Research Article

Cancer Epidemiology, Biomarkers & Prevention

Serum Metabolic Profiling Identified a Distinct Metabolic Signature in Bladder Cancer Smokers: A Key Metabolic Enzyme Associated with Patient Survival

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Abstract

Background: The current system to predict the outcome of smokers with bladder cancer is insufficient due to complex genomic and transcriptomic heterogeneities. This study aims to identify serum metabolite-associated genes related to survival in this population.

Methods: We performed LC/MS-based targeted metabolomic analysis for >300 metabolites in serum obtained from two independent cohorts of bladder cancer never smokers, smokers, healthy smokers, and healthy never smokers. A subset of differential metabolites was validated using Biocrates absoluteIDQ p180 Kit. Genes associated with differential metabolites were integrated with a publicly available cohort of The Cancer Genome Atlas (TCGA) to obtain an intersecting signature specific for bladder cancer smokers.

Results: Forty metabolites (FDR < 0.25) were identified to be differential between bladder cancer never smokers and smokers. Increased abundance of amino acids (tyrosine, phenylalanine, proline, serine, valine, isoleucine, glycine, and asparagine) and taurine were observed in bladder cancer smokers. Integration of differential metabolomic gene signature and transcriptomics data from TCGA cohort revealed an intersection of 17 genes that showed significant correlation with patient survival in bladder cancer smokers. Importantly, catechol-O-methyltransferase, iodotyrosine deiodinase, and tubulin tyrosine ligase showed a significant association with patient survival in publicly available bladder cancer smoker datasets and did not have any clinical association in never smokers.

Conclusions: Serum metabolic profiling of bladder cancer smokers revealed dysregulated amino acid metabolism. It provides a distinct gene signature that shows a prognostic value in predicting bladder cancer smoker survival.

Impact: Serum metabolic signature–derived genes act as a predictive tool for studying the bladder cancer progression in smokers.

Introduction

Urinary bladder cancer is the ninth most common malignant disease and the 13th most common cause of cancer-related death worldwide (1). Nearly 1.3 million people worldwide and 600,000 in the United States alone are diagnosed with bladder cancer each year contributing to a significant health care burden. Bladder cancer has a high recurrence rate and requires lifelong surveillance incurring major medical expenses (2–4). Occupational exposure to carcinogens has been long associated with increased bladder cancer risk. Tobacco smoke contains more than 60 carcinogens causing at least 18 different types of cancer including bladder cancer. Tobacco smoking is the best established risk factor for bladder cancer, with a population attributable risk for smoking of 50% for bladder cancer (5). More than 30% of people in the United States and Europe have a history of smoking (6), based on which the patients with bladder cancer can be categorized into former and current smokers. Current smoking also increases the risk of recurrence, drug resistance, and significantly increases the risk of death in bladder cancer compared with former and never smokers (7, 8). Previous studies have suggested that smoking

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status and the quantity of smoking is associated with higher tumor grade and stage in patients with bladder cancer (9).

Xenobiotic metabolizing enzymes expressed in the bladder detoxify the genotoxic compounds and excrete them through urine. It is known that alterations of xenobiotic enzymes are associated with bladder cancer development (10, 11). Environmental toxins, including the carcinogens from cigarettes, are cleared by xenobiotic pathways and pass through the body's excretion system. Over time, these products accumulate on the walls of the bladder, which, in turn, changes the genetic composition of the bladder wall. Our group has previously shown that tobacco-specific carcinogens alter metabolic pathways and the expression levels of xenobiotic enzymes in bladder cancer (12). Diagnostically, patients with bladder cancer are evaluated using cystoscopy, an endoscopic procedure performed by urologist (13). Also, cells in the urine (cytology) are inspected by trained cytopathologist to determine the presence of cancer lesions in the bladder (14). However, the sensitivity for bladder tumor detection with urine cytology surveillance is low. Noninvasive procedures are needed for facilitating diagnostic, therapeutic, and prognostic strategies. Unfortunately, to our knowledge, there are no diagnostic or surveillance serum- or plasma-associated metabolic biomarkers that have been identified in bladder cancer smokers.

The advent of metabolic profiling with LC/MS has opened the possibilities to identify dysregulated metabolic pathways and contributing markers. These, in turn, can serve as prognostic and diagnostic biomarkers in the identification of disease and its pathogenesis (15). Although prior studies have successfully utilized metabolomics for biomarker identification in bladder cancer sera (10, 16, 17), serum metabolic profiling for bladder cancer smokers is lacking. In this study, we applied targeted LC/MS approach to delineate serum-based biomarkers that differentiate patients with bladder cancer through smoking history (current smokers, former smokers) from never smoking patients with bladder cancer. This study provides initial evidence of serum-based metabolite prognostic markers in bladder cancer smokers.

Materials and Methods

Study population and serum profiling by LC/MS

In this study, serum samples from bladder cancer ($n = 67$) were used from two independent cohorts: (i) NCI ($n = 59$), (ii) Augusta University (Augusta, GA; $n = 8$), Georgia Cancer Center and healthy case controls ($n = 20$) from (iii) Baylor College of Medicine (Houston, TX) procured by a prior written informed consent under Institute review board–approved protocols. NCI patient cohorts are enrolled in a clinical study with monotherapy cabozantinib.

A flow chart depicting the study overview, sample processing, and analysis are illustrated in Figure 1.

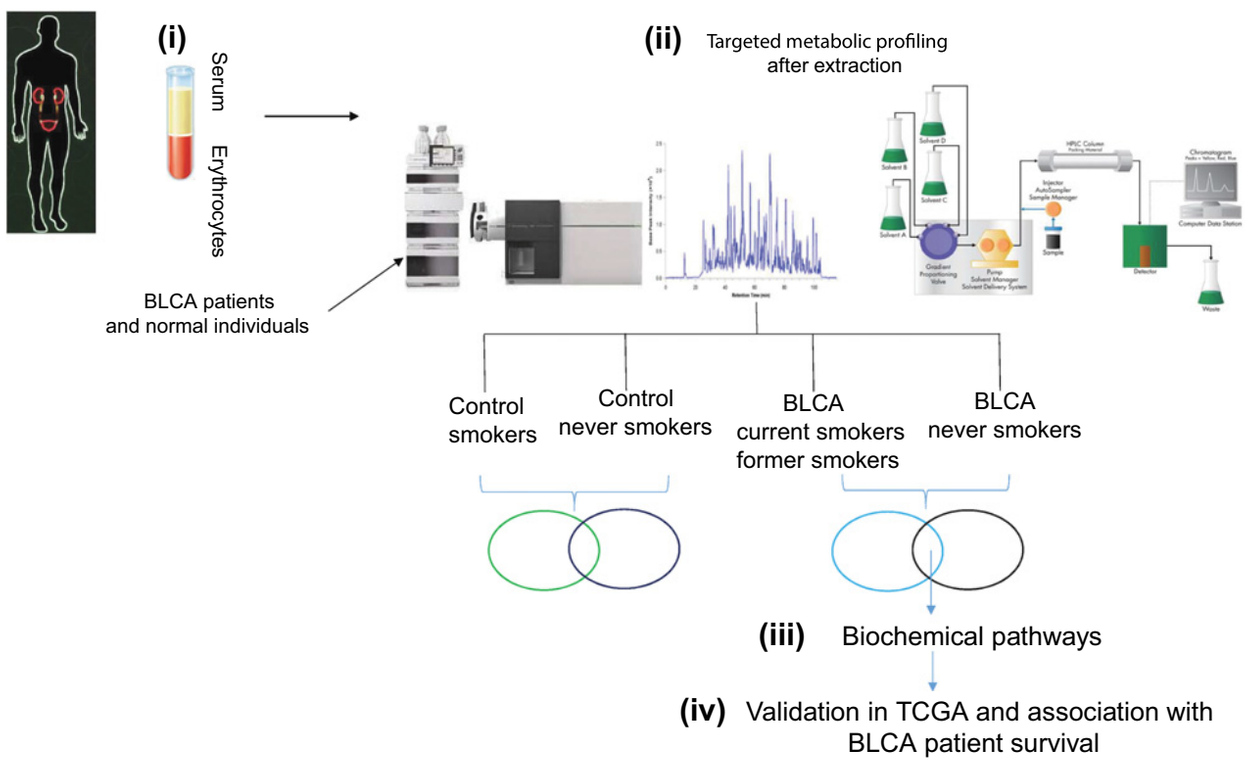

Figure 1.
Overview of global serum metabolic profiling in patients with bladder cancer (BLCA) and normal individuals. The methodology involves four important steps: (**i**) serum extraction from blood samples of patients with bladder cancer and normal individuals, (**ii**) filtration, metabolic extraction, and targeted metabolic profiling by LC/MS, (**iii**) identification of differential metabolites and deciphering the biochemical pathways involving the upregulated metabolites, and (**iv**) validation of genes involved in regulating the biochemical pathways and their clinical association in TCGA cohorts.

Targeted metabolomics using mass spectrometry

Reagents and internal standards. High-performance liquid chromatography (HPLC)-grade ammonium acetate was from Sigma, acetonitrile, methanol, chloroform, and water were procured from Burdick & Jackson. Mass spectrometry–grade formic acid was purchased from Sigma-Aldrich. Metabolite standards and internal standards, including N-acetyl Aspartic acid-d3, tryptophan-15N2, sarcosine-d3, glutamic acid-d5, thymine-d4, gibberellic acid, trans-zeatin, jasmonic acid, 15N anthranilic acid, and Testosterone-d3 were also purchased from Sigma-Aldrich.

Sample preparation for mass spectrometry and metabolomics analysis

Serum samples were stored at −80°C until processing. Metabolites were extracted from 40 μL of serum, control serum pool was used as quality controls, and the extraction procedure is as follows: briefly, each serum sample was resuspended in 750 μL of ice-cold methanol:water (4:1) containing 20 μL spiked internal standard mix. Ice-cold chloroform and water were added in a 3:1 ratio for a final proportion of 4:3:2 methanol:chloroform: water. Both the organic and aqueous layers were transferred into a new tube, dried, and resuspended with 50:50 methanol: water. The resuspended sample was then deproteinized using a 3-kDa molecular filter; (Amicon ultracel-3K Membrane; Millipore Corporation) and the filtrate was dried under vacuum (Genevac EZ-2plus). Prior to mass spectrometry, the dried extracts were resuspended in 50 μL of 1:1 methanol and water and were subjected to LC/MS.

Separation of metabolites

Two different analytic methods were used for the separation of targeted metabolomics to measure >300 metabolites.

Method 1. In ESI-positive mode, the HPLC column [(Waters X-bridge Amide 3.5 μm, 4.6 × 100 mm (PN: 186004868)] was used. Mobile phase A and B were 0.1% formic acid in water and acetonitrile, respectively. Gradient: 0–3 minutes 85% B, 3–12 minutes 30% B, 12–15 minutes 2% B, 16 minutes 95% B, followed by reequilibration at the end of the gradient 23 minutes to the initial starting condition 85% B. Flow rate: 0.3 mL/minute.

Method 2. In ESI-negative mode, the HPLC column [(Waters X-bridge Amide 3.5 μm, 4.6 × 100 mm (PN: 186004868, Waters). Mobile phase A and B were 20 mmol/L ammonium acetate in 95% acetonitrile and 5% water (pH 9.0) and 100% acetonitrile, respectively. Gradient: 0–3 minutes 85% B, 3–12 minutes 30% B, 12–15 minutes 2% B, 15–16 minutes 85% B followed by reequilibration at end of the gradient 23 minutes to the initial starting condition of 85% B. Flow rate: 0.3 mL/minute.

Data acquisition through LC/MS analysis

LC/MS analysis was performed using 6490 Triple Quadrupole Mass Spectrometer coupled to an Agilent 1290 series HPLC system (Agilent Technologies) with single-reaction monitoring. This liquid chromatography system is equipped with a degasser, binary pump, thermostatted auto sampler, and column oven. This single-reaction monitoring—based measurement of relative metabolite levels used normal phase chromatographic separation. Ten microliters of resuspended samples were injected and analyzed using source parameters as follows: gas temperature, 250°C; gas flow, 14 L/minute; Nebulizer, 20 psi; sheath gas temperature, 350°C; sheath gas flow, 12 L/minute; capillary, 3,000 V positive and 3,000 V negative; nozzle voltage, 1,500 V positive and 1,500 V negative. Approximately 8–11 data points were acquired per each detected metabolite.

Quantification of metabolites using the Biocrates AbsoluteIDQ Kit p180

Metabolite concentrations were obtained using the AbsoluteIDQ Kit p180 according to manufacturer's instructions on a QTRAP 6500 LC/MS/MS System. Ten microliters of the internal standard solution was added to each well of the 96-well plate, 10 μL of the serum samples, quality control samples, blank, and calibration standard were added to the appropriate wells. The plate was then dried and the samples were derivatized with phenyl isothiocyanate for the amino acids and biogenic amines. The samples were dried using GeneVac Vaccum system at 37°C and eluted with 5 mmol/L ammonium acetate in methanol. They were then diluted with 50:50 methanol: water for LC/MS analysis. The liquid chromatography column was Agilent Zorbax Eclipse XDB C18,3.0 × 100mm, 3.5 μm. Mobile phase A and B were 0.2% formic acid in water and acetonitrile, respectively. The injection volume was 10 μL, and the flow rate was 0.5 mL/minute. The samples were integrated and analyzed in AB Sciex Analyst Software to their specific labeled internal standards provided by the kit plate and exported the results file(.rdb). The plate was validated, and concentrations were calculated, exported by using this results file in MetIQ software. The final concentrations exported were in μmol/L units.

Statistical analysis

Agilent MassHunter Workstation Software - Quantitative analysis was used for manual review of chromatograms. Peak-area integration was accessed on the basis of the retention time. The normalization of each metabolite peak area was done by the peak area of the spiked internal standard, and then the data were $\log_2$-transformed, per method basis. For differential analysis, two-way sample *t* tests were conducted, and ANOVA was performed for three-way analysis. Differential metabolites were identified by adjusting the *P* values for multiple testing at a FDR threshold of <0.25.

Integration of metabolomics and transcriptomics and survival analysis

The panel of altered metabolites between smokers and never smokers was mapped to their corresponding genes using the Kyoto Encyclopedia of Genes and Human Metabolic Data Base (KEGG/HMDB), leading to the identification of 374 associated genes. We used the transcriptomic profiles of bladder cancer that were obtained from the Riester (18) and The Cancer Genome Atlas (TCGA) (19) with associated clinical information on smoking patients with bladder cancer. TCGA RNA expression data is based on mRNA sequencing, whereas Riester RNA expression data is microarray-based. Next, we generated the gene signatures of bladder cancer smokers versus never smokers using TCGA data. Following this methodology, we identified the common genes from metabolomics and transcriptomics and identified 17 intersecting genes. To avoid dataset-specific bias, we compared the 17 smoking signature genes by smoker status in the Riester dataset and used this fold change signature for patient survival in both TCGA patient cohort ($n = 365$ patients having smoking status)

and Riester cohorts ($n = 93$ patients having smoking status) by using Kaplan–Meier curves. We also analyzed the complete 17 gene signature for survival status in the same cohort.

Validation of identified genes in patient tissues and mouse xenografts

Six- to 8-week-old male NOD-SCID beige mice were injected subcutaneously with 1.5×10^6 UMUC3 bladder cancer cells. The control group ($n = 3$) mice with xenografts are maintained in normal cages. The experimental group ($n = 3$) was exposed to cigarette smoke from two cigarettes (Marlboro) every day for a period of 4 weeks. The tumor volume was measured every 4 days using vernier calipers and were dissected after reaching a volume of 1,500 mm^3. The dissected tumor were flash frozen and stored at $-80°C$ until analysis.

Ten milligrams of control and smoke-exposed mouse bladder cancer tissues were digested with a homogenizer in 500 μL Trizol (Ambion). Equal volumes of chloroform were added to the digested tissue and centrifuged at 15,000 rpm for 15 minutes. Next, the aqueous phase was taken in a new tube, and an equal volume of isopropanol was added and kept at $-20°C$ for 30 minutes. Later, the lysate was loaded on QIAGEN column (QIAGEN RNeasy Kit) for RNA purification. RNA was quantified using a Biotek plate reader. cDNA was transcribed from 1 μg of RNA by using qScript cDNA Supermix (Quantabio). Primers for the genes were obtained from Invitrogen and their sequences are available upon request. RT-PCR was performed using SYBR Green Master mix (Lifetech).

Results

Study population characterstics

The clinicopathologic characteristics are summarized in Table 1. Nearly, 95% of patients have T4 stage bladder cancer. More than 80% of the patients enrolled are LVI-positive and are older than 50 years of age. We characterized the metabolome of bladder cancer never smokers (39%) and smokers (former 46%; current 15%) and age-matched case–control serum (Table 1). In these two cohorts, the current smokers consume a range between <20 cigarette pack per year ($n = 5$) and >20 pack per year ($n = 5$). The cessation period for former smokers is around 2–20 years. Serum samples from healthy individuals ($n = 20$) were procured from Baylor College of Medicine out of which 55% ($n = 11$) are smokers, and 45% ($n = 9$) are never smokers. The characteristics of the healthy individuals are provided in Table 1.

Identification of altered metabolites and associated pathways

We targeted >300 metabolites (Supplementary Table S1) and detected 190 metabolites of different classes (amino acid and derivatives, free fatty acids, tricarboxylic acid, glycolysis/PPP pathway metabolites, nucleic acids/nucleotides) in serum samples (Fig. 2A). A repetitive analysis of pooled serum samples served as controls to ascertain the reproducibility and robustness of the profiling platform for both positive and negative ionization (Supplementary Fig. S1A and S1B) and showed <20 coefficient of variations for each metabolite. To identify the smoke-specific

Table 1. Baseline characteristics of patients with bladder cancer and healthy individuals involved in this study

Bladder cancer serum samples		
Bladder cancer patient characteristics		
Ethnicity	African American ($n = 17$; 25%)	
	European American ($n = 49$; 74%)	
	Unknown ($n = 1$; 1%)	
Smoking status	$n = 26$ (39%; Never smoker)	
	$n = 31$ (46%; Former-smoker)	
	$n = 10$ (15%; Current smoker)	
Gender	$n = 46$(69%; Male)	
	$n = 21$(31%; Female)	
Cohorts	NCI ($n = 59$; 88%)	
	Georgia ($n = 8$; 12%)	
T-stage	Ta = 1(1%)	
	T1 = 1(1%)	
	T2 = 2(2%)	
	T3 = 1(1%)	
	T4 = 62(95%)	
Age	≤50 years = 8(12%)	
	>50 years = 59 (88%)	
LVI[a]	LVI-Positive = 59(89%)	
	LVI-Negative = 8(11%)	
Number of packets per year	Current smokers	
	<20 Pack/year = 5(50%)	≥20 Pack/year = 5(50%)
Duration of cessation	Former smokers	
	Quit <20 years ago = 9(29%)	Quit ≥20 years ago = 22(71%)
Case–control serum samples		
Characteristics		
Ethnicity	African American ($n = 14$; 70%)	
	European American ($n = 3$; 15%)	
	Hispanic ($n = 3$; 15%)	
Smoking status	$n = 9$ (45%; Never smoker)	
	$n = 7$ (35%; Former-smoker)	
	$n = 4$ (20%; Current smoker)	
Gender	$n = 10$ (50%; Male)	
	$n = 10$ (50%; Female)	

[a]Lymphovascular invasion

serum metabolite differences among control, and bladder cancer smokers, two independent analyses were performed in which the groups were compared (i.e., healthy control never smokers vs. healthy control smokers, and bladder cancer never smokers vs. bladder cancer smokers). The comparison between control smokers ($n = 11$) and never smokers ($n = 9$) did not yield any differential metabolites (Supplementary Fig. S2). Interestingly, we have identified 40 metabolites (out of 190) that were significantly differential in bladder cancer smokers compared with bladder cancer never smokers. In the 40 differential metabolites, 37 were upregulated, and three were downregulated (Fig. 2B). Furthermore, most of the differential metabolites show higher levels in bladder cancer current smokers in comparison with bladder cancer former smokers (refer to the red box in Fig. 2B). We performed additional analysis to identify the metabolic signature specific to current smokers. A total of 62 differential metabolites were identified in this analysis (Supplementary Fig. S3A). The metabolic signature was compared with altered metabolic pool in Fig. 2A, and we identified 34 metabolites that were specific to bladder cancer current smokers (Supplementary Fig. S3A, indicated in red font). Pathway analysis was performed using Metaboanalyst 4.0 (20) for the differential metabolites between bladder cancer never smokers and bladder cancer smokers. We identified pathways that are involved in phenylalanine metabolism, tyrosine and tryptophan biosynthesis, and nitrogen metabolism (Fig. 2C). To graphically represent the differential metabolites and their coefficient of variance between bladder cancer smokers and bladder cancer never smokers, we used metaboanalyst 4.0 for the depiction of variability in a biplot. The length of arrow in the biplot represent the degree of variation of each metabolite. Significant changes of the differential metabolites are illustrated in the biplot (Fig. 2D). Observations indicate that these metabolic pathways could be dysregulated in bladder cancer smokers compared with never smokers.

A subset of differential metabolites was validated using Biocrates absoluteIDQ p180 Kit. However, the kit provides only a limited number of overlapping metabolites. Therefore, we could quantify only nine of the 40 differential metabolites. Interestingly, the glucogenic amino acids (proline, serine, valine, glycine, and asparagine) were found to be high in bladder cancer smokers compared with never smokers. Also, consistent with our earlier findings, phenylalanine, isoleucine, and tyrosine were also upregulated in bladder cancer smokers. The concentration of taurine, which is involved in the cysteine sulfinic acid pathway, is higher in bladder cancer smokers with respect to never smokers (Fig. 3).

Identification of a smoker-specific gene signature by integration of metabolomics and transcriptomics

We next integrated our metabolomics data with publicly available transcriptomics data using the TCGA (19) and Riester (18) cohorts in which a majority of the patients are high-grade bladder cancer (greater than 90%). Interestingly, our serum samples used for metabolite analysis are also high-grade bladder cancer (greater than 95%, Table 1). The panel of altered metabolites between bladder cancer smokers and never smokers were mapped to their corresponding genes using KEGG/HMDBs that led to the identification of 374 associated genes. Using TCGA (19), which contains patient smoking information, we found 1,249 genes were significantly altered between bladder cancer smokers and never smokers (Fig. 4A). The union of bladder cancer smoker gene signatures from the metabolomics mapped the gene set and TCGA-based gene set revealed 17 common genes (Fig. 4A). We checked the expression of these 17 genes for clinical association in bladder cancer smokers and never smokers. In this analysis, the time is the standard event time. Time is zero when observation began and measured to the last observed time point, and the analysis is the overall survival of the patient (21, 22).

For generation of this analysis, first, we analyzed the 17 genes in Riester cohort between bladder cancer smokers and never smokers. Second, we applied the Riester gene fold change in TCGA ($n = 261$ smokers and 104 never smokers) and Riester ($n = 75$ smokers and 18 never smokers) cohorts. We found that high expression of this 17 gene signature is associated with poor survival in only bladder cancer smokers in TCGA (Fig. 4B) cohort. A similar analysis was carried out on Riester cohort by classifying the data with bottom vs. top from bottom 5% to 95% with 5% increments, and we have observed that the 17 gene signature was significantly associated with poor survival only at the threshold of bottom 35% and top 65% (Supplementary Fig. S3B). Survival analysis in bladder cancer never smokers did not show any prognostic significance in either TCGA (Fig. 4C) or Riester datasets (Supplementary Fig. S3C).

To obtain additional insights, we further investigated the expression of these 17 (Fig. 4A) individual genes in bladder cancer smokers and never-smokers in the TCGA cohort. Here, 11 of 17 genes (Supplementary Table S2) show a significant difference between bladder cancer smokers and never smokers in TCGA. Cytidine uridine monophosphate kinase 2 (CMPK2), poly(ADP-ribose) polymerase family member 9 (PARP9) and 14 (PARP14), tubulin tyrosine ligase (TTL), procollagen-lysine, and 2-oxoglutarate 5-dioxygenase 2 (PLOD2) show higher expression levels in TCGA smokers, whereas catechol-O-methyltransferase (COMT), iodotyrosine deiodinase (IYD), aminomethyltransferase (AMT), aspartate aminotransferase (GOT2), solute carrier family 14 member 1 (SLC14A1), CMP-N-acetylneuraminate-β-galactosamide-α-2,3-sialyltransferase (ST3GAL4), cysteine sulfinic acid decarboxylase (CSAD) were downregulated (Fig. 5; Supplementary Fig. S4; Supplementary Table S2). To obtain additional intuitions into these 11 genes, we next performed individual gene survival analysis in the TCGA cohort. We found that two downregulated genes (COMT and IYD) have a significant clinical association (Fig. 5A and B). We next looked into the 4 upregulated genes (out of 11) in the same cohort and found that TTL had clinical associations with poor survival. Notably, COMT, IYD, and TTL showed a significant poor survival in TCGA. Kaplan–Meier survival analysis of COMT and IYD showed that low expression had significantly poor survival in TCGA bladder cancer smokers and did not show any significant difference in never smokers. Bladder cancer smokers with high expression of TTL showed the worst prognosis compared with bladder cancer never smokers (Fig. 5A and B).

We further validated the mRNA expression levels of these 3 genes in RNA isolated from bladder cancer smokers and bladder cancer never smokers patient tissues by RT-PCR. We observed increased levels of TTL and downregulation of COMT and IYD in bladder cancer smokers compared with never smokers (Fig. 6A). We developed a mouse xenograft model in which NOD-SCID beige mice were injected with UMUC3 bladder cancer cell line subcutaneously. Next, the mice were exposed to two cigarettes smoke a day for 4 weeks (Fig. 6B). Tumors were isolated from these mice, and we analyzed mRNA for the above genes in

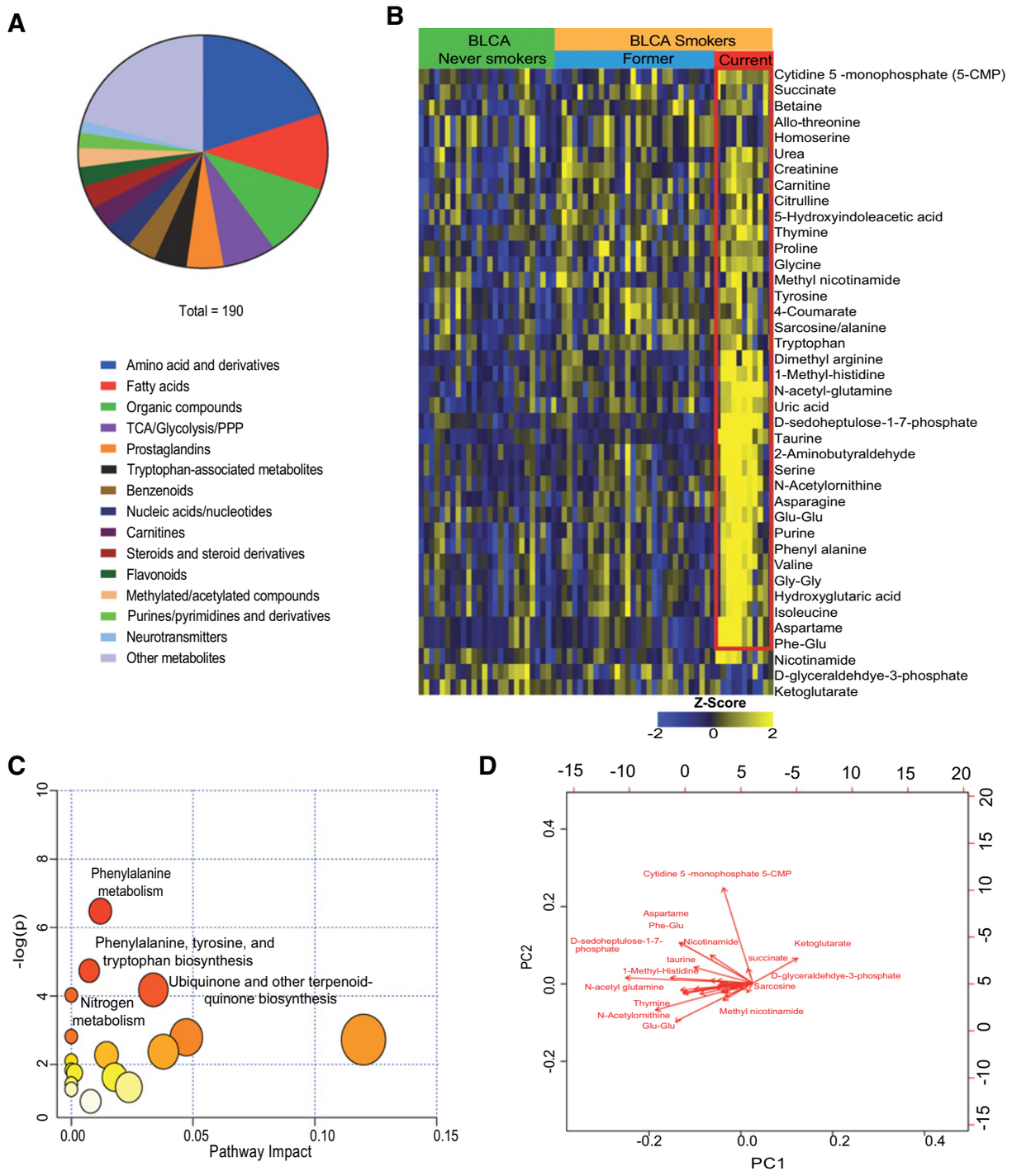

Figure 2.

Metabolic profiling of bladder cancer serum from smokers and nonsmokers and identification of key altered pathways. **A,** Pie chart illustrating the different classes of metabolites obtained in global targeted metabolic profiling of serum samples from patients with bladder cancer. A distinct color denotes each group of metabolites, and the arc length of each slice represents the percentage of that group in the obtained profile. **B,** Heat map visualization of 40 significantly altered metabolites in bladder cancer smokers compared with never smokers (FDR < 0.25). Shades of yellow and blue represent upregulation and downregulation of metabolites respectively, relative to median metabolic levels. **C,** Topology analysis of dysregulated metabolic pathways in association with bladder cancer smokers. The size of bubble area denotes the impact of each pathway, with a color representing the significance from highest in red to lowest in white. **D,** Biplot showing the upregulated differential metabolites in bladder cancer smokers and never smokers.

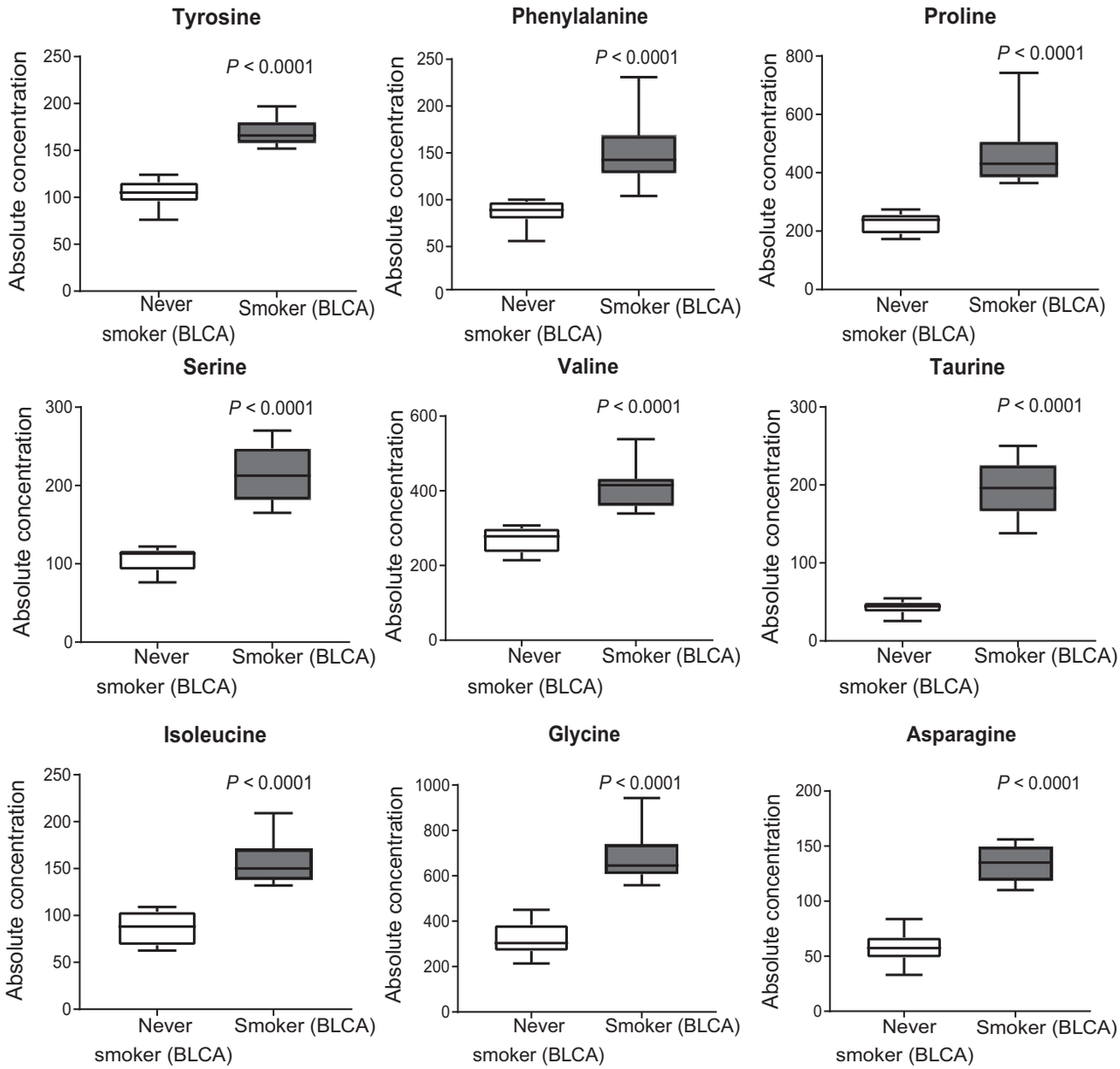

Figure 3.

Validation of a subset of metabolites using Biocrates absoluteIDQ p180 Kit. Box plots showing significant upregulation of amino acids and taurine levels in μmol/L in bladder cancer smokers ($n = 41$).

these tumors. Consistent with the patient data, we observed upregulation of TTL and downregulation of COMT and IYD expression levels in the cigarette smoke–exposed xenografts (Fig. 6C).

Discussion

Tobacco smoke is a major risk factor for the development of bladder cancer (23). Furthermore, patients with bladder cancer who smoke while undergoing treatment have decreased recurrence-free survival outcomes compared with their nonsmoking counterparts (8). Previously, we and others have reported metabolic profiling of bladder cancer using different mass spectrometry approaches (12, 24–27) and identified dysregulated metabolic pathways and gene signatures associated with them. However, to the best of our knowledge, no serum-based metabolic profiling was performed in bladder cancer smokers, although it has been studied in other types of cancers and diseases (28–31). This study focused on the identification of metabolic fingerprint that is unique to bladder cancer smokers when compared with never smokers. Our targeted mass spectrometry analysis resulted in detection of 190 metabolites of different classes: amino acids

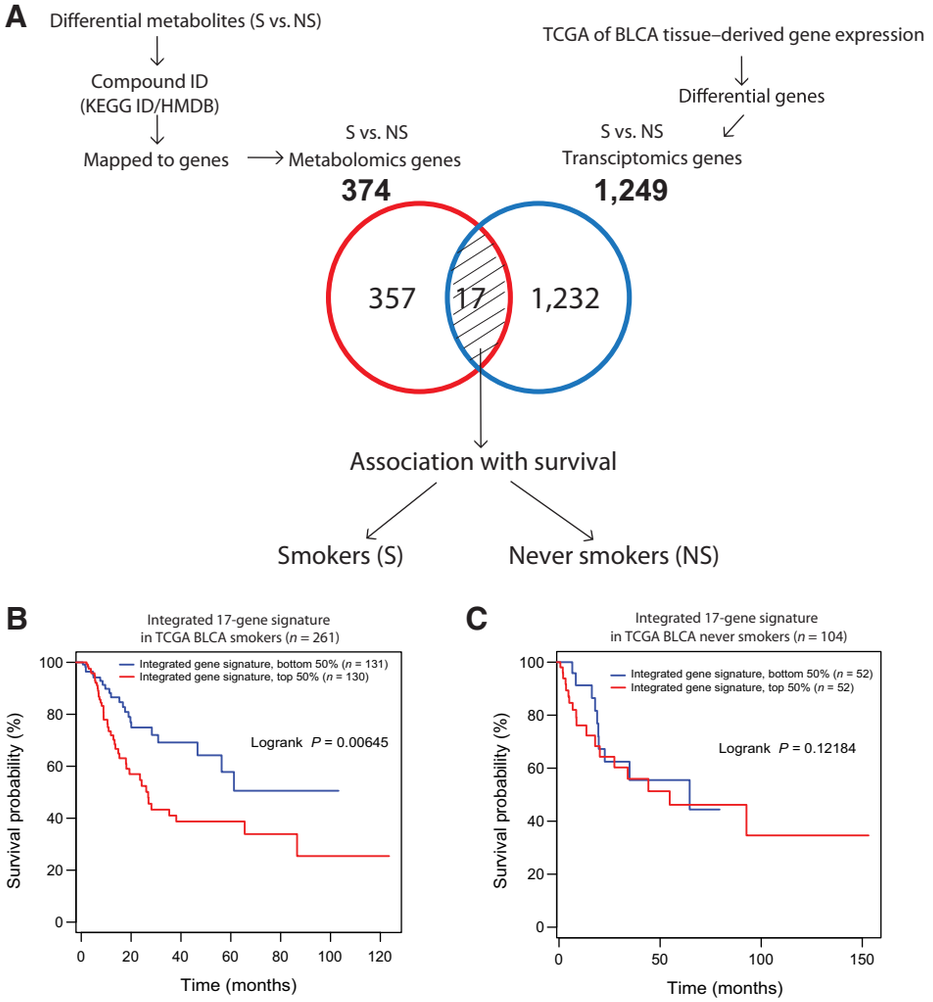

Figure 4.
Serum-based metabolomics and transcriptomics integration strategy. **A,** (i) Corresponding KEGG/HMDB ids of the 40 differential metabolites were mapped to genes. A total of 374 genes were obtained; (ii) these were further intersected with 1,249 genes that were significantly changed between bladder cancer never smokers and smokers in the TCGA cohort, resulting in a focused 17 gene signature. Kaplan–Meier survival plots of the 17 gene signature in bladder cancer cohorts, TCGA bladder cancer smokers (**B**) and TCGA bladder cancer never smokers (**C**). Smoking patients with Bladder cancer with a higher signature score ($n = 130$, Log-rank $P = 0.00645$) in TCGA.

and its derivatives, fatty acids, organic compounds, metabolites involved in TCA/glycolysis/PPP pathway and nucleic acid synthesis.

Forty differential metabolites between bladder cancer smokers and never smokers were identified. Most of these were amino acids suggesting the intricate dynamics of protein metabolism in the pathogenesis of bladder cancer smokers. One interesting observation is the upregulation of glycine, valine, isoleucine, and proline in the serum metabolic profile of bladder cancer smokers. These amino acids with leucine form the major constituents of elastin, a protein required for blood vessel formation (32, 33). Glycine amino acid makes nearly one-quarter of the elastin peptide sequence. This suggests that in bladder cancer smokers might have increased angiogenesis indicative of aggressive tumor formation.

Our data from serum metabolite profiling from bladder cancer smokers demonstrated increased serum asparagine levels. It has been shown that blocking the production of asparagine with a drug L-asparaginase and exposing the mice on a low aspargine diet greatly reduced the tumor metastasis (34). Thus increased asparagine levels may potentially be associated with tumor aggression in bladder cancer smokers. Another observation is an increase in the levels of taurine in bladder cancer smokers. Taurine has been shown to act as a possible urine biomarker for detection of nonmuscle invasive bladder cancer (35), but it was not established in bladder cancer serum smokers (36).

The integration of metabolic signature to the TCGA cohort led to the enrichment of 17 gene signatures that are specific to bladder cancer smokers. We found that high expression of this 17 gene signature is associated with poor survival in only bladder cancer smokers in both TCGA and Riester datasets. The gene signatures of the upregulated (TTL) or downregulated genes (COMT, IYD) showed a significant association with poor survival. COMT is a catalytic enzyme involved in the methylation of various endobiotic and xenobiotic substances (37, 38). Cigarette smoking can lead to exposure of the body organs to a high number of xenobiotic substances, and this may lead to disruption of the methylation process of endogenous substrates due to a lack of labile methyl groups. This phenomenon could be one of the reasons why the levels of COMT decrease in bladder cancer smokers.

Another enzyme that is significantly downregulated in bladder cancer smokers is IYD. The major role of this enzyme is to remove iodide from iodinated tyrosine residues in the thyroid gland (39). The removed iodide is recycled for the synthesis of thyroid hormones (40), which are required for the maintenance of homeostasis by regulating the metabolic rate, protein expression, and body temperature (41). One of the constituents

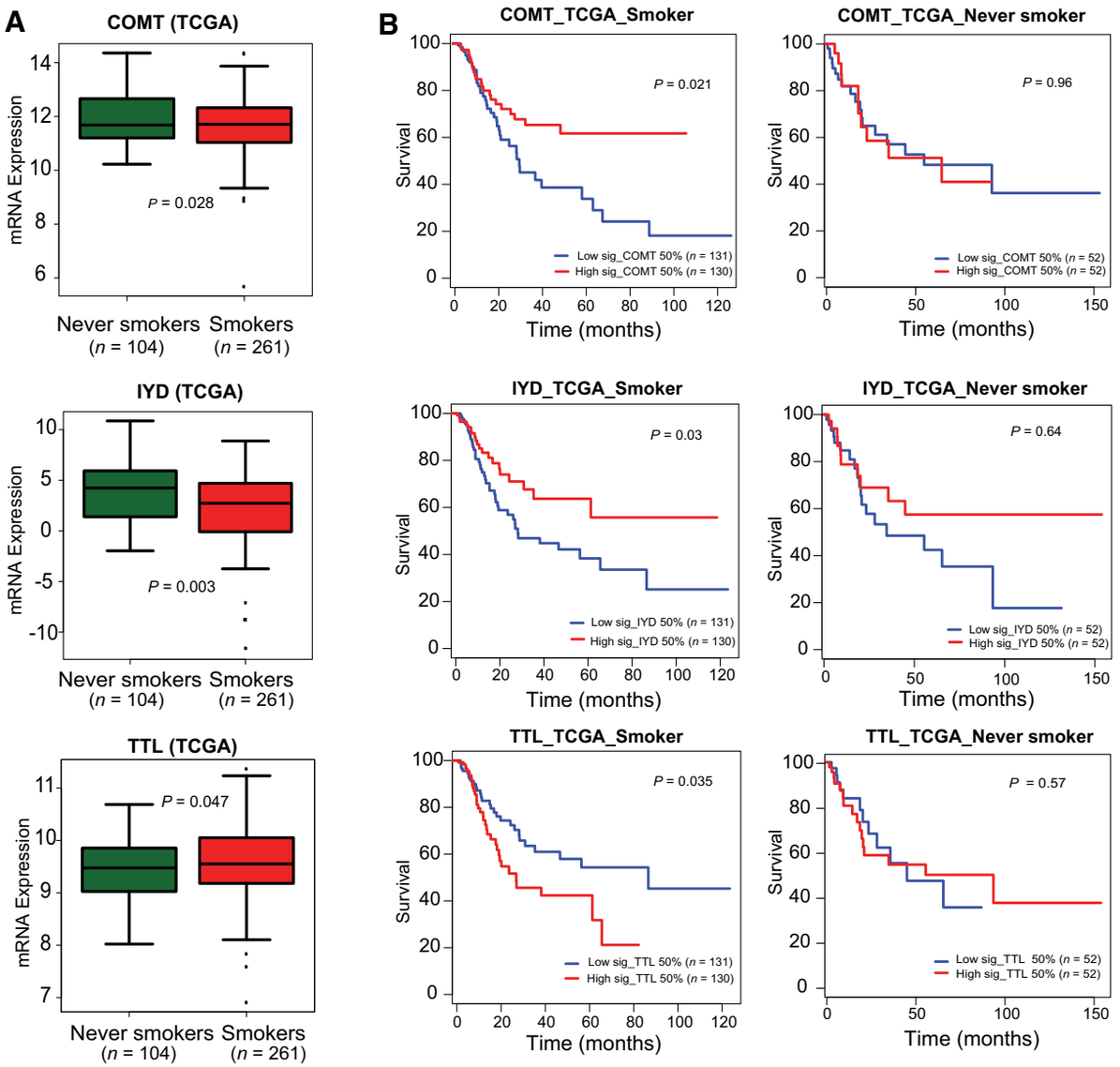

Figure 5.

mRNA alterations and clinical association of COMT, IYD, and TTL in publicly available cohort. **A,** Expression levels of COMT, IYD, and TTL genes in TCGA cohort. **B,** Survival analysis of these 3 genes in TCGA bladder cancer smokers cohort and their corresponding survival analysis in TCGA bladder cancer never smokers cohort.

of cigarette smoke is cyanide, which is converted to the antithyroid agent, thiocyanate. This agent decreases the absorption of iodine into the thyroid and lowers the production of thyroid hormones involved in maintaining body homeostasis (42). It could be inferred that smoking leads to a reduction of absorption of iodine perhaps due to reduced levels of IYD. A case study on one elderly patient suffering from primary bladder cancer reported thyroid metastases (43). The cytosolic enzyme TTL is involved in posttranslational modification of α-tubulin. α-Tubulin in assembled microtubules is detyrosinated at C-terminus. During microtubule disassembly, TTL plays an important role in restoring the tyrosine residues, thus participating in a cycle of tubulin detyrosination and tyrosination (44). It has been previously shown that cigarette smoke increases α-tubulin disassembly (45), which could result in increasing levels of tyrosine residues and hence, increase in TTL expression levels. Consistent with this observation, tyrosine is higher in bladder cancer smokers compared with bladder cancer never smokers. We have examined the expression of the genes TTL, IYD, and COMT independently in bladder cancer tissue samples and found that the expression levels concurs with observations from TCGA cohort. A validation on a large number of bladder cancer tissues and also stage-specific expression analysis of these genes would render significant analytical power for assessing the potential prognostic value of these genes.

In conclusion, we identified critical alterations of serum metabolites that could be a consequence of bladder cancer disease progression in smokers using advanced LC/MS–based

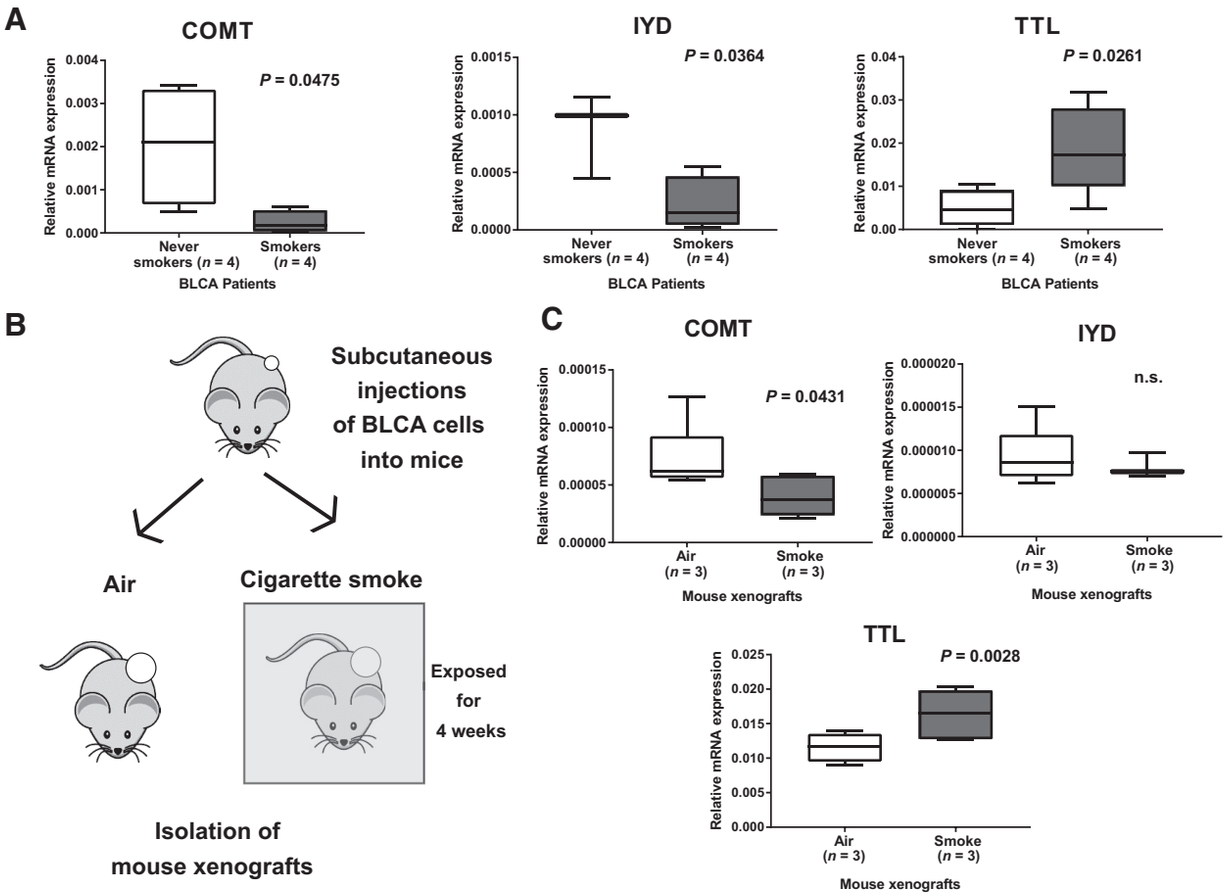

Figure 6.

mRNA expression levels of COMT, IYD, and TTL in patients with bladder cancer and mouse xenografts. **A,** Expression levels of COMT, IYD, and TTL genes in bladder cancer never smokers and bladder cancer smokers. **B,** Schematic diagram representing the mouse xenograft model. UMUC3 cells were subcutaneously injected into NOD-SCID mice. The mice were then exposed to cigarette smoke for a period of 4 weeks. **C,** Expression levels of COMT, IYD, and TTL in mouse xenografts exposed to cigarette smoke compared with air.

metabolomics in combination with bioinformatics. The integrated study of these platforms of bladder cancer smokers over never smokers provided numerous novel insights into disease biology and delineated multiple potential opportunities for therapeutic intervention. Importantly, a set of these gene signatures, especially COMT, IYD, and TTL, in bladder cancer smokers may play an important role in bladder cancer tumor progression, and this could provide potential prognostic value and future targets for therapeutic intervention.

Disclosure of Potential Conflicts of Interest

G. Chatta has received speakers' bureau honoraria from Medimmune and is a consultant/advisory board member for Astra Zeneca. A. Sreekumar reports receiving commercial research funding from Agilent Technologies. No potential conflicts of interest were disclosed by other authors.

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