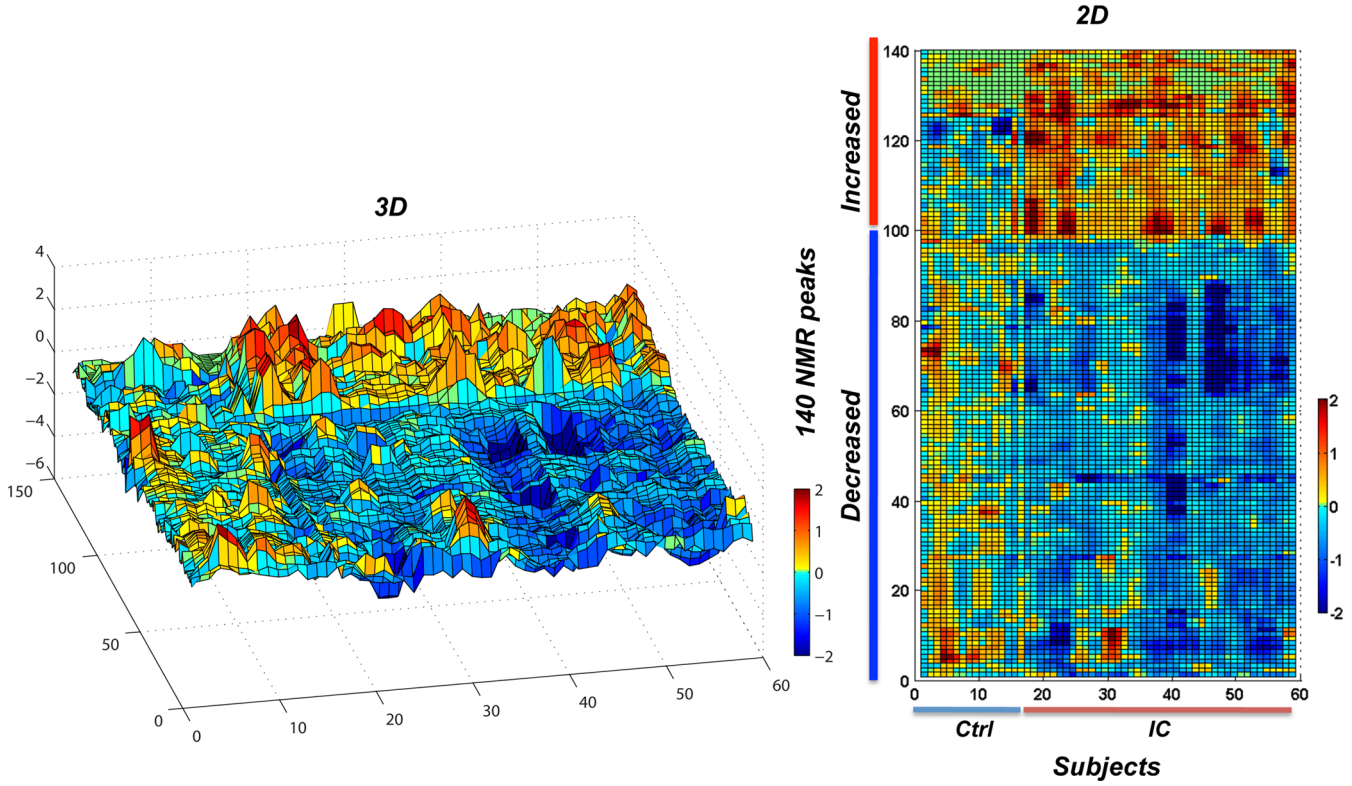

Figure 3. Surface plots (2D and 3D) of 140 quantified NMR peaks in IC and control groups. Among 4501 detected NMR peaks in total, 140 peaks were significantly altered in IC patients compared to that in controls (FDR < 0.05) (left, 3D; right, 2D).

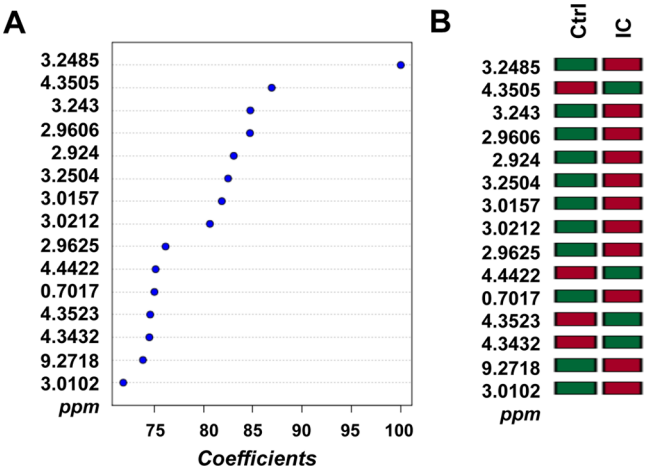

Figure 4. NMR spectra segregating IC from controls. (A) The major contributing NMR signals identified by PLS-DA. The regression coefficients represent the highest contributing signals from which the IC and control groups could be distinguished. (B) The colored boxes indicate the relative concentrations of the corresponding metabolite in each group. Red and green denote high and low concentrations, respectively. The top 15 NMR peaks are considered to be significantly different between the two groups based on their coefficients (>70).

reports suggest that 2-oxoglutarate exerts antitumor effects by inhibition of the cell cycle transition through regulation of p21 Waf1/Cip1, p27 Kip1, cyclin D1, and Rb.[19] 2-Oxoglutarate also inhibits angiogenesis-related proteins, such as HIF-1α, erythropoietin, and VEGF, under hypoxic conditions in tumor cells.[20] Bladder cell proliferation was not influenced by tyramine treatment (data not shown).

We also found that four NMR peaks, including those at 4.3505 and 4.4422 ppm, were significantly downregulated in patients compared to normal controls (Figure 6A). Two NMR peaks, at 4.4422 and 4.444 ppm, were annotated as trigonelline. The coefficient of the 4.444 ppm peak was just below 0.7. Box plots shown in Figure 6B suggest that levels of trigonelline might be significantly lower in urine samples of IC patients compared to those of controls.

DISCUSSION

In this study, we report, to our knowledge, the first global analysis of metabolic patterns in urine specimens derived from IC patients and healthy subjects. NMR-based metabolomics analysis identified 140 NMR peaks, which collectively distinguished the IC patient urinary profile from that of controls. The PLS-DA model using MetaboAnalyst software[14] revealed that 15 NMR peaks are significantly changed in urine of IC patients. Levels of tyramine and 2-oxoglutarate were significantly increased in the urine specimens from IC patients compared to those from controls. These compounds may be associated with bladder pathology; however, confirmatory studies are necessary.

Our metabolomic data suggest that tyramine might be concentrated in the urine of IC patients. Tyramine is a product of tyrosine metabolism and, like other trace amines,[21] is a neurotransmodulator.[22] Tyramine is detectable in plasma, serum, and urine, and the measured level is significantly altered in certain disorders characterized by pain, such as common headaches, migraines, urticaria, irritable bowel syndrome, and joint pain.[18,23,24] Thus, our findings suggest the interesting possibility that urine metabolites may elicit or reflect one's pain perception during bladder filling and discomfort associated with IC. A specific class of tyramine receptor, the trace amine associated

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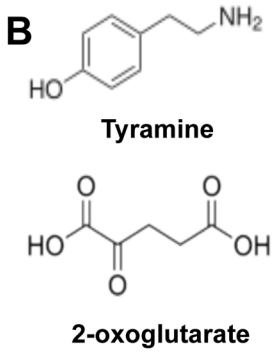
A

ppm	Assignment
3.2485	Tyramine
3.243	Tyramine
2.924	Tyramine
3.0157	2-oxoglutarate
3.0212	2-oxoglutarate



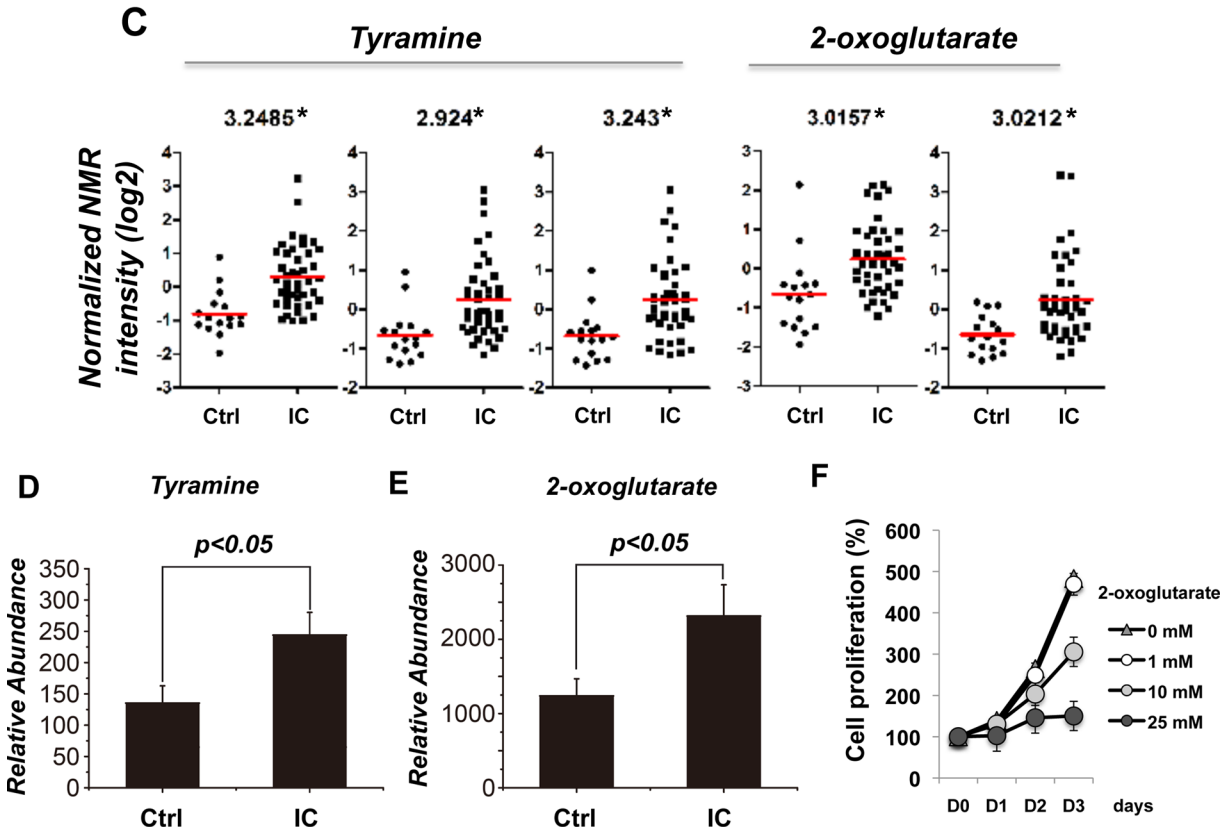


Figure 5. Upregulated metabolites that could be used to segregate IC patients from normal subjects. (A) NMR peaks at 3.0212 and 3.0157 ppm were annotated as 2-oxoglutarate, and those at 3.2485, 3.243, and 2.924 ppm were annotated as tyramine, using a 500 MHz machine (VNMRS500) at Varian Inc., Korea. No annotation was available for the other three peaks using our software. (B) Chemical structures of tyramine and 2-oxoglutarate are shown. (C) NMR peaks indicating that candidate metabolites, tyramine and 2-oxoglutarate, were significantly increased in IC patients compared to that in controls. Wilcoxon rank sum test of the relative difference of the marker signals for the IC and control groups. All signals showed statistical significance with *FDR < 0.05 (tyramine, 3.2485 ppm; 2.924 ppm; 3.243 ppm, 2-oxoglutarate, 3.0157 ppm; 3.0212 ppm). (D, E) LC−MS analysis showed the relative levels of two biomarker metabolites in urine from IC patients compared with those from controls. The bar graphs represent the relative peak area on LC−MS analysis for tyramine (D) and 2-oxoglutarate (E). Statistical analysis was performed using Student's *t*-test, and the resulting *p*-values are indicated. Error bars represent standard error. (F) Biological effects of 2-oxoglutarate on bladder cells. 2-Oxoglutarate treatment inhibited cell proliferation. Proliferation of TRT-HU1 cells treated with varying doses of 2-oxoglutarate (0, 1, 10, or 25 mM) was measured over time (days 0, 1, 2, and 3). Cell proliferation was determined by crystal violet assay. *, $p < 0.05$ (Student's *t*-test).

receptor (TAAR1),[25] is a G-protein coupled receptor (GPCR) expressed in the brain with a wide distribution in other organs.[26] One class of GPCR, the transient receptor potential (TRP) channel family, has been shown to regulate urothelial sensory perception and bladder function. TRPV (transient receptor potential channel subfamily V), located in the urothelium,[27] plays a role in the bladder sensor web.[28] Pharmacological antagonists against TRPV reduce bladder hyperactivity and urinary incontinence in mouse and rat cystitis models.[29] In addition, anticholinergic agents, which block the neurotransmitter acetylcholine in the central and the peripheral nervous system, have been shown to improve bladder cellular architecture and provide relief from pain and urgency.[30]

In the current study, we also found that the relative concentration of 2-oxoglutarate was increased in the urine of IC patients. 2-Oxoglutarate (also called α-ketoglutarate), which is an important player in the Krebs cycle, is known to be involved in the cellular detoxification of oxidative damage. Previous

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A

ppm	Assignment
4.3432	n.a
4.3505	n.a
4.3523	n.a
4.4422	Trigonelline
4.444	Trigonelline

B

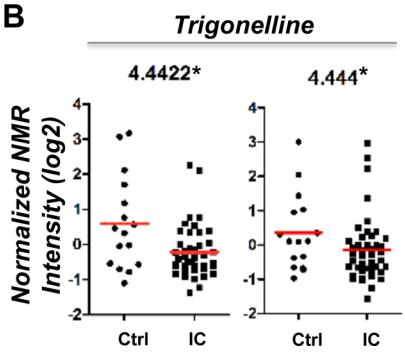

Figure 6. Downregulated metabolites differentially expressed in urine specimens of IC patients compared to those in normal subjects. (A) Two of five significantly downregulated NMR peaks, 4.4422 and 4.444 ppm, could be annotated as trigonelline. (B) Expression levels of trigonelline were determined in urine samples of IC and those of controls (*FDR < 0.05). (n.a, not assigned)

research has shown that 2-oxoglutarate converts to citrate during hypoxic states, resulting in cell growth and viability, suggesting the possibility of an antiproliferative and antiangiogenesis function of 2-oxoglutarate. However, no functional role of urinary 2-oxoglutarate has been proposed in the setting of bladder wall abnormalities or bladder diseases, and no correlations have been previously described.

In summary, our findings indicate that urinary metabolites may allow the segregation of IC patients from normal individuals and may reflect the underlying biology of IC, which is still largely unknown. Further attempts to validate the clinical relevance of urinary metabolites may provide novel insights into the etiology of IC and will identify urine metabolites as biomarkers of IC that have the potential to be employed clinically.

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Author Contributions

○H.W. and T.L. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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