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Original Article

Urine Metabolomic Analysis Identifies Potential Biomarkers and Pathogenic Pathways in Kidney Cancer

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Abstract

Kidney cancer is the seventh most common cancer in the Western world, its incidence is increasing, and it is frequently metastatic at presentation, at which stage patient survival statistics are grim. In addition, there are no useful biofluid markers for this disease, such that diagnosis is dependent on imaging techniques that are not generally used for screening. In the present study, we use metabolomics techniques to identify metabolites in kidney cancer patients' urine, which appear at different levels (when normalized to account for urine volume and concentration) from the same metabolites in nonkidney cancer patients. We found that quinolinate, 4-hydroxybenzoate, and gentisate are differentially expressed at a false discovery rate of 0.26, and these metabolites are involved in common pathways of specific amino acid and energetic metabolism, consistent with high tumor protein breakdown and utilization, and the Warburg effect. When added to four different (three kidney cancer-derived and one "normal") cell lines, several of the significantly altered metabolites, quinolinate, α-ketoglutarate, and gentisate, showed increased or unchanged cell proliferation that was cell line-dependent. Further evaluation of the global metabolomics analysis, as well as confirmation of the specific potential biomarkers using a larger sample size, will lead to new avenues of kidney cancer diagnosis and therapy.

Introduction

Although only the seventh most common cancer, kidney cancer (or renal cell carcinoma, RCC) is one of the few cancers that is steadily increasing in incidence due in part to the prevalence of adverse lifestyle changes resulting in metabolic syndrome, hypertension, and exposure to toxins such as cigarette smoke (Weiss and Lin, 2006). To make matters worse, RCC is frequently asymptomatic at presentation, such that it is often diagnosed incidentally during the workup of patients for unrelated kidney diseases. In a third of these cases, RCC is already metastatic at diagnosis, and at this stage prognosis is exceptionally grim. For these reasons, methods to diagnose the disease in its earliest incarnation are urgently needed.

The technique of metabolomics, in which the entire suite of metabolites produced by the body is examined, is ideally suited to the study of cancer of the urinary tract due to the severe and prevalent metabolic derangements that occur in this disease, as well as the fact that there exists communication between these epithelial tumors and the urinary space. However, when examining urine metabolomics, as opposed to serum or tissue metabolomics, it must be kept in mind that what appears in the urine is a complicated function of production, utilization, and kidney filtration, and thus necessarily an incomplete set.

For disorders of the urinary tract, urine evaluation by metabolomic techniques has considerable potential to result in the identification of useful biomarkers for kidney cancer, despite the incompleteness of the set of metabolites that appear in this biofluid. Despite the complexity of this (or any) cancer, it is possible that a panel of biomarkers will be sufficient to stratify patients into disease or nondiseased categories; thus, multiplexing in the clinic using a suite of biomarkers is more likely to yield diagnostic success given the high specificity that will be required for a clinically useful diagnostic assay. These data may also point to pathways that are disrupted in order to help elucidate pathophysiology of disease and to find novel drug targets. In previous studies we have shown that metabolomic analysis yields a set of previously unidentified urinary metabolites that have the ability to separate patients with kidney cancer from healthy individuals (Kim et al., 2009; Kind et al., 2007), but true chemical identification of metabolites and metabolic pathways affected by the disease was not

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accomplished in those studies due to relatively low numbers of patients and heterogeneity of collection methods.

It is now abundantly clear from the work from our and others' laboratories that locale of collection, as well as handling of samples after collection, can confound these metabolomic data and lead to inaccurate results (Kim et al., 2009; Saude et al., 2007; Saude and Sykes, 2007). For these reasons, we have in the current study examined a group of preoperative kidney cancer patients and matched controls collected and handled uniformly. We have used this data to identify metabolites that appear in the urine at different levels in RCC and non-RCC patients, and have begun to validate these metabolites as possibly oncogenic. In addition, we have utilized these metabolites to attempt to identify pathways which are likely important in kidney cancer pathogenesis, based solely on metabolites appearing in the urine. Ongoing work in our laboratory is directed at further study of the metabolites and pathways that are altered in RCC in order to yield clues about pathogenesis and serve as a starting point for novel drug development for this devastating disease.

Materials and Methods

Enrollment of patients

After approval by the appropriate institutional review boards, patients were consented for the study at their preoperative visit to the UC Davis or Sacramento VA urology clinics. Urine samples were obtained in a uniform fashion by one of three clinical coordinators in this project, as clean catch, midvoid specimens, and were aliquotted and frozen on dry ice or at -80°C within 30 min of collection. Control patients were matched as closely as possible for age, race, and gender, and were recruited from the same urology clinics among the pool of patients being evaluated for nonkidney cancer urological conditions. All urines were kept at -80°C until analyzed.

Materials

Three proximal tubule epithelial cancer cell lines, ACHN, A498, and 786-O, and one "normal"-derived kidney epithelial cell line, HK-2, were all obtained from the American Type Culture Collection (Rockville, MD). ACHN, A498, and HK-2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), and 100 units/mL streptomycin and 100 μg/mL penicillin. 786-O cells were maintained in RPMI supplemented with 10% FBS, and 100 units/mL streptomycin and 100 μg/mL penicillin. Cells were maintained at 5% CO_2 at 37°C. Quinolinic acid, gentisic acid, α-ketoglutarate, sulforhodamine B dye, doxorubicin, and trichloroacetic acid were all purchased from Sigma (St. Louis, MO, USA). Quinolinate was prepared by direct dissolution into culture media, gentisate was dissolved into 100% ethanol, and α-ketoglutarate was dissolved in water.

Cell proliferation assay

The sulforhodamine B (SRB) assay was performed to represent a measurement of the cell population for each cell line at the time of metabolite addition. The protocol is utilized by the NCI (http://dtp.nci.nih.gov/branches/btb/ivclsp.html) and was modified slightly per reference (Zheng et al., 2009). Briefly, 10^5 cells/mL were plated in a 96-well microtiter plate in media containing 1% FBS. The cells were incubated at 37°C overnight in 1% FBS-containing media. Twenty hours later, quinolinic acid (0.001, 0.01, 0.1, and 1 μM), gentisic acid (0.001, 0.01, 0.1, and 1 μM), or α-ketoglutarate (0.1, 1, and 10 μM) were added to four replicates for each dose in 10% FBS containing media added for 24 h. One plate containing cells in 1% FBS was fixed with 20% (w/v) cold trichloroacetic acid at 4°C for 90 min and stained with 0.4% (w/v) SRB in 1% acetic acid for 30 min. All wells were then washed twice with 1% acetic acid. The dye was solubilized with 10 mM Tris base and the absorbance read at 490 nm. The OD for this plate was used to estimate the number of cells at time zero (Tz). The above protocol was repeated for cells treated with the three different metabolites after incubation for 24 h. Percent proliferation was calculated using the following formula: percent proliferation = [(OD of dose after 24 h – Tz)/(Control – Tz)] * 100. For cells with inhibited proliferation, the following equation was used: percent Inhibition = [(OD of dose after 24 h – Tz)/(Tz)] * 100. Significance was determined by one-way analysis of variance (ANOVA) at a value of $p < 0.05$; the analysis was conducted using SAS 9.0.

Metabolomic profiling

The metabolomic platforms, including sample extraction process, instrumentation configurations and conditions, and software approaches for data handling, were previously described in detail (Evans et al., 2009). The major components of the process are summarized as follows:

Sample extraction. The samples were extracted using an automated MicroLab STAR® system (Hamilton Company, Salt Lake City, UT, USA) in 400 μL of methanol, containing the recovery standards. The samples were then separated into three equal aliquots for analysis in three independent platforms as described below.

Instrumentation platforms. The platform consisted of three platforms: ultrahigh performance liquid chromatography/tandem mass spectrometry (UHLC/MS/MS^2) optimized for basic species, UHLC/MS/MS^2 optimized for acidic species, and gas chromatography/mass spectrometry (GC/MS). The samples destined for GC/MS analysis were dried under vacuum desiccation for a minimum of 24 and then derivatized under dried nitrogen using bistrimethylsilyl-triflouroacetamide (BSTFA). The GC column was 5% phenyl and the temperature ramp was from 40 to 300°C in a 16-min period. Samples were analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization. UPLC/MS was carried out using a Waters Acquity UHPLC (Waters Corporation, Milford, MA, USA) coupled to an LTQ mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA. USA) equipped with an electrospray ionization source. Two separate UHPLC/MS injections were performed on each sample: one optimized for positive ions and one for negative ions. Chromatographic separation followed by full scan mass spectra was carried out to record retention time, molecular weight (m/z) and MS/MS^2 of all detectable ions presented in the samples.

Metabolite identification. Metabolites were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standard entries

that included retention time, molecular weight (*m/z*), preferred adducts, and in-source fragments as well as their associated MS/MS^2 spectra. This library allowed the rapid identification of metabolites in the experimental with high confidence. For ions that were not covered by the standards, additional library entries were added based on their unique ion signatures (chromatographic and mass spectral) and also by virtue of their recurrent nature among samples. The unknown biochemicals have the potential to be identified by future acquisition of matching purified standards or by classical structural analysis.

Statistical methods

Processing of the raw data yielded 298 known metabolites from 29 kidney cancer patients and 33 control patients. For samples with missing values for a metabolite, the minimum observed value of the metabolite was substituted for the missing values. Metabolites observed in fewer than 50% of the samples were excluded yielding 274 known metabolites for analysis. Prior to statistical analyses, metabolite values were first adjusted for osmolality by dividing by each sample's osmolality value for normalization. Metabolite intensities were log (base 2) transformed to meet underlying assumptions of normality with a constant variance and to reduce the dominant effect of extreme values. Then the standard separator normalization was applied to intensity measurements to have a mean of 0 and scaled to a variance of 1 for use in the following statistical analyses.

The primary objective of the statistical analysis was to identity metabolites whose concentrations differentiate between patients with and without kidney cancer that eventually could be used as diagnostic biomarkers for kidney cancer and to elucidate alterations of metabolite signals in pathways associated with the presence of kidney cancer. To identify metabolites as potential diagnostic biomarkers for patients with kidney cancer, we aimed to (1) identify metabolites that distinguish patients with and without kidney cancer (called "cancer" and "control" hereafter) using differential analysis, and (2) identify sets of relevant metabolites that act synergistically within functionally defined pathways, using functional score analysis. We accounted for potential confounding effects of differences in age, gender, and race between cancer and control groups.

Specifically, to identify metabolites whose expression was associated with the presence/absence of kidney cancer, we performed differential analysis based on a general linear model including the indicator of the presence of kidney cancer (the primary predictor of interest) and the other covariates (age, gender, race) to control for confounding effects on the association between metabolite expression and the presence/absence of kidney cancer. Multiple tests were controlled by the false discovery rate (FDR), the expected proportion of false positives among the tests declared significant (false plus true) (Benjamini and Hochberg, 1995). To identify sets of relevant metabolites that act synergistically within functionally defined pathways, we performed functional score analysis, based on all the metabolites (Pavlidis et al., 2004). To score metabolites by their tendency to show changes in expression with cancer status, we first modeled metabolite intensities as a function of age, gender, and race, and then used the residuals to test for a mean difference in the intensity of each metabolite between cancer and control patients with a *t*-test. We calculated each pathway's functional score in two ways: (1) as the median of the *t*-statistics of metabolites in the pathway, and (2) as the median of the squared *t*-statistics of the metabolites in the pathway. Using squared *t*-statistics favored identifying pathways with metabolites that differed according to cancer status irrespective of the direction of the difference, while the functional score analysis based on unsquared values accounted for the direction of the regulation of each metabolite associated with cancer status in a given pathway. All metabolites associated with a pathway were included regardless of whether the metabolite was found to differ significantly at a predefined threshold in the *t*-test and thus allows each metabolite to contribute its own merit of relevance with cancer status to a functional score. We used a permutation null distribution to identify pathways that were significantly altered by cancer status. To generate the null distribution, we permuted class labels (cancer status) 10,000 times and recalculated the functional scores for each pathway. This process yielded a null distribution of the functional scores for each pathway from which we calculated permutation *p*-values. Pathways with *p*-values less than 0.05 and 0.1 were considered to be significant and suggestive, respectively.

Results

Validation of technical reproducibility of metabolomic data

To assess technical reproducibility of urine metabolomic data, four urine samples were randomly selected and analyzed at two different times separated by several months. A total of 298 compounds were detected in both analyses, whereas 11 compounds were detected only in the first analysis and 28 were detected only in the second analysis. For all four analyzed samples, metabolite values were highly correlated between the technical replicates (Fig. 1), with correlation coefficients between intensity (concentration) measurements for the four samples ranging from 0.92 to 0.95. Thus, the analytical techniques and equipment used to generate the metabolomic data in this study yielded consistent results when operated at different and distinct times.

Identification of individual metabolites differentially associated with cancer status

In order to identify metabolites whose concentration level in the urine differs between kidney cancer and control patient groups, we compared the normalized intensity levels for individual metabolites between cancer and control patients. Among the 274 identified metabolites, 13 showed significant differences between the two groups (Table 1) and 10 of these were excreted in the urine at a lower quantity in cancer patients when compared to control patients. However, after adjusting for FDR, three of these metabolites (quinolinate, 4-hydroxybenzoate, and gentisate) were significantly differentially expressed between the two groups at an FDR of 0.26 (top 3 metabolites in Table 1), quinolinate being increased in cancer patients' urine compared to urine of control patients, and 4-hydroxybenzoate and gentisate being decreased. Results for all metabolites are provided in the Supplementary Table 1.

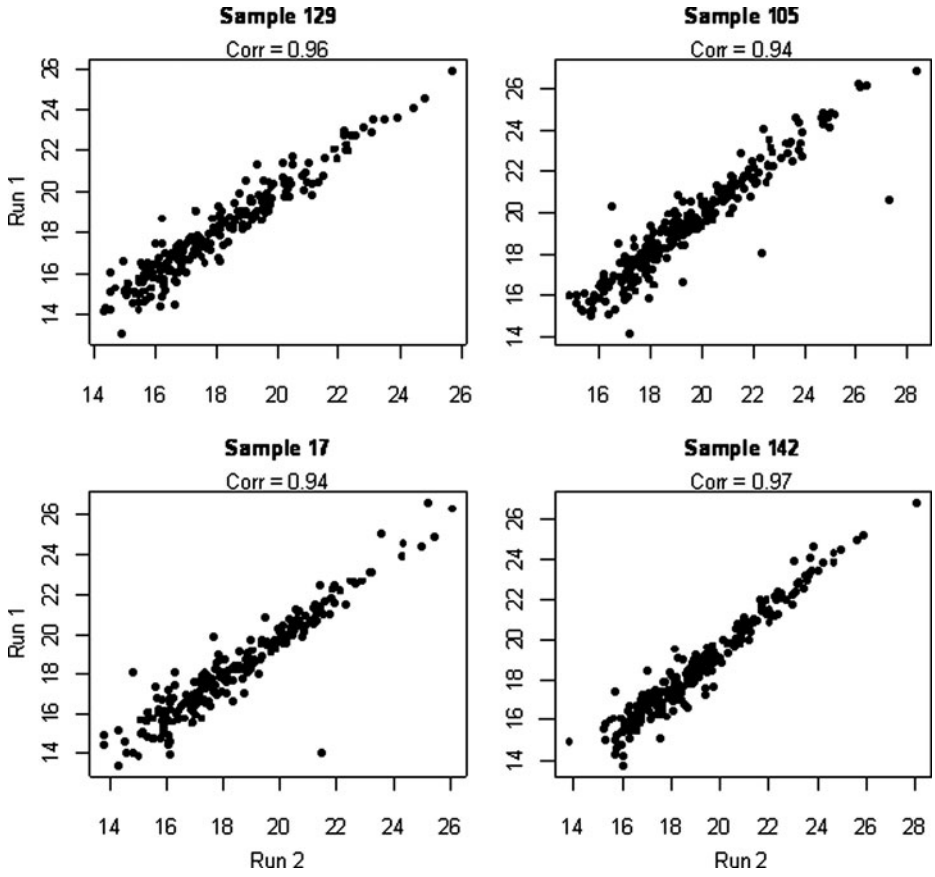


FIG. 1. Validation of technical reproducibility of analytical chemistry. Four randomly selected urine samples were analyzed as described in Materials and Methods on 2 separate days separated by several months. Metabolite values were log (base 2) transformed and normalized to have a mean of 0 and variance 1.

Table 1. Results of Differential Analysis

Top 13 most significant metabolite in order	*Diff. Analysis (cancer vs. control)* p-*value*	*Mean levels*[a]		*Direction*
		Cancer	*Control*	
1. quinolinate	0.0026[b]	0.315	−0.571	up
2. *4-hydroxybenzoate*	0.0034[b]	−0.378	0.239	down
3. gentisate	0.0039[b]	−0.605	0.227	down
4. *galactitol (dulcitol)*	0.0085	−0.401	0.234	down
5. N-(2-furoyl)glycine	0.0087	−0.306	0.269	down
6. alpha-ketoglutarate	0.0100	−0.205	−0.960	up
7. *fructose*	0.0140	−0.257	0.226	down
8. *thymol sulfate*	0.0176	−0.578	−0.012	down
9. tryptophan betaine	0.0179	−0.481	0.070	down
10. *hexanoylglycine*	0.0332	0.134	−0.529	up
11. 1,6-anhydroglucose	0.0376	−0.239	0.210	down
12. 4guanidinobutanoate	0.0390	−0.197	0.102	down
13. 3-hydroxyphenylacetate	0.0488	−0.729	−0.250	down

Metabolites whose expression differed significantly (at $p < 0.05$) between cancer and control patients are shown. Direction indicates whether cancer patients have lower (down) or higher (up) levels of the metabolite compared to control patients. Metabolites in *italic* indicate those involved in significant or suggestive pathways.

[a]Expression levels were normalized and log transformed as described in the text.

[b]*p*-value as significant at FDR of 0.26.

Identification of metabolic pathways from urine metabolites which differ by cancer status

The metabolites in this study were limited to those that appear in the urine, so this set of compounds is obviously incomplete and a subset of those that would likely be found in serum or tissue. Furthermore, increased amounts of specific metabolites in the urine in people with normal kidney function may represent increased production (if urinary levels are elevated compared to controls), increased utilization (if urinary levels are decreased), or altered glomerular filtration. Nevertheless, it is likely that metabolites that are massively overproduced by the renal tumor and fit the criteria of normal glomerular filtration (all patients in this study had serum creatinine values in the normal range) will enter the urinary space and find their way into the final urine. For this reason, examination of these metabolites in aggregate can result in metabolic pathway information, albeit incomplete, which can in turn be useful in generating hypotheses for RCC disease mechanisms and possible therapeutic targets.

The significant metabolites and their directions of change were mapped into general biochemical pathways according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/), and this assignment was used for pathway analysis. Those metabolites that differed significantly in expression between cancer and control patients were identified with a functional score analysis using all the 274 known metabolites associated with 57 KEGG or Human Metabolome Database (HMDB, www.hmdb.ca) pathways. Of the 57 assigned pathways, 2 were significant at a permutation value of $p < 0.05$, although three more pathways were suggestive of significance (red pathways in Table 2). Two (thymol sulfate and hexanoylglycine) of the 13 most significant metabolites belonged to pathways found to

Table 2. Results of Functional Score Analysis

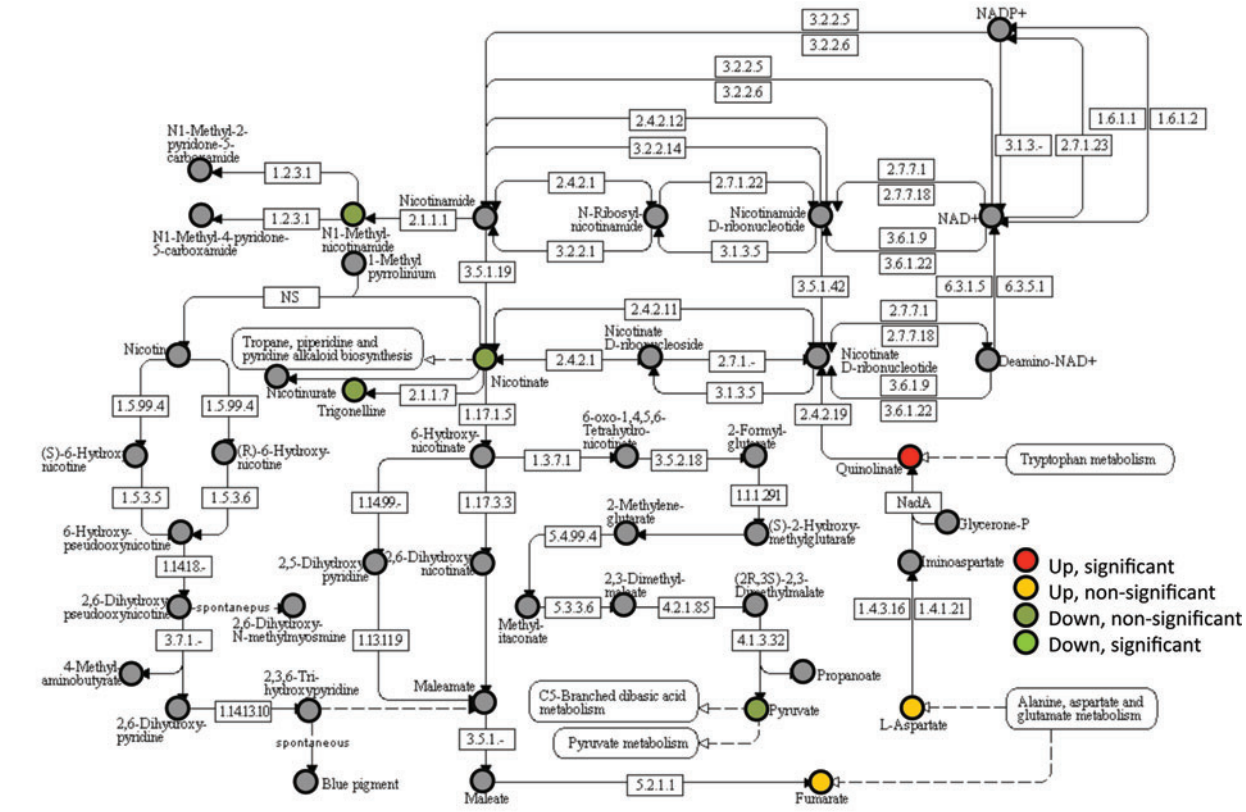
Top 13 most significant in order	*Pathway*	*KEGG ID*	*HMDB ID*	# *Met*	# *Up*	# *Down*	% *Up*	p-*Value*[a] *partial* t-*statistics*	p-*Value*[a] *squared partial* t-*statistics*
1. quinolinate	Nicotinate and nicotinamide metabolism	C03722	HMDB00232	4	1	3	25%	0.363	0.403
2. 4-hydroxybenzoate	Benzoate metabolism	C00156	HMDB00500	10	0	10	0%	0.053[b]	0.110
3. gentisate	Phenylalanine and tyrosine metabolism	C00628	HMDB00152	19	2	17	11%	0.225	0.582
4. galacticol (dulcitol)	Fructose, mannose, galactose, starch and sucrose metabolism	C01697	HMDB00107	8	2	6	25%	0.092[b]	0.094[b]
5. N-(2-furoyl)glycine	Glycine, serine and threonine metabolism	NA	HMDB00439	9	1	8	11%	0.223	0.285
6. α-ketoglutarate	Krebs cycle	C00026	HMDB00208	10	7	3	70%	0.437	0.535
7. fructose	Fructose, mannose, galactose, starch and sucrose metabolism	C00095	HMDB00660	8	2	6	25%	0.092[b]	0.094[b]
8. thymol sulfate	Food component/ Plant	C09908	NA	6	1	5	17%	0.007[c]	0.027[c]
9. tryptophan betaine	Tryptophan metabolism	C00078	HMDB00929	11	3	8	27%	0.508	0.654
10. hexanoylglycine	Fatty acid, beta-oxidation metabolism	NA	HMDB00701	1	1	0	100%	0.032[c]	0.032[c]
11. 1,6-anhydroglucose	Glycolysis, gluconeogenesis, pyruvate metabolism	NA	HMDB00640	6	2	4	33%	0.473	0.785
12. 4-guanidinobutanoate	Guanidino and acetamido metabolism	C01035	HMDB03464	3	2	1	67%	0.498	0.654
13. 3-hydroxyphenylacetate	Phenylalanine and tyrosine metabolism	C05593	HMDB0040	19	2	17	11%	0.225	0.582

Pathways associated with the 13 most significant metabolites (Table 1) are shown. #Met is the number of metabolites identified through GC/MS or LC/MS in each pathway. % Up is the percentage of metabolites upregulated in each pathway. *p*-Value partial *t*-statistic is the *p*-value obtained by using unsquared *t*-statistics that accounted for the direction of regulation, and *p*-value squared partial *t*-statistics is the *p*-value irrespective of the direction of regulation in each pathway.

[a]*p*-Values were calculated from a permutation null distribution based on 10,000 permutations.

[b]*p*-value significant at 0.1; [c]*p*-value significant at 0.05.

a NICOTINATE AND NICOTINAMIDE METABOLISM



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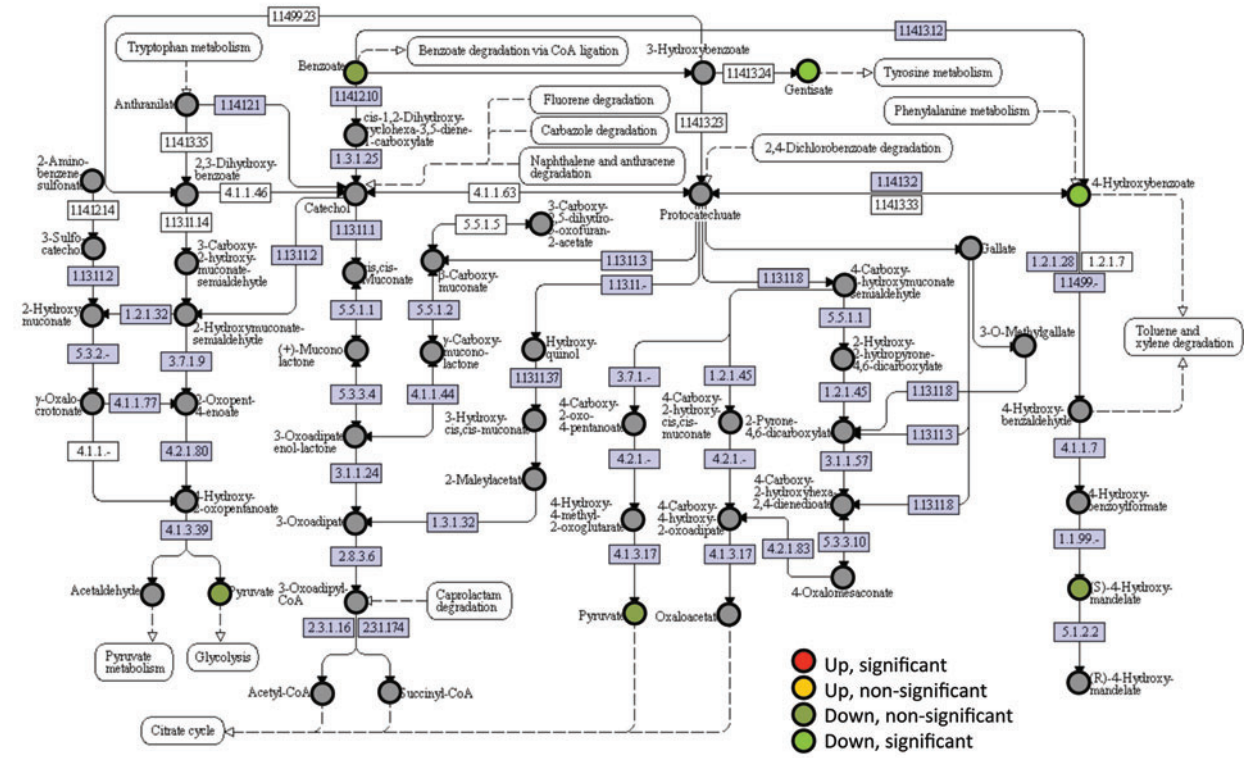
b BENZOATE DEGRADATION VIA HYDROXYLATION



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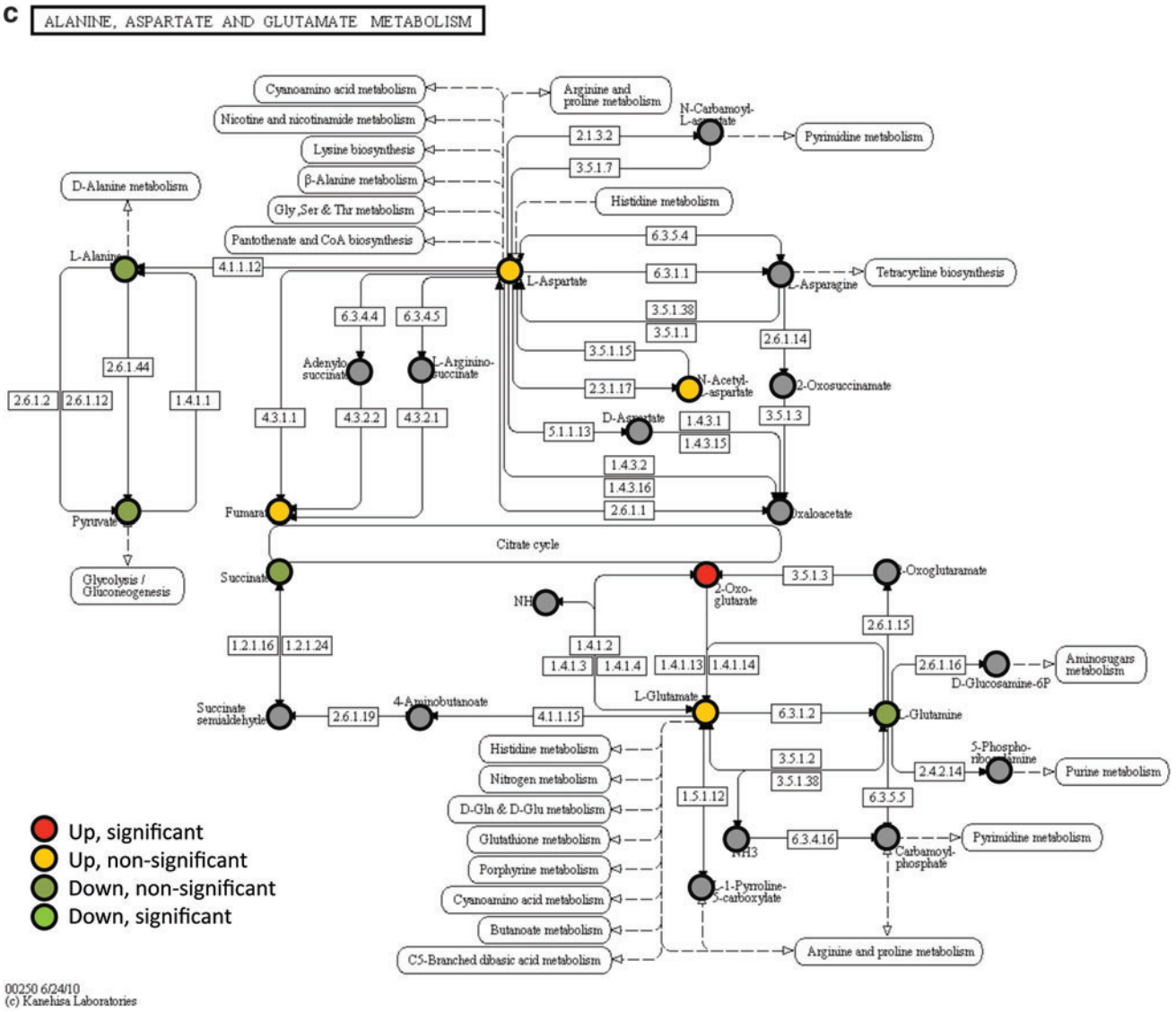

FIG. 2. (Continued).

be significant by functional score analysis (Table 2). The significant pathways were xenobiotic or dietary component pathway and fatty acid β-oxidation metabolism.

Specifically, the xenobiotic or dietary component pathway containing the significant metabolite thymol sulfate was identified as the most significantly altered pathway in terms of individual metabolites' regulation between cancer and control patients at a value of $p < 0.05$. The reason this pathway received a significant functional score p-value can be understood by observing that five out of the six metabolites in the pathway were expressed in the same direction and downregulated in cancer patients compared to controls. Thus, it is likely that metabolism of xenobiotics or dietary components does not simply reflect dietary intake but is also reflective of patient and/or tumor physiology.

The additional pathway found to be significant among cancer and control patients (fatty acid, β-oxidation metabolism) contained the metabolite hexanoylglycine. Hexanoylglycine is likely derived from hexanoyl-CoA, which is an intermediate in fatty acid β-oxidation. However, it should be pointed out that the significant result of this pathway reflects only a single metabolite in our dataset, so it is less likely to provide any useful information about the involvement of the fatty acid β-oxidation metabolism pathway as a

FIG. 2. KEGG pathway diagrams containing significantly altered metabolites. Significant urinary metabolites are shown on KEGG pathway diagrams with direction of change indicated. For completeness, identified metabolites with nonsignificant changes are also shown. Gray circles represent unobserved metabolites in this study, but in the KEGG pathway libraries. (**a**) Nicotinate and nicotinamide metabolism. (**b**) Benzoate degradation via hydroxylation. (**c**) Alanine, aspartate, and glutamate metabolism.

whole in kidney cancer. Although not significant at $p < 0.05$, a few pathways are "suggestive" of significance, including benzoate metabolism and fructose, mannose, galactose, starch, and sucrose metabolism pathways. Interestingly, benzoate metabolism became close to significant (p-value = 0.0532) when direction of regulation in expression was taken into account. For this pathway, all the metabolites were excreted at a lower quantity in cancer patients' urine than control patients.

Experimental validation of individual metabolites

To ascertain whether those significant urinary metabolites that ultimately may turn out to be biomarkers for RCC have biological significance, we examined three metabolites that lie in nodes of KEGG pathways: quinolinate (nicotinate and nicotinamide metabolism pathway; Fig. 2a), gentisate (benzoate degradation pathway, Fig. 2b), and α-ketoglutarate (also known as 2-oxoglutarate on KEGG diagram; alanine, aspartate, and glutamate metabolism pathway; Fig. 2c).

When added at physiologic (Grootveld and Halliwell, 1986; Wagner et al., 2010; Yoshimura et al., 2009) or supraphysiologic doses to four distinct RCC cell lines, all three of these metabolites resulted in increased or unchanged cell proliferation (Fig. 3a–c). Interestingly, after the addition of quinolinate (Fig. 3a) and α-ketoglutarate (Fig. 3b), both of which were increased in the urine of cancer patients, there was substantially more growth stimulation, which in several cell lines reached statistical significance, than when gentisate, which is decreased in the urine of cancer patients, was added (Fig. 3c). None of the cell lines showed statistically increased cell growth upon addition of gentisate. As a control for sensitivity of the TCA assay for cell growth inhibition (given that this phenomenon did not occur with the metabolites examined), the DNA chemotherapeutic agent doxorubicin was added separately and showed the expected anti-proliferative effect (Fig. 3d).

Discussion

In this study, we examined the urinary metabolites that appear differentially in kidney cancer versus nonkidney

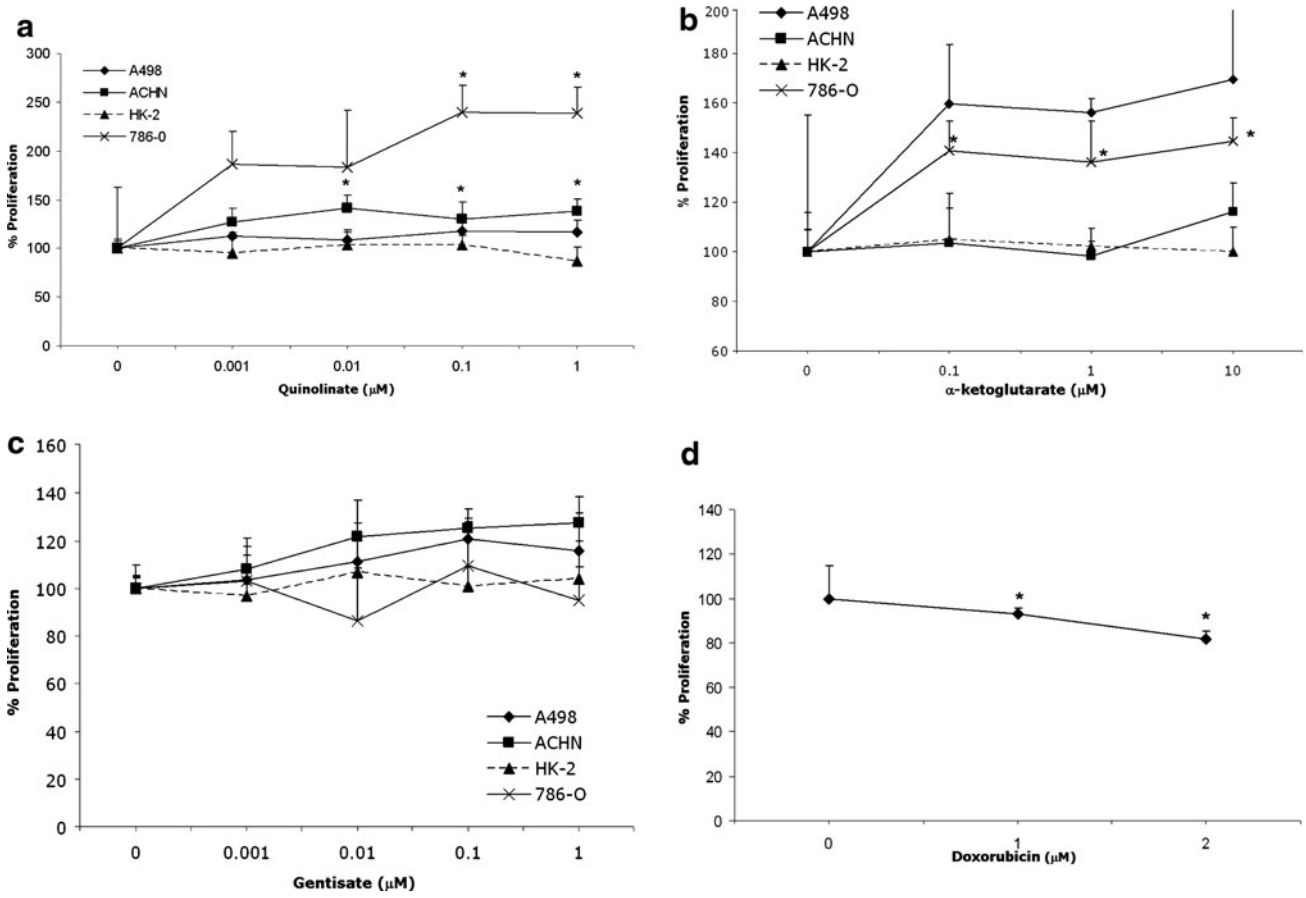

FIG. 3. Changes involving increases in several significant urinary metabolites associated with kidney cancer and tubular epithelial-derived cell proliferation. Four different cell lines, three RCC-derived (ACHN, 786-O, and A498), and one normal renal tubular epithelial derived (HK-2), were grown to confluence and serum starved for 20 h. Subsequently, (**a**) quinolinate, (**b**) α-ketoglutarate, and (**c**) gentisate were applied to the cells in 10%-serum containing media at the concentrations indicated. After 24 h, cell proliferation was assessed as described in Materials and Methods. (**d**) Doxorubicin was added to A498 cells, and proliferation was assessed above as a control for proliferation inhibition. *$p < 0.05$ compared to cells with no addition of metabolite or doxorubicin (0 μM). Shown is a representative experiment out of a total of at least three repeats except for the doxorubicin experiment, which was performed twice.

cancer patients. We have previously shown that RCC patients are separable from non-RCC patients using metabolomic analyses, but in the previous studies we did not identify specific metabolites as possible biomarker candidates. Due to our (Kim et al., 2009) and others' (Saude et al., 2007; Saude and Sykes, 2007) findings that metabolomics analyses can be dependent on conditions of collection, the current analysis was undertaken in a group of patients from two hospitals who were enrolled and whose urine samples were taken by the same clinical coordinators, such that consistency of collection, storage, and processing were tightly controlled. We were able to identify several metabolites that were significantly different between these groups, and validation efforts demonstrated that these metabolites have important cell proliferative properties *in vitro*. However, because our primary goal was to discover potential disease biomarkers in the urine, and because we have examined only compounds that are filtered by the kidney, it is important to realize that metabolic pathway analysis is necessarily incomplete.

Although all of the significant biomarkers that were most significantly different between cancer and noncancer groups have diagnostic potential, only those that have potential biological relevance would be of practical use. Quinolinate is of special interest, as it was the most significantly altered metabolite, being present in higher concentrations in cancer patients' urine in our study. Quinolinate lies upstream of NAD+ (see Fig. 2a), which is key cofactor for glycolysis. Thus, because gluconeogenesis and glycolysis are reciprocally regulated, quinolinate, being increased in cancer patients' urine, is likely being overproduced as an intermediary that promotes glycolysis, a manifestation of the Warburg effect (Kim and Dang, 2006; Warburg, 1956). This increased signature of glycolysis seen in the urine is also consistent with proteomic analysis of RCC tissues undertaken in our laboratory (Perroud et al., 2009; Unwin et al., 2003). In addition, quinolinate is a catabolite of tryptophan through the kynurenine branch, which eventually leads to the production of nicotinamide. Quinolinate has been known to associate with various disease conditions, such as infection and neurodegenerative disease (reviewed in Schwarcz, 2004). Interestingly, one recent study further suggested a link between quinolinate and kidney disease (Yoshimura et al., 2009), with similar findings of increased proliferation when kidney mesangial cell were exposed to this compound *in vitro*, similar to what was observed in RCC cell lines in the present study. The mechanism of its deleterious (and potentially oncogenic) effect is not fully understood, although it has been proposed that quinolinate can induce oxidative stress through iron-catalyzed Fenton reactions (Stone et al., 2007). In addition, quinolinate was described many years ago as an inhibitor of gluconeogenesis and promoter of glycolysis in multiple tissues including the kidney (Klahr and Schoolwerth, 1972). Both oxidative stress (Stone et al., 2007) and accelerated glycolysis have been well established to be hallmarks of cancer progression via the Warburg effect, as discussed earlier. Another plausible explanation for the elevated quinolinate level is that it is an indication of the rate of *de novo* nicotinamide biosynthesis. Whatever the cause, quinolinate could be a viable biomarker that may be mechanistically associated with kidney cancer; further validation of this concept is currently underway in our laboratory.

Quinolinate lies at the intersection of several pathways, including the KEGG pathway "nicotinate and nicotinamide metabolism" (Fig. 2a). Quinolinate is a key molecule linking in the pathways "Tryptophan metabolism" as well as "Alanine, aspartate, and glutamate metabolism" through L-aspartate. Quinolinate is itself then converted into nicotinate D-ribonucleotide, a precursor to NAD+. Although not reaching significance, two upstream metabolites of quinolinate, L-aspartate and fumarate, are also increased in cancer patients' urine, suggesting augmented production of NAD+ and thus increased transit through the citric acid cycle. It is interesting that the above amino acids were not significantly differentially expressed between cases and controls (e.g., not up- or downregulated), while the key molecule quinolinate did reach statistical significance. This could be an indication that nicotinate-nucleotide pyrophosphorylase (carboxlating), the enzyme that acts on quinolinate, is itself downregulated to reign in production of NAD+.

Another key significantly altered metabolite, α-ketoglutarate (also known as 2-oxoglutarate), is also within the "alanine, aspartate, and glutamate metabolism" pathway (Fig. 2c). This metabolite is the entry point to the citric acid cycle, which is increased in glycolysis, again consistent with the Warburg effect if in fact it is a result of increased production by the tumor. Both citric acid cycle metabolites α-ketoglutarate and fumarate are increased in the urine (though the latter not significantly), consistent with an increased glycolysis signature in the urine of cancer patients. Other metabolites in the citric acid cycle were observed (isocitrate, citrate, and *cis*-aconitate), but they did not appear at differential concentrations in the urine examined in this study.

Two of the significantly altered metabolites, 4-hydroxybenzoate and gentisate, which are differentially expressed in the urine at an FDR of 0.26, are linked to each other in the pathway "Benzoate degradation via hydroxylation" (Fig. 2b). Only two metabolites lie between the two: protocatechuate and 3-hyroxybenzoate. Neither of these two intermediates is observed in our samples likely due either to their being below the detection limit or lack of excretion into the urine (or possibly their lack in the GC/LC compound library and hence an "unknown" metabolite). Other metabolites in this pathway, benzoate, pyruvate, and 4-hydroxymandelate, were decreased in the urine in our analysis but did not reach statistical significance. These metabolites are upstream of the citric acid cycle and their decrease could be a means of shunting benzoate degradation into acetyl-CoA, and hence, the citric acid cycle, again consistent with the Warburg effect. Gentisate has also been shown to have electron-scavenging properties and may prevent oxidation of lipoproteins (Ashidate et al., 2005). Because cancer cells are highly metabolic, increasing gentisate levels within the cell may prevent oxidation of both cellular and organelle membranes. In this study, we showed gentisate levels in urine from our cancer patients is lower than the noncancer patients, suggesting that gentisate is rapidly metabolized resulting in a lower excreted value.

Biological validation was initially undertaken by examining the effect of several of the highly significant identified urinary metabolites on both cancer and "normal" renal epithelial cell lines in culture. We found that quinolinate and α-ketoglutarate were increased in cancer urines; because

these metabolites play key roles in glycolysis, which would be expected to increase in a cancer cell, it is logical that addition of these compounds would increase proliferation by facilitating glycolysis in at least some cancer as well as "normal" cell lines (Fig. 3a and b). By contrast, the minimal and nonsignificant increase of growth of some RCC cell lines upon addition of gentisate, a metabolite that we found to be decreased in cancer urine, suggests that this metabolite may emanate from the tumor and have growth suppressive or growth-neutral properties. It is important to keep in mind, as mentioned previously, that the appearance of metabolites in the urine is not simply a representation of increased production of the compound, but rather represents a complicated equation of production, utilization, and glomerular filtration. For this reason, further investigations are being undertaken with all of these metabolites to determine their signaling mechanisms *in vitro*.

In light of the controversy surrounding a recent study promoting sarcosine as a potential urinary biomarker for prostate cancer (Jentzmik et al. 2010; Sreekumar et al. 2009; Struys et al., 2010), it is prudent to not overstate the case for our finding of potential biomarkers for RCC. Rather, our study is suggestive of urinary signatures of the tumor (e.g., increased glycolysis) or even perhaps a homeostatic response of the body to the presence of the cancer. Another limitation of our data is the lack of information as to whether these metabolites are specific to kidney cancer, or will be found to be markers for cancer in general, especially given that the processes that they represent have been found to occur in a variety of malignancies; it is likely, although not proven, that the urinary appearance of the metabolites increases sensitivity for kidney as opposed to other organ malignancy. Clearly, additional validation using many more patients and controls will be necessary before claims of specific diagnostic biomarkers can be made. However, the data provided here serve as a starting point for further investigation of urinary metabolites that are altered in kidney cancer and may prove to be viable biomarkers for the disease.

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Author Disclosure Statement

No competing financial interests exist.

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