Urinary Metabotyping of Hepatocellular Carcinoma in a UK Cohort Using Proton Nuclear Magnetic Resonance Spectroscopy

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Background: **Discriminatory metabolic profiles have been described in urinary ^{1}H nuclear magnetic resonance (NMR) spectroscopy studies of African patients with hepatocellular carcinoma (HCC). This study aimed to assess similarities in a UK cohort, where there is a greater etiological diversity.** ***Methods:*** **Urine from cirrhosis and HCC patients was analyzed using a 600 MHz ^{1}H NMR system. Multivariate analysis and median group MR spectra comparison identified metabolite alterations between groups. Metabolite identification was achieved through literature reference and statistical total correlation spectroscopy. Diagnostic accuracy was compared to serum alpha-fetoprotein (AFP).** ***Results:*** **Of the 52 patients recruited, 13 samples from HCC and 25 from cirrhosis patients were selected. At 200 IU mL^{-1}, diagnostic sensitivity of AFP was 27%. Multivariate analysis of urinary spectra generated diagnostic models with a sensitivity/specificity of 53.6%/96%. p-Cresol sulfate (P = 0.04), creatinine (P = 0.03), citrate (P = 0.21) and hippurate (P = 0.52) were reduced in the HCC patients. Carnitine (P = 0.31) and formate (P = 0.44) were elevated.** ***Conclusion:*** **Diagnostic sensitivity was lower than previous African studies, but still outperformed serum AFP. Reduced creatinine, citrate and hippurate and elevated carnitine are comparable with the African studies. p-Cresol sulfate alteration is a novel finding and may indicate an altered sulfonation capacity of the liver in patients with HCC.** **(J Clin Exp Hepatol 2016;6:186–194)**

Hepatocellular carcinoma (HCC) is the commonest primary liver cancer and the second global cause of cancer mortality. Although the heaviest disease burden is in sub-Saharan Africa and Eastern Asia,[1] recent evidence suggests HCC incidence is rising in previously low-risk countries such as the United Kingdom.[2]

The 5-year mortality for HCC in the developed world is poor.[3] The Surveillance Epidemiology and End Results (SEER) database from the United States documents a 9.9% HCC 5-year survival, compared to over 60% for all tumors.[4] The current HCC surveillance guidelines in the developed world recommend 6-monthly hepatic ultrasonography (US) in patients with cirrhosis, but requirement for serum alpha-fetoprotein (AFP) measurement has been dropped, owing to poor sensitivity and specificity of this test.[5,6] However, a retrospective SEER-database based study between 1994 and 2001 reported 6-monthly regular surveillance rates of only 17% in patients over 65 years with cirrhosis.[7] This highlights problems concerning patient attendance under surveillance, in particular those who live greater distances from medical centers.

''Metabonomics'', the study of metabolic responses to physiological, drug and disease stimuli, may be utilized to identify profiles of biomarkers that characterize HCC. The

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Abbreviations: ^{1}H NMR: proton nuclear magnetic resonance; AFP: alpha-fetoprotein; ALT: alanine transaminase; BCLC: Barcelona Clinic Liver Cancer; BMI: body mass index; HBV: hepatitis B virus; HCC: hepatocellular carcinoma; HCV: hepatitis C virus; HIV: human immunodeficiency virus; INR: International Normalized Ratio; NASH: non-alcoholic steatohepatitis; PCA: principal component analysis; PLS-DA: partial least squares discriminant analysis; SEER: surveillance Epidemiology and End Results; STOCSY: statistical total correlation spectroscopy; TSP: trimethyl-silyl phosphate; US: ultrasonography
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most common metabonomic technique is proton nuclear magnetic resonance (^{1}H NMR) spectroscopy.[8] Previous ^{1}H NMR studies performed in Nigerian, Egyptian and Gambian patients have identified a number of altered metabolites in the urine, implicating changes in hepatic function according to Warburg's hypothesis of altered metabolism.[9–11] Applying this theory, a simple, non-invasive test that identifies tumor profiles from the urine would be invaluable for cirrhosis patients, potentially allowing surveillance in the community by local general practitioners or even patients themselves.

The etiological variation of HCC patients in the UK is greater than African patients, including viral hepatitis, alcohol and non-alcoholic steatohepatitis (NASH). The collection of samples in the UK also allowed detailed patient and disease demographics to be collated.

The main aims of this study were to identify tumor-specific urinary metabolite changes in patients with HCC of varying etiology and to compare findings to previous African studies with larger tumors, caused solely by viral hepatitis.

MATERIALS AND METHODS

Patient Selection

Patients were recruited at six hospital sites around the United Kingdom: London, Manchester, Newcastle, Nottingham, Plymouth and Southampton. All subjects gave written, informed consent, in accordance with local Research Ethics Committee approval (LREC reference no. 06/Q041/10).

HCC was diagnosed with two confirmatory imaging modalities and cirrhosis with histological and/or radiological confirmation. Tumor staging was according to the Barcelona Clinic Liver Cancer (BCLC) staging system,[5] based upon tumor size and multiplicity, patient performance status, presence or absence portal vein invasion, presence or absence of portal hypertension, and Child–Pugh scores of liver function.[12,13] Patients with cirrhosis were graded according to the Child–Pugh score. Participant height, weight, medical, drug, smoking, and alcohol intake history were recorded. A 24-h dietary history was also recorded, paying attention to foodstuffs known to produce prominent nuclear magnetic resonance (NMR) signals, such as vanilla (vanillin) and vinegar (acetic acid). Exclusion criteria included those patients not meeting the diagnostic criteria cited above for HCC and cirrhosis, those patients with HCC who had undergone curative resection or transplant, patients co-infected with human immunodeficiency virus (HIV) and those samples identified as outliers on principal component analysis.

Sample Collection

15 mL of random, non-fasted urine were collected into preservative-free universal containers and placed immediately on ice and centrifuged within 2 h at 4 °C, 1000 rpm for 10 min. The supernatant was then transferred as 2 mL aliquots into 2 mL microvial tubes and stored at −80 °C until analysis.

Sample Preparation

Urine samples were thawed at room temperature and 400 µL were transferred to 1.5 mL microvial tube to which 200 µL of phosphate buffer solution (pH 7.4), containing 1 mM trimethyl silyl phosphate (TSP), sodium azide 3 mM (as a bacterial biocidal agent to inhibit bacterial growth and contamination) and D_2O 20%. Samples were centrifuged for 5 min at 13,000 rpm and 550 µL of supernatant were transferred to 5 mm NMR tubes (Norell, Landisville, NJ, USA).

Proton Nuclear Magnetic Resonance Spectroscopy

Samples were run in random non-grouped order under automation on a Bruker DRX-600 NMR system operating at 600.44 Hz ^{1}H frequency (Bruker Biospin, Rheinstetten, Germany). The system was tuned, matched and frequency locked to deuterated hydrogen as the nucleus of interest. A representative sample was utilized to set shim gradients to ensure a homogenous magnetic field across the sample, a 90° pulse length and water suppression offset parameters.[14] Spectra were acquired at 300 K using a one-dimensional (1-D) noesypr1d pulse sequence with water presaturation during relaxation delay (RD) and mixing time (t_m) using the following pulse program: -RD-90°_t-90°-t_m-90°-acquire; where RD = 2.0 s and t_m = 0.1 s. For each sample, 128 FIDs were collected into 32,000 data points with a spectral width of 20 ppm. A line broadening function of 0.3 Hz was applied prior to Fourier transformation. Spectra were manually phased, baseline corrected and referenced to TSP at 0 ppm using TOPSPIN v2.0 (Bruker Biospin, Rheinstetten, Germany). Spectral peaks were assigned with reference to the literature.[15–17]

Spectral Processing

Proton NMR spectra were exported to MATLAB R2010 (MathWorks, Natick, MA, USA) and to avoid influence on analyses from water suppression aberration, the water region from 4.5 ppm to 6 ppm was excluded. Spectra were aligned to negate the effect of pH dependent variation in metabolite resonances which may occur despite sample buffering. To remove the effect of differential urine concentrations, data were normalized using median fold-change normalization. Median spectra for both clinical groups were generated to allow direct visual comparison of average spectra and allow the selection of regions that were visually divergent, in addition to those identified by multivariate analysis, for peak integration.