The Plasma and Serum Metabotyping of Hepatocellular Carcinoma in a Nigerian and Egyptian Cohort using Proton Nuclear Magnetic Resonance Spectroscopy

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Background/Aims: **Previous studies have observed disturbances in the ^{1}H nuclear magnetic resonance (NMR) blood spectral profiles in malignancy. No study has metabotyped serum or plasma of hepatocellular carcinoma (HCC) patients from two diverse populations. We aimed to delineate the HCC patient metabotype from Nigeria (mostly hepatitis B virus infected) and Egypt (mostly hepatitis C virus infected) to explore lipid and energy metabolite alterations that may be independent of disease aetiology, diet and environment.** ***Methods:*** **Patients with HCC (53) and cirrhosis (26) and healthy volunteers (19) were recruited from Nigeria and Egypt. Participants provided serum or plasma samples, which were analysed using 600 MHz ^{1}H NMR spectroscopy with nuclear Overhauser enhancement spectroscopy pulse sequences. Median group spectra comparison and multivariate analysis were performed to identify regions of difference.** ***Results:*** **Significant differences between HCC patients and healthy volunteers were detected in levels of low density lipoprotein ($P = 0.002$), very low density lipoprotein ($P < 0.001$) and lactate ($P = 0.03$). *N*-acetylglycoproteins levels in HCC patients were significantly different from both healthy controls and cirrhosis patients ($P < 0.001$ and 0.001).** ***Conclusion:*** **Metabotype differences were present, pointing to disturbed lipid metabolism and a switch from glycolysis to alternative energy metabolites with malignancy, which supports the Warburg hypothesis of tumour metabolism.** **(J Clin Exp Hepatol 2017;7:83–92)**

Hepatocellular carcinoma (HCC) is the second commonest cause of cancer-related death and bears a poor prognosis in developing countries due to late diagnosis.[1–3] Curative treatment options, namely orthotopic liver transplantation and surgical resection, are limited to low-grade cancers that are identified early.[4] The widely accepted HCC screening using serum alpha-fetoprotein (AFP), a foetal glycoprotein, has shown evidence of improvement in mortality and morbidity.[5] Although most HCC tumours secrete AFP, the tumour marker has poor sensitivity and specificity of less than 70%.[6–8] Furthermore, serum AFP testing is unavailable in many parts of Africa, where HCC is most prevalent.

"Metabonomics" is the study of global metabolic responses to physiological, drug and disease stimuli. The most commonly used method of metabolite characterisation is proton nuclear magnetic resonance (^{1}H NMR) spectroscopy.[9] There is a paucity of data concerning the value of blood profiling using ^{1}H NMR in HCC, but previous studies have identified a number of altered metabolites, implicating changes in hepatic function, lipid metabolism and bile acid metabolism.[10–13] Heterogeneity in genotype, diet, environment, co-morbid status and liver disease aetiological factors in man, may influence the ability to translate these findings to human disease.[14]

Keywords: hepatocellular carcinoma, Nigeria, Egypt, proton nuclear magnetic resonance spectroscopy, serum metabotype
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Abbreviations: ^{1}H NMR: proton nuclear magnetic resonance; HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NOESY: Nuclear Overhauser enhancement spectroscopy; LDL: Low density lipoprotein; JUTH: Jos University Teaching Hospital; US: Ultrasonography; CT: Computed Tomography; MRI: Magnetic resonance imaging; WHO: World Health Organisation; EDTA: Ethylenediaminetetraacetic acid; ALT: Alanine transaminase; ALP: Alkaline phosphatase; AFP: α-fetoprotein; IQR: Interquartile ranges; 1-D: One-dimensional; RD: Relaxation delay; t_m: Mixing time; FID: Free induction decays; PCA: Principal components analysis; PLS-DA: Partial least squared discriminant analysis; HBsAg: Hepatitis B surface antigen; ELISA: Enzyme-linked immunosorbent assay; VLDL: Very low density lipoprotein; ppm: Parts per million; PC: Principal component; PPARα: Peroxisome proliferator-activated receptor α; IDL: Intermediate density lipoprotein
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One previous study, performed in a Chinese population utilised ^{1}H NMR of serum to discriminate patients with HCC (n = 39) from patients with cirrhosis (n = 36).[15] In this study, alterations were observed in levels of lipoproteins, amino acids, *N*-acetylglycoproteins, ketoacids and lipids. Unfortunately, no information was provided on age, gender or liver disease aetiology of the participants, which is particularly relevant when utilising this method to distinguish patients with cancer to those without. In 1986, Fossel and colleagues proposed using the line widths of methyl (CH_3) and methylene ($(CH_2)_n$), measured by 400 MHz ^{1}H NMR spectroscopy, as a sensitive test for cancer.[16] Levels of these metabolites were found to be significantly elevated in patients with a variety of tumours (n = 81). A number of validation studies performed on similar cohorts of patients using similar or higher magnetic field strengths, refuted this finding, citing age, triglyceride content and number of freeze thaw cycles as confounding variables that were likely to have contributed to Fossel's original findings.[17–20]

We have previously found discriminatory metabolites for HCC using urinary metabolic profiling with ^{1}H NMR spectroscopy in Nigerian, Egyptian and Gambian populations.[21–23] The aim of the study presented here was to investigate whether serum and plasma ^{1}H NMR profiles, collected in parallel with the published urinary studies, are different in patients with HCC compared to patients with cirrhosis and healthy volunteers in well-characterised populations from Nigeria and Egypt. These study populations were subject to widely different environmental, dietary and aetiological factors.

METHODS

Patient and Healthy Volunteer Selection

Subjects were recruited in two cohorts from Jos University Teaching Hospital (JUTH) in Nigeria and The National Liver Institute, Menoufiya University, Shebeen El Kom, Egypt. The Nigerian study protocol was approved by the research ethics committee of JUTH, Nigeria and the Egyptian protocol by Menoufiya University, Egypt. The metabolic profiling protocol was approved by the research ethics committee of Imperial College London, UK. All volunteers provided informed, signed consent.

Hepatocellular carcinoma was diagnosed by radiological measures: ultrasonography (US) and/or computed tomography (CT) in Nigeria, while in Egypt, CT or magnetic resonance imaging (MRI) was used. Cirrhosis was diagnosed on clinical findings, by the presence of portal hypertension (esophageal varices or ascites) and US or CT confirmation. Tumours were staged according to the Okuda system, which includes tumour size, the presence of ascites, bilirubin and albumin levels as its criteria.[24] This scoring method was chosen out of necessity as other, more comprehensive scoring tools, such as the Barcelona Clinic Liver Cancer staging algorithm, require World Health Organisation (WHO) performance status, presence of portal vein invasion and encephalopathy as criteria, which were not recorded for most of the Nigerian patients in this study, owing to lack of axial imaging resources.

Sample Collection

5 mL fasted blood samples were venesected into either plain serum or ethylenediaminetetraacetic acid (EDTA)-containing sterile tubes and placed immediately on ice or into a refrigerator at 4 °C. Samples were centrifuged within 1–2 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 undergoing no freeze thaw-cycles until analysis. Forty-eight of 56 Nigerian samples were collected into tubes containing EDTA as an anticoagulant. The remainder of samples were collected into plain serum tubes. All of the Egyptian samples were collected into plain serum tubes, with no additives. Previous studies have reported similar ^{1}H NMR metabolic profiles from serum and plasma, allowing the two to be compared with relative assurance.[25–27] These studies highlight the fact that clinical differences between groups were profoundly more influential than spectral differences between EDTA plasma and plain serum samples.

Blood Laboratory Tests

For the Nigerian samples, serum urea, creatinine, alanine transaminase (ALT), alkaline phosphatase (ALP), total bilirubin and albumin levels were measured using automated techniques (Abbott™ Architect Ci16200 Analyser, UK) at St Mary's Hospital, London. Serum AFP was measured using an automated Siemens™ Immulite 2500 Analyser, (Deerfield, USA). For the Egyptian samples, serum AFP, creatinine, ALT, aspartate aminotransferase (AST), bilirubin and albumin were measured at the time of collection in Egypt using a Cobas Integer 400-Autoanalyzer, (Roche, Germany). Median and interquartile ranges (IQR) were calculated for each assay and median levels were compared using unpaired Mann–Whitney tests of significance.

Sample Preparation

Samples were prepared according to standard validated protocols.[28] Samples were thawed at room temperature and 200 µL were transferred into 1.5 mL Eppendorf (Cambridge, UK) tubes to which 400 µL NaCl/D_2O (90%/10%) were added. External reference standards, such as 3-trimethylsilyl-(2,2,3,3-$^{2}H_4$)-1-propionate (TSP), were not added, as in blood they may bind to protein, resulting in a final NMR signal that is reduced and has a very broad linewidth.