Research Article

Cancer Epidemiology, Biomarkers & Prevention

Urinary Metabolites Diagnostic and Prognostic of Intrahepatic Cholangiocarcinoma

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Abstract

Background: Liver cancer is the second leading cause of cancer-related deaths worldwide. With a predicted 2.4-fold rise in liver cancer incidence by 2020, there is an urgent need for early, inexpensive diagnostic biomarkers to deploy in the clinic.

Methods: We employed ultraperformance liquid chromatography tandem mass-spectrometry (UPLC/MS-MS) for the quantitation of four metabolites, creatine riboside (CR), *N*-acetylneuraminic acid (NANA), cortisol sulfate, and a lipid molecule designated as 561+, in urine samples from the NCI-MD cohort comprising 98 hepatocellular carcinoma (HCC) cases, 101 high-risk subjects, and 95 controls. Validation was carried out in the TIGER-LC cohort [$n = 370$ HCC and intrahepatic cholangiocarcinoma (ICC) cases, 471 high-risk subjects, 251 controls], where ICC, the second most common primary hepatic malignancy, is highly prevalent. Metabolite quantitation was also conducted in TIGER-LC tissue samples ($n = 48$ ICC; $n = 51$ HCC).

Results: All profiled metabolites were significantly increased in liver cancer when compared with high-risk subjects and controls in the NCI-MD study. In the TIGER-LC cohort, the four-metabolite profile was superior at classifying ICC than a clinically utilized marker, CA19-9, and their combination led to a significantly improved model (AUC = 0.88, $P = 4E\text{-}8$). Metabolites CR and NANA were significantly elevated in ICC when compared with HCC cases in both urine and tissue samples. High levels of CR were associated with poorer prognosis in ICC.

Conclusions: Four metabolites are significantly increased in HCC and ICC and are robust at classifying ICC in combination with the clinically utilized marker CA19-9.

Impact: Noninvasive urinary metabolite biomarkers hold promise for diagnostic and prognostic evaluation of ICC.

Introduction

Liver cancer is the second leading cause of cancer-related deaths worldwide. A tremendous burden of this disease is evident through an estimated 2.4-fold rise in liver cancer incidence by 2020 in the United States (1). In this context, early diagnostic biomarkers could offer significant improvements in patient survival, since the 5-year relative survival rate for people with localized disease is approximately 30.5% and for regional stage is only 10.7% (2–7). Among the various types of primary liver cancers, hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC) are the most common, accounting for roughly 80% and 15% of cases, respectively (2).

While there are many common risk factors, such as hepatitis B virus (HBV), hepatitis C virus (HCV), and obesity, the magnitude of their impact differs in the two most common intrahepatic cancer types. It has been well established that the most important risk factors for the development of HCC are chronic liver disease and cirrhosis. The main contributors to those risk factors worldwide are viral hepatitis and excessive alcohol intake (8). Chronic viral hepatitis can lead to cirrhosis and/or HCC. Hepatitis B and C are the most common causes of chronic hepatitis in the world. For ICC, epidemiologic and experimental evidence strongly indicate that the liver fluke (*Opisthorchis viverrini*) is a major etiologic agent for this disease (9). Liver fluke is a fish-borne trematode endemic in Southeast Asia, including Thailand, where 10 million people are estimated to be infected (10). While recent studies indicate that certain subtypes of HCC and ICC share similar molecular biology profiles, ICC is histopathologically distinct from HCC, and its etiology is less well-known (11–13). Numerous studies have characterized ICC as having very poor prognosis and poor response to current therapies (14–16). Considering the limited benefit of conventional chemotherapy in the management of

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Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (http://cebp.aacrjournals.org/).

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Cancer Epidemiol Biomarkers Prev 2019;28:1704–11

doi: 10.1158/1055-9965.EPI-19-0453

unresectable or metastatic ICC, identifying biomarkers of this form of liver cancer will assist in the early diagnosis of stage I disease amenable to surgery and guiding emerging molecular-targeted therapies and personalized medicine to metastatic disease in which efficacy of therapy and cancer recurrence can be monitored.

The pathogenesis of ICC involves multiple molecular alterations at the level of genome, epigenome, and stromal environment, resulting in several deregulated signal transduction pathways (11, 14, 17–19). Carbohydrate antigen 19-9 (CA19-9), a sialylated Lewis blood group antigen, is the most common serum marker utilized as an aid in the diagnosis of ICC in clinical practice (20). Protein CA19-9 is produced by several types of cells: normal human pancreatic cells, biliary ductular cells, and gastric and colonic epithelial cells. Because of its widespread expression, CA19-9 is nonspecific to ICC, as it may be elevated in benign biliary diseases (e.g., cholangitis and primary biliary cirrhosis). This protein has also been shown to be elevated in other gastrointestinal cancers (e.g., pancreatic and gastric cancers). Not surprisingly, CA19-9′s utility as a biomarker of ICC has been reported with suboptimal selectivity (21, 22).

In the search for robust biomarkers, metabolomics is emerging as the method of choice (23–28). Metabolomics technology holds promise to increase the diagnostic utility of biomarkers in diseases and conditions that would benefit from an increase in diagnostic sensitivity and specificity. Considering that metabolites serve as a comprehensive chemical fingerprint of cell metabolism, metabolomics holds further promise to be able to discover metabolite biomarkers that can be developed for early cancer detection, response to treatment, and monitoring of cancer recurrence. Metabolites are not only end products of gene and protein expression but also a consequence of the mutual relationship between the genome and the internal environment (29, 30). While the first observations regarding metabolic alterations characteristic of all tumors were made nearly a century ago, namely the Warburg effect (31), the field of cancer metabolism has become a topic of renewed interest in the past decade. One of the most notable hallmarks of cancer cell metabolism is the ability of cancer cells to acquire crucial nutrients from nutrient-poor environments, which are then utilized to maintain viability and build new biomass.

We have previously leveraged the high sensitivity and specificity of ultraperformance liquid chromatography coupled to mass spectrometry (UPLC-MS) to conduct a large metabolomics study in the U.S. lung cancer subjects from the greater Baltimore area (NCI-MD cohort) and uncovered noninvasively measured urinary metabolites: creatine riboside (CR), *N*-acetylneuraminic acid (NANA), cortisol sulfate (CS), and lipid metabolite 561+ (Met 561+). The aforementioned metabolite markers were found to be associated with early stage I and II lung cancer status and poorer prognosis (27). These findings were subsequently validated in the well-characterized prospective Southern Community Cohort Study comprising subjects from the Southeastern United States (26). Because of commonalities in metabolic changes between different cancer types, we hypothesized that the four-metabolite profile previously described as a robust diagnostic indicator of lung cancer may also be predictive of liver cancer. We were specifically interested in identifying sensitive and specific markers that can aid in the detection of ICC, which currently suffers from a lack of clinically relevant diagnostic markers. In that regard, we utilized liver cancer specimens from the NCI-MD study for the initial observations regarding the potential diagnostic utility of the four metabolites in HCC, and then subsequently validated those findings in the well-characterized TIGER-LC cohort (11), as well as made novel observations in ICC.

Materials and Methods

Study subjects

NCI-MD cohort. Urine samples from 95 HCC cases and 101 high-risk subjects (hepatitis virus B– and/or C–infected subjects) were recruited from the pathology department of the University of Maryland Medical Center (Baltimore, MD) from 2011 to 2014. Population controls ($n = 98$) were identified from the Department of Motor Vehicles (DMV) lists, and frequency matched to cases by age, gender, and self-reported race. All cases were determined to be HCC by pathologic assessment. This study was approved by the institutional review boards of the participating institutions, and all patients had signed written informed consents. Urine samples were collected at the time of interview when possible. If collected at a different time, a brief intake questionnaire was administered. In each case, urine was collected in a plain, sterile 50-mL container, split into 10-mL aliquots, and stored at −80°C until used. Urines were thawed on wet ice at the time of use. Subjects were not required to fast or undergo any other preparatory procedure before urine collection. The time of interview and subsequent urine collection was recorded with the questionnaire data. Demographic and clinical data of the NCI-MD cohort are presented in Supplementary Table S1.

TIGER-LC cohort. A set of urine specimens from HCC cases ($n = 117$), HCC high-risk controls ($n = 228$; chronic liver disease, HBV or HCV infections or both, alcoholic liver disease, patients with fatty liver disease, at high risk of developing HCC), ICC cases ($n = 253$), ICC high-risk controls ($n = 243$; liver fluke infections, associated with the development of ICC), and 251 healthy controls from the TIGER-LC cohort were analyzed in this study. Tumor diagnosis was independently confirmed by pathologic assessment by resident pathologists at each participating center in Thailand as well as a surgical pathologist in the United States. Clinical and demographic data were abstracted from comprehensive questionnaires and medical chart records. Staging of liver cancer cases was performed by the pathologists at participating institutions and based on the 7th edition of the American Joint Committee on Cancer (AJCC) staging manual. Details regarding clinical and demographic characteristics of the TIGER-LC subjects included in this study are presented in Table 1. Informed consent was obtained from all patients included in this study and approved by the institutional review boards of the respective institutions. The TIGER-LC cohort has been previously described in more detail (11).

UPLC/MS-MS

UPLC/MS-MS was performed as described previously (27). Briefly, metabolite quantitation was performed by multiple reaction monitoring (MRM) using a Waters Acquity UPLC system coupled to a Waters Xevo TQ Triple Quadrupole Mass Spectrometer (Waters) operated by MassLynx software. Urine samples were processed with an equal volume of 50% aqueous acetonitrile

Table 1. TIGER-LC clinical and demographic characteristics

Characteristics	HCC cases	HCC high risk	ICC cases	ICC high risk	Controls
N	117	228	253	243	251
Age; mean ± SD	54.6 ± 10.2	45.0 ± 12.5	59.7 ± 8.5	58.2 ± 8.7	53.9 ± 9.6
Gender; *n* (%)					
Female	23 (20)	98 (43)	86 (34)	78 (32)	75 (30)
Male	91 (78)	59 (26)	155 (61)	164 (67)	64 (64)
Unknown	3 (2)	71 (31)	12 (5)	1 (1)	16 (6)
HBV status; *n* (%)					
Negative	30 (26)	7 (3)	135 (53)	133 (55)	207 (82)
Positive	70 (60)	138 (61)	65 (26)	109 (45)	27 (11)
Unknown	17 (14)	83 (36)	53 (21)	1 (1)	17 (7)
HCV status; *n* (%)					
Negative	75 (64)	140 (61)	193 (76)	236 (97)	228 (91)
Positive	22 (19)	8 (4)	3 (1)	4 (2)	0 (0)
Unknown	20 (17)	80 (35)	57 (23)	3 (1)	23 (9)
Stage; *n* (%)					
I–II	73 (62)		49 (19)		
II–IV	24 (21)		78 (31)		
Unknown	20 (17)		126 (50)		

containing chloropropamide and aminopimelic acid as internal standards and chromatographed on a 50 × 2.1 mm Acquity BEH 1.7 μm C18 column (reverse-phase chromatography for the quantitation of CS and metabolite 561+). In addition, samples were analyzed using hydrophilic interaction chromatography (HILIC) columns (Acquity UPLC BEH Amide 1.7 μm 50 × 2.1 mm) for the quantitation of CR and NANA. Metabolite levels were quantitated using commercially available standards (CS and NANA), whereas CR was semiquantitated to creatine equivalents, and 561+ to CS equivalents due to lack of commercially available standards.

For analysis of tissue metabolite levels, fresh-frozen liver tumor specimens were pulverized by cryogenic grinding using a 5-mm stainless steel ball bearing (Cryomill) before being extracted in a monophasic mixture of 2:5:2 water:methanol: chloroform with aminopimelic acid internal standard. Extracted samples were centrifuged at maximum speed at 4°C for 20 minutes, and the supernatant was dried down by vacuum evaporation (Speedvac). Samples were reconstituted in 70% aqueous acetonitrile, of which 5 μL was injected for LC/MS-MS analysis. LC/MS-MS was performed as described above by employing HILIC chromatography and electrospray ionization in positive mode (Waters, Xevo TQ-S micro triple quad) on a C18 BEH Amide 1.7 μm 50 mm × 2.1 mm Column (Waters). Metabolite peak areas were normalized to internal standard, quantified using a standard curve, and reported normalized to the total tissue weight (Peirce BCA Assay). Samples with metabolite concentrations below the lower limit of detection were excluded from further quantitative analysis. Out of 51 analyzed HCC tissue samples, 5 samples for CR and 2 samples for NANA assays were below the lower limit of detection. Out of 48 analyzed ICC tissue samples, 1 sample for CR and 1 sample for NANA assays were below the lower limit of detection.

All data were processed using TargetLynx Software (Waters) to generate results from the acquired chromatographic data, permitting accurate quantitation and review of results, including evaluation of data quality and analyte confirmation. Internal standard-normalized areas under the peak from authentic standard solutions were used to build calibration curves, which were then used for the quantitation of metabolites in urine samples.

Statistical analysis

All analyses were performed using Stata Software, version 13 (Stata Statistical Software Release 13.1). All reported *P* values were two sided, and all *P* values less than or equal to 0.05 were considered statistically significant. One-way ANOVA with *post hoc* analysis was used to assess difference in metabolite levels across three groups (cases, high-risk subjects, and controls). Mann–Whitney (two-sample Wilcoxon rank-sum) test was used to analyze differences of metabolite levels between HCC and ICC cases. Reproducibility of duplicate measurements was assessed by intraclass coefficient analysis.

Unconditional logistic regression models were used to estimate ORs and 95% confidence intervals (CI) for the association of metabolites with liver cancer (when compared with controls and high-risk subjects), categorized into high and low based on the 75th percentile of abundance in controls. Multivariable models were adjusted for age, gender, and HBV and HCV infection status.

In exploratory analyses, we used the ROC modeling to assess the predictive value of identified metabolites in HCC and ICC using the roccomp function. Models were built using logistic regression on the continuous abundances of four metabolites, CA19-9 (ICC only), AFP (HCC only), and combined models (four metabolites + CA19-9 in ICC and four metabolites + AFP in HCC). Differences between the predictive abilities of the models containing only four metabolites and models containing metabolites with clinically established biomarkers (CA19-9 for ICC and AFP for HCC) were assessed using rocreg function.

Cox proportional hazards model was used to estimate HRs for the association of metabolites with liver cancer, categorized into high and low based on the 75th percentile of abundance in controls.

Results

Deregulated metabolite levels in the NCI-MD liver cancer cohort

We initially conducted a pilot study in 95 HCC cases, 101 high-risk subjects (hepatitis virus B– and/or C–infected subjects), and 98 population controls. Liver cancer cases in the NCI-MD cohort are predominantly HCC intrahepatic type. The goal was to initially assess whether four urinary metabolites

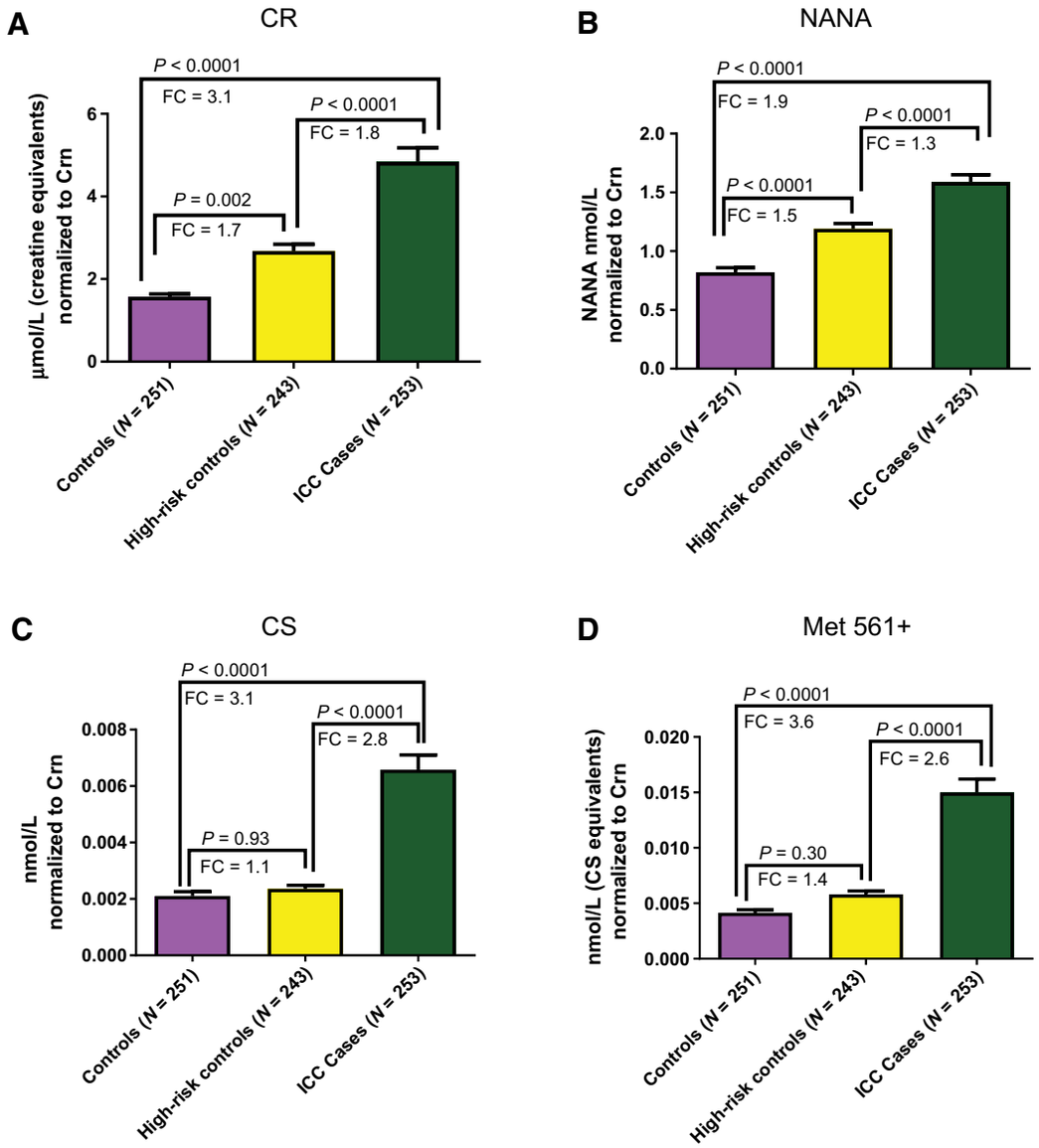

Figure 1.
Bar graphs of urinary metabolite levels of CR (**A**), NANA (**B**), CS (**C**), and metabolite 561+ (**D**) quantitatively measured by UPLC/MS-MS in ICC cases in comparison with unaffected population controls and high-risk subjects in the TIGER-LC cohort. Crn, creatinine.

previously identified in lung cancer could have any diagnostic utility in liver cancer before we embarked on a validation process in a larger, independent cohort. Controls and high-risk subjects were frequency matched to cases on age, gender, and race (Supplementary Table S1). This initial pilot study resulted in findings that all four urinary metabolites are increased in HCC cases compared with both population controls and, more importantly, high-risk subjects in the NCI-MD case–control cohort ($P < 0.0001$ for all four metabolites; Supplementary Fig. S1). These promising results prompted a validation study and a more thorough investigation of the four-metabolite profile's diagnostic potential in the independent TIGER-LC cohort, where ICC is highly prevalent.

Deregulated metabolite levels in the TIGER-LC liver cancer cohort

We next evaluated whether our previously described findings from the NCI-MD liver case–control study can be validated in the TIGER-LC cohort. As we observed elevated metabolites levels in HCC in the NCI-MD cohort, we further hypothesized that the four metabolites are elevated in ICC and may be able to aid traditionally utilized diagnostic methodology to increase the sensitivity and specificity of the ICC diagnosis. The study design is described in Table 1. High-risk patients for the HCC group comprised those who had hepatitis virus B, hepatitis virus C infection, or both (HBV and HCV, respectively), alcoholic liver disease, and fatty liver disease, as these are factors that predispose individuals for

Table 2. Logistic regression analysis of four metabolites in ICC cases when compared with population controls in the TIGER-LC cohort

	Univariable analysis				Multivariable analysis[a]			
Metabolite[b]	**Controls *N* (%)**	**Cases *N* (%)**	**OR (95% CI)**	***P***	**Controls *N* (%)**	**Cases *N* (%)**	**OR (95% CI)**	***P***
$Referent_{low}$			1.0				1.0	
CR_{high}	63 (25)	202 (80)	11.8 (7.8–18.0)	<0.0001	61 (26)	193 (81)	10.3 (6.0–16.8)	<0.0001
CS_{high}	62 (25)	171 (68)	6.4 (4.3–9.4)	<0.0001	55 (23)	160 (67)	7.2 (4.5–11.5)	<0.0001
$NANA_{high}$	63 (25)	167 (66)	5.8 (3.9–8.5)	<0.0001	61 (26)	157 (66)	5.8 (3.6–9.4)	<0.0001
$561+_{high}$	62 (25)	178 (70)	7.2 (4.9–10.7)	<0.0001	61 (26)	169 (71)	6.5 (4.1–10.3)	<0.0001

[a]Multivariable logistic regression analysis adjusted for age, gender, and HBV and HCV infection status.
[b]Metabolite levels dichotomized on the basis of the 75th percentile of abundance in controls.

HCC development (8). High-risk patients for the ICC group comprised those who had a known liver fluke infection, a factor associated with the development of ICC (9).

Data were first evaluated for sufficient quality. Our initial quality control (QC) assessment included an assessment of reproducibility of 13% of duplicates included in the study. As seen in Supplementary Table S2, intraclass correlation coefficient analysis shows that the duplicated measurements are highly correlated and reproducible, as indicated by significant intraclass coefficients above 0.97. Furthermore, we included a QC sample designated as a "pool" after every 20 injections. This pooled sample was contrived in such way that equal urine volumes from 200 subjects were combined and mixed. This sample was utilized to track retention time shifts and reproducibility of measurements. As indicated in Supplementary Fig. S2, coefficients of variation of pooled when compared with the remaining urine samples for each metabolite of interest measured in this study were found to be significantly smaller ($P < 0.0001$), indicating a high reproducibility of metabolite measurements using UPLC/MS-MS.

Assured of high quality of the acquired data as a result of the QC assessment, we then evaluated whether the four metabolite levels are significantly increased in liver cancer. The results of this analysis indicated that the four metabolites were found to be elevated in ICC when compared with unaffected controls and high-risk subjects ($P < 0.0001$; Fig. 1). We observed similar findings in HCC, thus directly validating findings from the NCI-MD liver cancer cohort, with significant differences of the four metabolites when HCC cases were compared with unaffected controls and high-risk subjects ($P < 0.003$; Supplementary Fig. S3). We were additionally interested whether the levels of four metabolites differ between HCC and ICC cases. When we compared HCC to ICC cases in TIGER-LC, we found that three of the four metabolites, CR, NANA, and metabolite 561+, are significantly increased in ICC. CS did not display any observable differences in levels between the two types of primary intrahepatic carcinomas (Supplementary Fig. S4).

Associations with liver cancer status

We next assessed whether the four metabolites are associated with liver cancer when compared with both unaffected population controls and high-risk subjects in logistic regression analysis before and after adjusting for a number of putative confounders (age, gender, and HBV and HCV infection status). Metabolite levels were dichotomized on the basis of the 75th percentile of abundances in unaffected controls. While the comparisons of metabolite levels in ICC cases when compared with the unaffected controls were all significant and showed large effect sizes (Table 2), the more relevant analysis was conducted in comparison with high-risk subjects. In the comparison of ICC cases and high-risk subjects, high levels of all four metabolites were significantly associated with the disease status even after the adjustment for putative confounders (Table 3). We observed similar associations when HCC cases were compared with unaffected controls and high-risk subjects (Supplementary Tables S3 and S4). These results indicated that high levels of each of the four metabolites may be robust biomarkers of intrahepatic liver cancer and most significantly of ICC. The ability of the combination of the four metabolites to aid currently available molecular methods for the detection of ICC was evaluated next.

ROC analysis

We utilized ROC analysis to assess the ability of the urinary four-metabolite profile to classify ICC and found that the four-metabolite profile is superior to that of CA19-9 (AUC = 0.81 vs. 0.77, respectively; Fig. 2). Performances of the metabolites, CA19-9, and their combination were evaluated with respect to ICC high-risk controls. The selected cutoff point of 100 U/L for CA19-9 was based on the prior literature indicating a diagnostic clinical significance of such cutoff point (32). The selected cutoff point for the four-metabolite profile was chosen based on a point at which the sensitivity and specificity of the model were the highest. Most importantly, the combination of the four metabolites and CA19-9 led to a significantly improved classifier ($P = 4E\text{-}8$) with high sensitivity (71%), specificity (92%), positive predictive value (PPV = 89%), and negative predictive value (NPV = 78%). These findings suggest a strong possibility that the four metabolites may have a robust potential to aid in diagnosis of ICC as an adjunct to CA19-9, the most commonly utilized clinical biomarker of ICC. In comparison, the four-metabolite profile did not add significantly to the model containing a clinically utilized

Table 3. Logistic regression analysis of four metabolites in ICC cases when compared with high-risk subjects in the TIGER-LC cohort

	Univariable analysis				Multivariable analysis[a]			
Metabolite[b]	**Controls *N* (%)**	**Cases *N* (%)**	**OR (95% CI)**	***P***	**Controls *N* (%)**	**Cases *N* (%)**	**OR (95% CI)**	***P***
$Referent_{low}$			1.0				1.0	
CR_{high}	136 (56)	202 (80)	3.1 (2.1–4.6)	<0.0001	135 (56)	160 (80)	3.2 (2.1–5.1)	<0.0001
CS_{high}	53 (22)	171 (68)	7.5 (5.0–11.2)	<0.0001	53 (22)	136 (68)	7.9 (5.1–12.2)	<0.0001
$NANA_{high}$	83 (34)	167 (66)	3.7 (2.6–5.4)	<0.0001	83 (34)	132 (66)	3.7 (2.4–5.6)	<0.0001
$561+_{high}$	74 (46)	178 (70)	5.4 (3.7–8.0)	<0.0001	74 (31)	139 (70)	5.5 (1.4–5.0)	<0.0001

[a]Multivariable logistic regression analysis adjusted for age, gender, and HBV and HCV infection status.
[b]Metabolite levels dichotomized on the basis of the 75th percentile of abundance in controls.

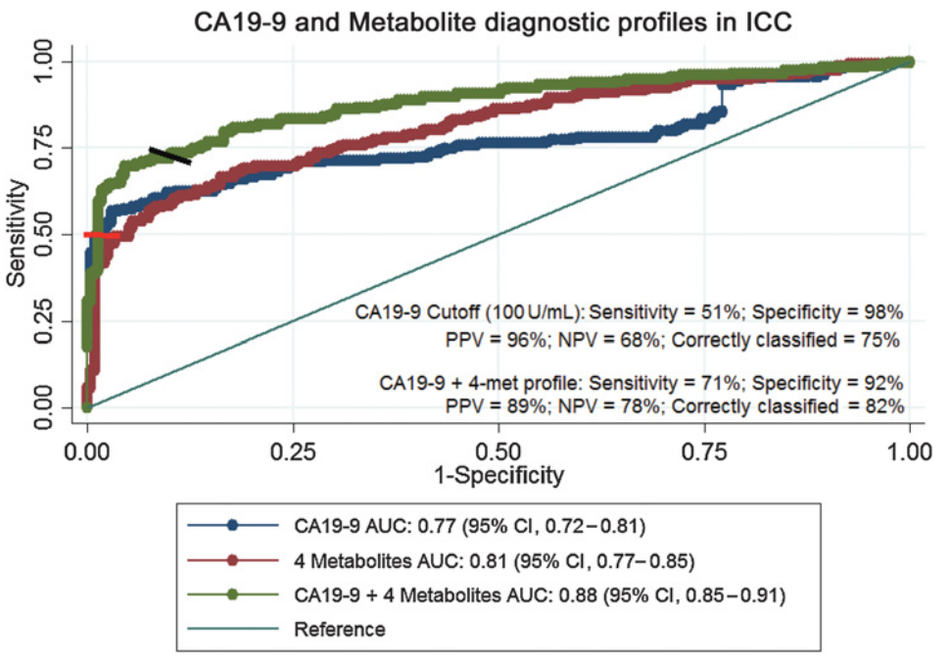

Figure 2.
ROC analysis of the models comprising four urinary metabolites, CR, NANA, CS, and metabolite, 561+, in comparison with and in addition to a clinically utilized biomarker of ICC, CA19-9, in the TIGER-LC cohort.

marker for HCC, alpha-fetoprotein (AFP; Supplementary Fig. S5). Performances of the metabolites, AFP, and their combination were evaluated with respect to HCC high-risk controls.

Deregulated metabolite levels in cancer tissue in the TIGER-LC liver cancer cohort

We hypothesized that urinary CR and NANA may derive from tumor tissue, as has been previously observed in lung cancer (27). To that extent, we measured CR and NANA in a tissue set from the TIGER-LC cohort comprising 48 ICC and 51 HCC cases, with a goal of detecting these metabolites in tumor tissue, as well as evaluating their levels when ICC was compared with HCC. Consistent with our observations in urine, we found that both CR and NANA were significantly elevated in ICC when compared with HCC ($P = 0.002$ and $P = 0.02$, respectively; Fig. 3).

Association with liver cancer survival

We finally interrogated the association of metabolites with liver cancer survival. We observed an association between high levels of CR and ICC poorer survival, as illustrated by the Kaplan–Meier survival estimates in Supplementary Fig. S6A (HR = 1.7; $P = 0.001$). We then evaluated whether the observed association between CR and higher ICC mortality persists after adjusting for age, gender, stage, and ALT and ALP liver enzyme concentrations, with the latter to control for liver function. Metabolite levels were normalized to creatinine to control for kidney function. After the adjustment for the aforementioned putative confounders in ICC cases comprising all stages, the observed association was borderline significant, although the effect magnitude and direction remained similar (HR = 1.7, $P = 0.001$ vs. $HR_{adjusted} = 1.4$, $P = 0.06$). We then stratified the analysis by stage (stages I–II vs. stages III–IV). The association between CR and higher ICC mortality was significant in early ICC stages I through II, even after the adjustment for the aforementioned putative confounders (HR = 2.1, $P = 0.05$ vs. $HR_{adjusted} = 2.2$, $P = 0.04$; Supplementary Fig. S6B). The association between CR and ICC mortality was not significant in ICC stages III through IV (HR = 1.3, $P = 0.36$ vs. $HR_{adjusted} = 1.1$, $P = 0.74$; Supplementary Fig. S6C).

Discussion

We previously identified novel urinary metabolite biomarkers in lung cancer, which proved to be robust in distinguishing lung cancer cases from nondiseased controls, even prior to

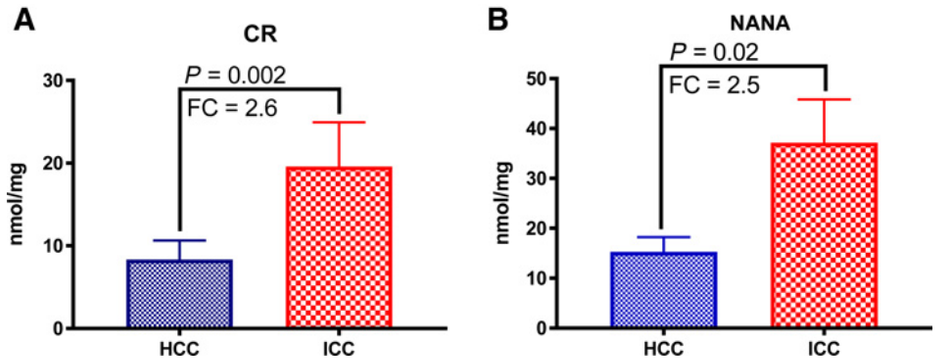

Figure 3.
Bar graphs of tissue metabolite levels of CR (**A**) and NANA (**B**) compared between HCC and ICC cases in the TIGER-LC cohort.

diagnosis (26, 27). Considering that one of the common colonization sites for metastatic lung cancer is liver (33–36), pointing to a favorable environment for cancer cells to thrive, we wondered whether the four urinary metabolite biomarkers may be able to distinguish liver cancer cases from high-risk patients (e.g., harboring HBV and HCV infections)—individuals who would be subject to liver cancer screening. While the two cancer types share some of the mutual metabolic characteristics, such as deregulated lipid metabolism (23, 37, 38), not enough is known about the similarities between lung and liver cancer metabolism or about overlapping products of metabolism that may serve as robust biomarkers in both cancer types. We therefore hypothesized that the four metabolites previously described as robust markers of lung cancer (27), even 2 or more years prior to diagnosis (26), may demonstrate a significant diagnostic and prognostic potential in liver cancer. Our initial findings from a pilot study conducted in the NCI-MD cohort showed that four metabolites, CR, NANA, CS, and metabolite 561+, are indeed significantly increased in HCC. This was true not only for the comparison between HCC and population controls but more importantly, for the comparison between HCC and high-risk controls (subjects with HBV and HCV infections). The latter comparison was more important because liver cancer screening is not conducted in the general population but rather in those who are at high risk for developing this disease. The initial results from the pilot study pointed to a strong possibility that the four-metabolite profile may have utility in the diagnosis of liver cancer, which required a validation in a larger and independent cohort. Most importantly, we were interested in evaluating these metabolites in ICC, a less well-characterized type of intrahepatic liver cancer that suffers from lack of robust diagnostic markers, with a goal of identifying biomarkers that may be able to eventually aid in the detection of this aggressive disease.

We validated the findings of four metabolites being significantly increased in HCC in an independent TIGER-LC cohort in the Thai population, as well as made novel observations of the same metabolites being significantly elevated in ICC. Because of a sufficiently powered study, we were able to investigate categorical associations of the four metabolites with HCC and ICC status. While the metabolites displayed significant and large effect sizes for both types of intrahepatic liver cancer, the effect sizes were larger and associations stronger in ICC. A stronger association in ICC is consistent with the elevated levels of these metabolites in ICC compared with HCC. This is a significant finding because there are currently no robust clinical biomarkers to aid in the diagnosis of ICC. While serum marker CA19-9 is utilized, its clinical utility is variable as it is often falsely elevated in benign biliary disease and/or cholangitis, with levels falling after relief of biliary obstruction and sepsis. We further showed that the four urinary metabolites have the potential to be used as an adjunct to CA19-9 as an aid in diagnosis of ICC. The combination of the four metabolites and CA19-9 led to a substantial diagnostic potential with a sensitivity of 71% and specificity of 92%. A significant increase in sensitivity of the model comprising four metabolite biomarkers and CA19-9 versus CA19-9 alone, 71% versus 51% respectively, suggests a possible improvement in early ICC detection.

In this study, we further showed that CR and NANA can be measured in liver cancer tissue, and that these metabolites are also elevated in ICC when compared with HCC. This is a novel finding that can help characterize ICC, a less well-characterized type of primary intrahepatic carcinoma. It remains to be investigated in future studies whether these metabolite biomarkers present a therapeutic opportunity for monitoring disease progression in ICC given their expression in the tumor tissue and detection in noninvasively collected urine specimens.

We finally hypothesized that levels of metabolites measured in noninvasively collected urine may serve as prognostic biomarkers. We only observed an association with CR, the high levels of which were found to be associated with poorer prognosis in ICC. This association appeared to be driven by early stage I through II ICC cases, indicating that CR may be a useful early prognostic marker of ICC when therapeutic intervention may be possible and more effective. This presents a novel finding and a potentially useful *in vitro* diagnostic assay that may be able to select those patients who would benefit from more aggressive treatments. This result warrants further investigation in an independent cohort.

The findings of this study remain to be investigated in a prospective cohort in the intended-use population, namely high-risk subjects who would be screened for liver cancer. The results of such study may further indicate whether the four-metabolite profile has future clinical utility for diagnosis and prognostic evaluation of ICC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M. Haznadar, C. Mahidol, X.W. Wang, C.C. Harris
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Haznadar, E.D. Bowman, M. Forgues, A. Budhu, X.W. Wang
Study supervision: M. Haznadar, F.J. Gonzalez, X.W. Wang, C.C. Harris
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Received April 20, 2019; revised June 6, 2019; accepted July 23, 2019; published first July 29, 2019.

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