Published in final edited form as:

Clin Cancer Res. 2017 September 01; 23(17): 5302–5310. doi:10.1158/1078-0432.CCR-16-3156.

Unique and novel urinary metabolomic features in malignant versus benign adrenal neoplasms

Dhaval Patel¹, Matthew D. Thompson², Soumen K. Manna³, Kristopher W. Krausz², Lisa Zhang¹, Naris Nilubol¹, Frank J. Gonzalez², and Electron Kebebew¹

¹Endocrine Oncology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

²Laboratory of Metabolism, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

³Biophysics and Structural Genomics Division, Saha Institute of Nuclear Physics, Kolkata-700064, India

Abstract

Purpose—Adrenal incidentalomas must be differentiated from adrenocortical cancer (ACC). Currently, size, growth, and imaging characteristics determine the potential for malignancy but are imperfect. The aim was to evaluate whether urinary small molecules (<800 Da) are associated with ACC.

Experimental Design—Preoperative fasting urine specimens from patients with ACC (n=19) and benign adrenal tumors (n=46) were analyzed by unbiased ultra performance liquid chromatography/mass spectrometry. Creatinine-normalized features were analyzed by Progenesis, SIMCA, and unpaired t-test adjusted by false discovery rate. Features with an AUC >0.8 were identified through fragmentation patterns and database searches. All lead features were assessed in an independent set from patients with ACC (n=11) and benign adrenal tumors (n=46) and in a subset of tissue samples from patients with ACC (n=15) and benign adrenal tumors (n=15) in the training set.

Results—Sixty-nine features were discovered and four known metabolites identified. Urinary creatine riboside was elevated 2.1-fold (p=0.0001) in patients with ACC. L-tryptophan, Ne,Ne,Ne-trimethyl-L-lysine and 3-methylhistidine were lower 0.33-fold (p<0.0001), 0.56-fold (p<0.0001), and 0.33-fold (p=0.0003) in patients with ACC, respectively. Combined multivariate analysis of the four biomarkers showed an AUC of 0.89 (sensitivity 94.7% (CI 73.9–99.1%), specificity 82.6% (CI 68.6–92.2%), PPV 69.2% (CI 48.2–85.6%), and NPV 97.4% (CI 86.5–99.6%)) for distinguishing ACC from benign tumors. Of the four, creatine riboside and four unknown features were validated. Creatine riboside, Ne,Ne,Ne-trimethyl-L-lysine, and two unknown features were elevated in ACC tumors.

Corresponding author and person to whom reprint requests should be addressed: Dhaval Patel, M.D., Endocrine Oncology Branch, National Cancer Institute, NIH, Clinical Research Center, Building 10-CRC, Room 4W-5950, Bethesda, MD 20892. Phone: 301-827-4989, dhaval.patel@nih.gov.

Additional Information: The authors declare no potential conflicts of interest.

Conclusions—There are unique urinary metabolic features in patients with ACC with some metabolites present in patient tumor samples. Urinary creatine riboside can differentiate benign adrenal neoplasms from ACC.

Keywords

adrenocortical cancer; metabolites; metabolomics; urine; biomarkers

Introduction

Adrenal masses detected incidentally by computed tomography (CT) scan are highly prevalent with a range from 4 to 10% (1–3). Management is guided by tumor size and growth, patient and imaging characteristics, and biochemical findings (4). Although these characteristics may help differentiate benign adrenal incidentalomas from the rare adrenocortical carcinoma (ACC) with a poor prognosis (5–7), patients frequently undergo adrenalectomy to exclude a cancer diagnosis. Thus, identification of biomarkers that distinguish benign from malignant adrenal neoplasm are needed.

ACCs can be hormonally active, thus targeted urine metabolomic approaches using gas chromatography mass spectrometry have been utilized to differentiate patients with benign adrenal tumors and patients with ACC. This approach was able to discriminate between patients with benign adrenal tumors and ACC with a sensitivity and specificity of 90% (8). Given these promising results, there may be smaller molecular biomarkers that may improve the sensitivity and specificity in combination, which could be discovered via an untargeted metabolomics approach. Growing evidence also suggests that the metabolome has an important role in cancer initiation and/or progression, thus an untargeted metabolomic investigation of ACC may provide new information in the pathogenesis of ACC and other cancers.

Metabolomics, the study of small molecule metabolites, was successfully used to identify biomarkers in urine of patients with ovarian cancer (9), colorectal cancer (10), bladder cancer (11), lung cancer (12), and hepatocellular carcinoma (13). Metabolomic analysis of body fluid samples focuses on identifying metabolites related to tumor biology (14). The two major analytical tools used in metabolomics are nuclear magnetic resonance (NMR) and mass spectrometry (MS) coupled to a separation technique. Unlike NMR, MS provides semi-quantitative data with high sensitivity and the ability to identify low abundance metabolites (15). Ultra performance liquid chromatography (UPLC) MS provides high-throughput analysis and reliable data quality (16). High-throughput UPLC/MS analysis of serum and urine samples is thus a platform that could help discover new biomarkers for ACC.

The objective of this study was to perform an untargeted metabolomic analysis by UPLC/MS to determine if there is a specific urinary metabolomic signature that can discriminate patients with benign adrenal tumors and patients with ACC. Secondly, the identified markers were assessed in tumor tissue to provide further insight and validation into the origin of these metabolites.

Methods

Urine samples

Fasting urine samples from patients undergoing adrenal surgery were obtained the morning of surgery and stored at -80°C . Urine samples from patients undergoing experimental treatment for ACC were obtained the morning of drug treatment and stored at -80°C . Tissue samples from patients undergoing adrenal surgery were procured at the time of surgery and flash frozen in liquid nitrogen and stored at -80°C . Demographic, clinical, and pathologic information and samples were collected under an Institutional Review Board (IRB) approved protocol (NCT01005654). Clinical characteristics of the study cohorts are summarized in Table 1, Supplementary Data, Table 1 and Table 2. Tumors were classified as ACC if the Weiss criteria was >3 at the initial surgery and/or they developed locoregional recurrence after complete resection or distant metastasis at follow up. Tumors were classified as benign if the Weiss score was <3 (17) and there was no recurrent or metastatic disease that developed during follow up (median follow-up 32.8 months, range 1.0–83.6 months). Three patients had follow-up of less than one year with final pathology showing adrenal hyperplasia. Tissue samples were confirmed to be either malignant or benign by hematoxylin and eosin staining by a pathologist in entirety.

Untargeted global urinary metabolomic analysis and biomarker identification

Urine samples were deproteinated (dilution of 1:5) using a solution of isopropanol/ acetonitrile/water (65/30/5) containing 10 μM chloropropamide or α -aminopimelic acid as internal standards for reverse-phase (RP) or hydrophilic interaction liquid chromatography (HILIC), respectively. Supernatants were transferred into 96-well sample plates. All pipetting and dilution were performed using a MICROLAB STARLET automated liquid handler (Hamilton Robotics, Reno, NV). For HILIC analysis, 5 μL aliquot of samples were injected in a randomized fashion into a 2.1×50 mm Acquity UPLC BEH amide column (1.7 μm) connected to a Waters XEVO G2 ESI-QTOF mass spectrometer (Waters Corporation, Milford, MA). Chromatographic separation was achieved by using a mixture of (A) 10 mM ammonium acetate in 90% acetonitrile (pH = 9.0) and (B) 10 mM ammonium acetate in 10% acetonitrile (pH = 9.0) as mobile phase. The gradient elution was performed over 10 min using: 1–60% B in 4 min, 60–80% B at 8 min, holding at 80% B to 8.5 min, returning to initial conditions for column equilibration. Flow rate was maintained at 0.4ml/min and total run time for each sample was 12.5min. Column temperature was maintained at 40°C . For RP analysis, samples were further diluted with an equal volume of water before a 5 μL aliquot of samples was injected into a 2.1×50 mm Acquity UPLC BEH C18 column (1.7 μm). Chromatographic separation was achieved by using a mixture of (A) water containing 1% formic acid and (B) acetonitrile containing 1% formic acid as mobile phase. The gradient elution was performed over 6 min at a flow rate of 0.4 ml/min using: 1–99% B in 4 min, holding at 99% B up to 5.0 min, returning to initial conditions for column equilibration (total run time of 10 min.). Column temperature was maintained at 40°C . The column was re-equilibrated with 98% A at the end of each run prior to injection of the next sample. Mass spectrometric analysis (for both RP and HILIC chromatography) was performed in both positive and negative ionization modes. Sulfadimethoxine was used as the lock mass (m/z 311.0814+) for accurate mass calibration in real time. MassLynx software

(Waters Corporation, Milford, MA) was used to acquire mass chromatograms and mass spectral data in centroid format. Chromatographic separation (for both RP and HILIC conditions) was performed on a Waters Acquity H-class UPLC system consisting of a quaternary solvent manager, FTN-solvent manager, and a column manager, all controlled by MassLynx software.

Targeted urinary metabolite quantitation

Metabolites in the deproteinated urine samples were quantified in multiple reactions monitoring mode on a Waters XEVO TQ-S triple quadrupole mass spectrometer (Water Corporation, Milford, MA). α -Aminopimelic acid (0.5 μ M) was used as internal standard. The following metabolites were quantified by monitoring characteristic fragmentation reactions (in bracket); α -aminopimelic acid (176 $\rightarrow$ 112, ESI+), 3-methylhistidine (170 $\rightarrow$ 96, ESI+), Ne,Ne,Ne-trimethyllysine (189 $\rightarrow$ 84, ESI+), L-tryptophan (205 $\rightarrow$ 118, ESI-), creatine (132 $\rightarrow$ 90, ESI+), and creatine riboside (264 $\rightarrow$ 132, ESI+). Creatine was used to standardize creatine riboside due to lack of purified standard.

Chromatographic separation was achieved on a 2.1 $\times$ 50 mm Acquity UPLC BEH amide column using the mobile phase as mentioned above. All data were processed using Waters TargetLynx software. Internal standard-normalized area under the peak (response) from serially diluted authentic standard solution was used to build calibration curve for each metabolite. The concentration of metabolite was determined from the calibration curve and divided by creatinine concentration to determine creatinine-normalized excretion of the urine metabolite.

Targeted tissue metabolite quantitation

A subset of tissue samples from patients with ACC (n=15) and patients with benign adrenal disease (n=15) from the training set were evaluated for metabolites. Metabolites in the deproteinated and homogenized tissue samples were quantified in multiple reactions monitoring mode on a Waters XEVO TQ-S triple quadrupole mass spectrometer (Water Corporation, Milford, MA). α -aminopimelic acid (0.5 μ M) was used as internal standard. The following metabolites were quantified by monitoring characteristic fragmentation reactions (in bracket); α -aminopimelic acid (176 $\rightarrow$ 112, ESI+), 3-methylhistidine (170 $\rightarrow$ 96, ESI+), Ne,Ne,Ne-trimethyllysine (189 $\rightarrow$ 84, ESI+), L-tryptophan (205 $\rightarrow$ 118, ESI-), creatine (132 $\rightarrow$ 90, ESI+), creatine riboside (264 $\rightarrow$ 132, ESI+), Var_0001 (334 $\rightarrow$ 85), and Var_0012 (306 $\rightarrow$ 85). All tissue samples were normalized to the internal standard α -aminopimelic acid.

Chromatographic separation was achieved on a 2.1 $\times$ 50 mm Acquity UPLC BEH amide column using the mobile phase as mentioned above. All data were processed using Waters TargetLynx software. Internal standard-normalized area under the peak (response) from serially diluted authentic standard solution was used to build calibration curve for each identified metabolite. The concentration of metabolite was determined from the calibration curve and divided by α -aminopimelic acid concentration to determine normalized levels of the tissue metabolites.

Quality Control and Normalization

The order of sample injection was randomized to avoid order artifact using the rand function in Microsoft Excel (Redmond, WA USA). Quality control involved running a standard mix prior to samples to monitor instrument performance over time. Internal standards (α -aminopimelic acid, chlorpropamide) were used for HILIC and reverse phase chromatography, respectively. A set of pooled samples was injected at regular intervals during the analysis. The data was assessed with unsupervised principal component analyses. Creatinine concentration was determined by the Jaffe method (18). In brief, creatinine was assayed colorimetrically after reaction of creatinine with picrate to generate a chromophore in alkaline solution. The absorbance of the creatinine-picrate chromophore was measured at 500 nm in a 96 well microplate.

Statistical analyses

Samples were classified as ACC or benign adrenal tumor. Data acquired was aligned and deconvoluted by Progenesis software (Durham, NC USA). Urine data was normalized to externally measured creatinine by the Jaffe method (see above). Data was exported and analyzed using a minimal detection rate to eliminate spurious human and instrument variation. Minimal detection rate was defined as a feature that must be present in at least 40% of samples in each group *a priori*. The data was analyzed in three separate and complementary methods for discovery (Figure 1). Method 1 used the Progenesis software to compare the groups and a one way ANOVA with a false discovery rate (FDR) of $q < 0.05$ (19). Method 2 consisted of a two-tailed T-test and Benjamini-Hochberg test for FDR 5% (19). Method 3 consisted of using soft independent modelling of class analogy (SIMCA) with the construction of principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) plots with a p correlation of 0.5 for intergroup discrimination.

Potential features that were statistically significant by any of the three methods were then analyzed using online MetaboAnalyst 3.0 software (<http://www.metaboanalyst.ca/>) with an area under the curve (AUC) cutoff of > 0.8 for each feature (20). The multivariate analysis was completed utilizing the online MetaboAnalyst software by calculating predict class probabilities based on each sample and the biomarker panels tested. The multivariate algorithm utilized was a random forest algorithm that performed repeated random sub-sampling cross validations (CV). The predicted accuracies were then plotted with increasing number of features. The sensitivities, specificities, positive predictive values, and negative predictive values were calculated using the predicted class probabilities for identifying the biomarker panel ability to correctly diagnose patients as either benign or malignant. Each potential feature was then verified by using TargetLynx software (Waters Corporation, Milford, MA). In order to eliminate features related to mitotane administration, PCA and OPLS-DA plots were constructed using SIMCA software. S-plots that did not show a correlation to mitotane ingestion were considered as related to cancer and were included in further analysis. To account for unequal variance, the urine and tissue features were compared by the Mann-Whitney U test and correlated by Spearman rank test. A p -value of less than 0.05 was considered significant.

Results

Unsupervised PCA plots show discrimination and clustering between patients with ACC and benign adrenal tumors in reverse phase positive, reverse phase negative, HILIC positive, and HILIC negative modes (Figure 2A, 2B, 3A, and 3B). Supervised OPLS-DA analysis showed separation and features associated with ACC and benign adrenal tumors (Figure 2C, 2D, 3C, and 3D). S-plot analysis showed features associated with ACC and benign adrenal disease for each mode, respectively (Figure 2E, 2F, 3E, and 3F). These data show that patients with ACC and benign adrenal tumors have significantly different urinary metabolites.

Preoperatively, patients with recurrent and metastatic ACC were treated with mitotane. Given the long half-life (18 to 159 days) and the possibility that metabolites discovered may be mitotane metabolites or related to mitotane metabolism, further drug analysis was performed. Unsupervised PCA plots show clustering between patients with and without mitotane therapy (Supplementary Data, Figure 1A, 1B, 2A, and 2B). OPLS-DA plots and S plots showed separation and features associated with mitotane use, which were excluded from further analysis (Supplementary Data, Figure 1C-F, and 2C-F). To verify that these were metabolites of mitotane, MS/MS fragmentation pattern of a top hit was identified as a mitotane metabolite.

Metabolites not associated with mitotane ingestion were identified and further studied. The significant features as discovered by different methods are summarized (Supplementary Data, Table 3). Sixty-nine features were discovered. Specific m/z masses and retention times that are significantly different between the two groups are summarized in Supplementary Data, Table 4). The significant features of unique parent compounds were combined in a multivariate random forest model to classify benign and malignant adrenal tumors. Of the 69 features, 46 unique features were parent compounds and not related to mitotane ingestion. A combination of the 46 unique features included in the model showed that the AUC was 0.928 with the inclusion of all features (Supplementary Data, Figure 3A). The predicted accuracy of classification by urinary metabolic analysis utilizing these features was 84% (Supplementary Data, Figure 3C).

Among the features discovered, four features were identified through MS/MS fragmentation and online metabolomics database searches. The four metabolites identified through fragmentation patterns and database searches were creatine riboside, Ne,Ne,Ne-trimethyllysine, 3-methylhistidine, and L-tryptophan (Supplementary Data, Figure 4A, 4B, 4C, and 4D). These four metabolites were quantitated on a Waters Xevo triple quadrupole mass spectrometer (Waters Corporation, Milford, MA) in multiple reaction monitoring (MRM) mode. Urinary creatine riboside was elevated over two-fold in patients with ACC compared to patients with benign adrenal tumors (Figure 4A, 2.1 fold change, $p=0.0001$, AUC=0.793). Urinary Ne,Ne,Ne-trimethyllysine (Figure 4B, 0.56-fold change, $p<0.0001$, AUC=0.820), 3-methylhistidine (Figure 4C, 0.33-fold change, $p=0.0003$, AUC=0.782), and L-Tryptophan, (Figure 4D, 0.33-fold change, $p<0.0001$, AUC=0.860) were each lower in patients with ACC compared to patients with benign adrenal tumors. Using the four identified metabolites as a panel, the AUC was 0.89 (Figure 5A). Use of the four features further showed a sensitivity of 94.7% (CI 73.9 to 99.1%) and specificity of 82.6% (CI 68.6

to 92.2%). The positive predictive value was 69.2% (CI 48.2 to 85.6%) and negative predictive value was 97.4% (CI 86.5 to 99.6%). The model was predicted to be 81.9% accurate in diagnosing malignancy when using a cross validation model (Figure 5C).

The four identified metabolites and 65 unknown features were analyzed in an independent set of urine samples consisting of eleven patients with ACC and forty-six patients with benign adrenal tumors (Table 1 and Supplementary Data, Table 2). Creatine riboside was validated and continued to show a significant difference between the two groups (Figure 4E). L-Tryptophan, Nε,Nε,Nε-trimethyllysine, and 3-methylhistidine were found to be not significantly different. Ten of the 65 unknown features were validated and found to be significantly different between the two groups. Of the ten features, the top four unknown features (Supplementary Data, Table 4, **denoted by an asterisk**) included m/z 334.186 (Supplementary Figure 5A, 3.9 fold change, p=0.0001, HILIC Positive), m/z 306.153 (Supplementary Figure 5B, 5.0 fold change, p=0.0001, HILIC Positive), m/z 209.030 (Supplementary Figure 5C, 2.5 fold change, p=0.0281, HILIC Negative), and m/z 427.231 (Supplementary Figure 5D, 4.8 fold change, p=0.0002, RP Negative). The top four unknown features and creatine riboside were combined and differentiated between the two groups with an area under the curve of 0.875 (Supplementary Data, Figure 6A). A subset of patients who presented with initial surgery for ACC was compared to patients with benign adrenal tumors for clinical data including size of the mass on imaging and initial clinical presentation. Clinical presentation was not predictive of malignancy. Therefore, size on imaging and the validated marker creatine riboside were utilized to create a composite model. Quantified creatine riboside data and size improved the area under the curve 0.96 (Supplementary Data, Figure 6B). The use of size and creatine riboside showed a sensitivity of 80.0% and specificity of 95.5%. The positive predictive value was 57.1% and negative predictive value was 98.4%.

In order to determine whether these biomarkers originated from the tumor, a subset of available tissue samples were selected and targeted metabolic studies were conducted. Creatine riboside, feature m/z 334.186, and feature 306.153 were identified and optimized in adrenal tissue samples. Feature m/z 209.030 and feature m/z 427.23 were not identified in the tissue. L-Tryptophan and 3-methylhistidine were not found to be different between patients with malignant and benign adrenal tumors. Levels of creatine riboside (p<0.0001), feature m/z 306.153 (p=0.0063), and feature m/z 334.186 (p<0.0001), and Nε,Nε,Nε-trimethyllysine (p=0.0357) in ACC tumor tissue samples were compared to benign adrenal tumors (Supplementary Data, Figure 7A, 7B, 7C and 7D). The levels of creatine riboside in the tissue were compared to the levels of creatine riboside in paired urine samples, and there was a significant positive association (Supplementary Data, Figure 4FC, r=0.5681, p=0.0016).

Discussion

This study is the first untargeted metabolomics examination of the urine of patients with benign adrenal tumors and ACC. An unbiased examination of the data shows that the two groups can be discriminated by a urinary metabolomics approach. Furthermore, mitotane and mitotane associated metabolites were identified in patients that could be used to direct

steroid replacement therapy and measure compliance. Analysis of specific features identified that patients with ACC had higher levels of creatine riboside and lower levels of L-tryptophan, Ne,Ne,Ne-trimethyllysine and 3-methylhistidine when compared to patients with benign adrenal tumors. Creatine riboside and four unknown features were validated in an independent set of samples. The combination of creatine riboside and size of adrenal tumor had a high degree of discrimination between ACC and benign adrenal tumor. Furthermore, targeted tissue analysis showed elevated levels of creatine riboside, feature m/z 306.186, feature m/z 334.153, and Ne,Ne,Ne-trimethyl-L-lysine to be higher in patients with ACC compared to patients with benign adrenal tumors. Levels of creatine riboside in the tissue and urine were positively associated.

Creatine riboside, which was significantly elevated in patients with ACC, was recently described as a noninvasive urinary marker in patients with lung cancer. In addition to elevated levels in the urine, increased creatine riboside was found in the tumors of patients with lung cancer compared to normal adjacent tissue (12). Creatine riboside, therefore, may not be specific to lung cancer or ACC, but may increase the index of suspicion of a potential malignant neoplasm. Although the function of creatine riboside has yet to be elucidated, the fact that both lung and ACC have elevated levels may indicate that this metabolite is involved in tumorigenesis. In the validation set, creatine riboside was the only known metabolite that was validated. Furthermore, creatine riboside was found to be elevated in the tissue of patients with ACC compared to patients with benign adrenal tumors. This may indicate that creatine riboside is produced in the tumor and secreted in the urine. In support of this, creatine riboside levels in the tissue and urine were positively associated.

The three other identified metabolites, L-tryptophan, Ne,Ne,Ne-trimethyllysine, and 3-methylhistidine were not validated. These metabolites may have been false-positives or possibly related to lack of fasting in the validation cohort. Each of these three metabolites is associated with consumption of food, and therefore, may still be valid with further testing. Further analysis of the 65 features in the validation cohort revealed four features that were significantly different in both the training and validation sets. These validated features were searched in metabolite databases and were not known metabolites. These features may be novel metabolites that will require future characterization, as they may be specific to ACC. Two of the four features were detected in the tissue of patients with adrenal tumors. These two features were elevated in ACC tissue compared to benign adrenal tumor. These unknown features may have a role in adrenal cancer and will require further study.

Although urinary Ne,Ne,Ne-trimethyllysine was not validated and may have been affected by diet in the independent set, the difference in tissue levels indicates that it may have a role in adrenal cancer. Ne,Ne,Ne-trimethyllysine, is associated with trimethylation of lysine residues on histone complexes. Histone lysine methylation is critical in the regulation of gene expression, cell cycle, genomic stability and nuclear architecture (21). Immunohistochemistry studies have shown that a decrease in trimethylation of lysine 27 at histone H3 by the methyltransferase EZH2 was associated with poor prognosis in patients with breast cancer regardless of estrogen receptor (ER) status. The present data showed that the metabolite Ne,Ne,Ne-trimethyllysine, was decreased in urine of patients with ACC. However, in the subset of tissue samples Ne,Ne,Ne-trimethyllysine showed an elevated level

in patients with ACC compared to benign adrenocortical neoplasms. Drelon and colleagues recently reported that EZH2 to be overexpressed in ACC and was possibly the result of inactivation of the P53/RB/E2F pathway. High levels of expression were associated with poor prognosis and increased proliferation. In vitro studies showed knockdown of EZH2 to lead to decreased cellular growth, inhibition of migration and clonogenic growth, and apoptosis. The increased Ne,Ne,Ne-trimethyllysine would be explained by these findings, however the paradoxical levels in the urine may indicate that the metabolite may have other sources as well such as muscle (22).

The annual number of adrenalectomies has been increasing in the United States. Murphy and colleagues reported that the number of both open and laparoscopic adrenalectomies has increased from 3,241 in 1998 to 5,323 adrenalectomies in 2006. Furthermore, the majority of adrenalectomies were performed for benign disease (83%) (23). Biomarkers able to discriminate between malignant and benign adrenal disease are needed to avoid surgery and reduce the morbidity of adrenalectomy for patients with benign adrenal disease. Given the high negative predictive value of the discovered metabolites and features in combination with adrenal tumor size, clinicians may be able to avoid an adrenalectomy in both large and small adrenocortical tumors or stratify patients for further work-up including functional imaging (24). However, prior to clinical adaptation, the biomarkers will have to be validated in independent cohorts seen at high volume adrenal surgery centers as well as patients undergoing active surveillance for incidentally discovered adrenal tumors.

There are several limitations to this study, including sample size. The limited sample size is due to the rarity of ACC (two cases per million). In order to overcome this limitation, a training set and validation set were used to reduce a Type 1 error of discovered metabolites. A second limitation to this study is that not all patients in the validation set had fasting urinary samples available for analysis. This limitation may have resulted in a Type 2 error of features that were not validated. However, this finding does emphasize that the validated features/metabolites are not affected by diet and are truly different between the two groups. A third limitation is that many patients were on mitotane as either adjuvant therapy or for disease progression. The authors attempted to control for steroid metabolite alterations by analyzing samples of patients who were treated with mitotane. In addition, steroid supplementation may have affected the urinary metabolome. Although an important limitation, the impact may be limited given that both benign and malignant cohorts in both the learning and independent set had patients with hypercortisolism or patients receiving steroid supplementation.

In this study, urine samples from patients with benign adrenal tumors and ACC were analyzed using a metabolomic approach in an untargeted unbiased fashion. Unique urinary metabolic features were identified in benign and malignant adrenocortical tumors. Urinary creatine riboside may provide additional diagnostic information on adrenal incidentalomas. Unique unknown features may be novel metabolites that would be able to differentiate patients with malignant adrenocortical tumors and benign adrenal disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by the Intramural Research Program of the Center for Cancer Research, National Cancer Institute, National Institutes of Health.

References

1. Hammarstedt L, Muth A, Wangberg B, Bjorneld L, Sigurjonsdottir HA, Gotheerstrom G, et al. Adrenal lesion frequency: A prospective, cross-sectional CT study in a defined region, including systematic re-evaluation. *Acta radiologica*. 2010; 51:1149–56. [PubMed: 20969508]
2. Terzolo M, Stigliano A, Chiodini I, Loli P, Furlani L, Arnaldi G, et al. AME position statement on adrenal incidentaloma. *European journal of endocrinology / European Federation of Endocrine Societies*. 2011; 164:851–70.
3. Bovio S, Cataldi A, Reimondo G, Sperone P, Novello S, Berruti A, et al. Prevalence of adrenal incidentaloma in a contemporary computerized tomography series. *Journal of endocrinological investigation*. 2006; 29:298–302. [PubMed: 16699294]
4. Bittner, JGt, Brunt, LM. Evaluation and management of adrenal incidentaloma. *Journal of surgical oncology*. 2012; 106:557–64. [PubMed: 22623268]
5. Terzolo M, Ali A, Osella G, Mazza E. Prevalence of adrenal carcinoma among incidentally discovered adrenal masses. A retrospective study from 1989 to 1994. *Gruppo Piemontese Incidentalomi Surrenalici. Archives of surgery*. 1997; 132:914–9. [PubMed: 9267279]
6. Fassnacht M, Kroiss M, Allolio B. Update in adrenocortical carcinoma. *The Journal of clinical endocrinology and metabolism*. 2013; 98:4551–64. [PubMed: 24081734]
7. Aufforth RD, Nilubol N. Emerging therapy for adrenocortical carcinoma. *International journal of endocrine oncology*. 2014; 1:173–82. [PubMed: 25635221]
8. Arlt W, Biehl M, Taylor AE, Hahner S, Libe R, Hughes BA, et al. Urine steroid metabolomics as a biomarker tool for detecting malignancy in adrenal tumors. *The Journal of clinical endocrinology and metabolism*. 2011; 96:3775–84. [PubMed: 21917861]
9. Zhang T, Wu X, Ke C, Yin M, Li Z, Fan L, et al. Identification of potential biomarkers for ovarian cancer by urinary metabolomic profiling. *Journal of proteome research*. 2013; 12:505–12. [PubMed: 23163809]
10. Cheng Y, Xie G, Chen T, Qiu Y, Zou X, Zheng M, et al. Distinct urinary metabolic profile of human colorectal cancer. *Journal of proteome research*. 2012; 11:1354–63. [PubMed: 22148915]
11. Pasikanti KK, Esuvaranathan K, Ho PC, Mahendran R, Kamaraj R, Wu QH, et al. Noninvasive urinary metabolomic diagnosis of human bladder cancer. *Journal of proteome research*. 2010; 9:2988–95. [PubMed: 20337499]
12. Mathe EA, Patterson AD, Haznadar M, Manna SK, Krausz KW, Bowman ED, et al. Noninvasive urinary metabolomic profiling identifies diagnostic and prognostic markers in lung cancer. *Cancer research*. 2014; 74:3259–70. [PubMed: 24736543]
13. Chen T, Xie G, Wang X, Fan J, Qiu Y, Zheng X, et al. Serum and urine metabolite profiling reveals potential biomarkers of human hepatocellular carcinoma. *Molecular & cellular proteomics : MCP*. 2011; 10:M110 004945.
14. Spratlin JL, Serkova NJ, Eckhardt SG. Clinical applications of metabolomics in oncology: a review. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2009; 15:431–40. [PubMed: 19147747]
15. Vermeersch KA, Styczynski MP. Applications of metabolomics in cancer research. *Journal of carcinogenesis*. 2013; 12:9. [PubMed: 23858297]
16. Yin P, Xu G. Metabolomics for tumor marker discovery and identification based on chromatography-mass spectrometry. *Expert review of molecular diagnostics*. 2013; 13:339–48. [PubMed: 23638817]

17. Aubert S, Wacrenier A, Leroy X, Devos P, Carnaille B, Proye C, et al. Weiss system revisited: a clinicopathologic and immunohistochemical study of 49 adrenocortical tumors. *Am J Surg Pathol*. 2002; 26:1612–9. [PubMed: 12459628]
18. Thompson HJ, Heimendinger J, Sedlacek S, Haegeler A, Diker A, O'Neill C, et al. 8-Isoprostane F2alpha excretion is reduced in women by increased vegetable and fruit intake. *The American journal of clinical nutrition*. 2005; 82:768–76. [PubMed: 16210705]
19. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate - a Practical and Powerful Approach to Multiple Testing. *J Roy Stat Soc B Met*. 1995; 57:289–300.
20. Xia J, Sinelnikov IV, Han B, Wishart DS. MetaboAnalyst 3.0--making metabolomics more meaningful. *Nucleic Acids Res*. 2015; 43:W251–7. [PubMed: 25897128]
21. Black JC, Van Rechem C, Whetstine JR. Histone lysine methylation dynamics: establishment, regulation, and biological impact. *Molecular cell*. 2012; 48:491–507. [PubMed: 23200123]
22. Huszar G. Methylated lysines and 3-methylhistidine in myosin: tissue and developmental differences. *Methods in enzymology*. 1984; 106:287–95. [PubMed: 6436641]
23. Murphy MM, Witkowski ER, Ng SC, McDade TP, Hill JS, Larkin AC, et al. Trends in adrenalectomy: a recent national review. *Surg Endosc*. 2010; 24:2518–26. [PubMed: 20336320]
24. Tessonnier L, Sebag F, Palazzo FF, Colavolpe C, De Micco C, Mancini J, et al. Does 18F-FDG PET/CT add diagnostic accuracy in incidentally identified non-secreting adrenal tumours? *Eur J Nucl Med Mol Imaging*. 2008; 35:2018–25. [PubMed: 18566816]

Translational Relevance

Adrenal incidentalomas are a common clinical dilemma and a diagnosis of adrenocortical carcinoma (ACC) must be excluded in all cases. There are currently no reliable clinical, imaging or biochemical features to exclude the rare and often fatal diagnosis ACC. Preoperative fasting urine specimens from patients with ACC and benign adrenal tumors were analyzed by unbiased ultra performance liquid chromatography/mass spectrometry to discover four known metabolites (L-tryptophan, 3-methylhistidine, Ne,Ne,Ne-trimethyl-L-lysine, and creatine riboside) and sixty-five unknown features that differentiate patients with ACC and patients with benign adrenal neoplasms. Creatine riboside and four unknown features were validated in an independent set of urine samples. This data indicates that patients with ACC can be differentiated from benign adrenal disease with fasting spot urine specimens leading to improved patient selection for adrenalectomy.

Figure 1.

Workflow and approach to identifying potentially discriminating metabolites between ACC and benign adrenal tumors.

Figure 2.

PCA plots showing discrimination between patients with ACC and benign adrenal tumors in reverse phase (RP) in both (A) ESI+ and (B) ESI- mode. Supervised OPLS-DA plots showing differences between the two groups in both (C) ESI+ and (D) ESI- mode. S-Plot correlative analysis showing significant variables correlated with patients with ACC or benign adrenal tumors (highlighted in red) in both (E) ESI+ and (F) ESI- mode.

Figure 3.

PCA plots showing discrimination between patients with ACC and benign adrenal tumors in HILIC phase in both (A) ESI+ and (B) ESI– mode. Supervised OPLS-DA plots showing differences between the two groups in both (C) ESI+ and (D) ESI– mode. S-Plot correlative analysis showing significant variables correlated with patients with ACC or benign adrenal tumors (highlighted in red) in both (E) ESI+ and (F) ESI– mode.

Figure 4.

A comparison between patients with ACC and benign adrenal tumors with respect to the four identified biomarkers: (A) creatine riboside, (B) Ne, Ne, Ne trimethyllysine, (C) 3-methylhistidine, and (D) tryptophan. (E) A comparison of patients with ACC and benign adrenal disease in an independent set of samples. Levels were quantified for creatine riboside. Red denotes patients not fasting. (F) Correlation between tissue and urine levels of creatine riboside in patients with benign and malignant adrenal tumors.

Figure 5. (A) Multivariate ROC curve analysis showing higher AUC with a combination of creatine riboside, L-tryptophan, Ne Ne,Ne-trimethyllysine, and 3-methylhistidine. (B) Predictive ability of the features to differentiate between patients with ACC (filled circles) and patients with benign adrenal tumors (open circles). (C) Predictive accuracy of the combined features (81.9%).

Table 1

Clinical Characteristics of Study Cohorts

	Training Set		Independent Set	
	Adrenocortical carcinoma	Benign adrenal tumor	Adrenocortical carcinoma	Benign adrenal tumor
No. of patients	19	46	11	46
Age (average $\pm$ SD)	55.68 $\pm$ 10.78	47.85 $\pm$ 15.99	47.33 $\pm$ 15.65	41.94 $\pm$ 17.54
Gender (female/male)	11/8	26/20	6/5	30/16
Size (cm) *	15.40 $\pm$ 4.74	2.43 $\pm$ 1.37	13.7 $\pm$ 11.31	2.50 $\pm$ 1.90
Syndrome[†]				
Cushing's	7	24	4	21
Subclinical Cushing's	0	0	0	3
Conn's	1	20	0	14
Hyperandrogenism	0	0	0	2
Nonfunctioning	11	2	7	6

* Size measured in longest dimension for patients with measurable masses undergoing initial surgery for an adrenocortical tumor.

[†] Functional status at initial presentation.