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Metabolomic profiling of human urine in hepatocellular carcinoma patients using gas chromatography/mass spectrometry

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ABSTRACT

With the technique of metabolomics, gas chromatography/mass spectrometry (GC/MS), urine or serum metabolites can be assayed to explore disease biomarkers. In this work, we present a metabolomic method to investigate the urinary metabolic difference between hepatocellular carcinoma (HCC, $n = 20$) male patients and normal male subjects ($n = 20$). The urinary endogenous metabolome was assayed using chemical derivatization followed by GC/MS. After GC/MS analysis, 103 metabolites were detected, of which 66 were annotated as known compounds. By a two sample t-test statistics with $p < 0.05$, 18 metabolites were shown to be significantly different between the HCC and control groups. A diagnostic model was constructed with a combination of 18 marker metabolites or together with alphafetoprotein, using principal component analysis and receiver–operator characteristic curves. The multivariate statistics of the diagnostic model yielded a separation between the two groups with an area under the curve value of 0.9275. This non-invasive technique of identifying HCC biomarkers from urine may have clinical utility.

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most prevalent malignant tumour worldwide and ranks the third as a cancer killer, causing more than half a million deaths annually in the world [1,2]. The prognosis of HCC largely depends on the stage of the tumor. Early diagnosis can be obtained with surveillance of patients at risk using diagnostic modalities such as ultrasound (US) [3]. The commonly used serology tests for screening such as alphafetoprotein (AFP) are no longer considered as powerful screening tools in patients with chronic liver disease due to high false positive and false negative rates [4,5]. The ideal biomarker should be the one that can be detected with good sensitivity and specificity in biological samples from the patient in a minimally invasive manner (e.g., blood, saliva, or urine). Recently, metabolomic profiling approaches have been increasingly used to elucidate significant changes in tumor metabolism and to explore candidate "biomarkers" from such variance within a huge number of endogenous metabolites.

Blood and urine are the most frequently used samples for exploring the systematic alteration in human metabolome. Compared with blood sample, utilization of urine samples is preferred as it enables non-invasive monitoring of metabolomic changes.

Metabolomics, defined as the quantitative measurement of all low-molecular-weight metabolites in an organism at a specified time under specific environmental conditions [6], has been shown to be an effective tool for disease diagnosis [7,8], biomarker screening [9,10], and characterization of biological pathways [11]. Metabolomic studies generally employ such techniques as nuclear magnetic resonance (NMR), high-performance liquid chromatography/mass spectrometry (HPLC/MS, and LC/MS/MS), Fourier transform infrared (FT/IR) spectroscopy, and gas chromatography/mass spectrometry (GC/MS) [12]. Among them, GC/MS has been described as a sensitive and reproducible method, which *have been proposed as an* ideal tool for metabolomic profiling of urine *samples* [13]. Metabolic profiling of urine has been successfully performed by GC/MS using bis-(trimethylsilyl)–trifluoroacetamide (BSTFA) as the derivatization agent [13,14].

The aims of this study were to compare metabolite profiling from urine samples between HCC patients and healthy subjects using GC/MS and chemical derivatization and to establish a diagnotic model from these metabolic biomarkers to distinguish HCC from the normal subjects, using principal components analysis (PCA).

2. Experimental

2.1. Chemicals and reagents

L-2-chlorophenylalanine as internal standard was purchased from Shanghai Intechem Tech. Co. Ltd. (Shanghai, China). Methanol

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(pesticide residue grade), bis-(trimethylsilyl)–trifluoroacetamide (BSTFA) plus 1% trimethylchlorosilane (TMCS) and amino acid standard solution were purchased from Sigma–Aldrich (St Louis, MO,USA). All other chemicals and reagents were purchased from Ampu Company (Shanghai, China). Distilled water was produced by the Milli-Q ReagentWater System (Millipore, MA, USA).

2.2. Patient recruitment and sample collection

Ethics Committee of Zhongshan Hospital approved the study. Informed consent was obtained from each participant. Twenty hospitalized male patients aged 30–53 years with histopathologically confirmed HCC from November 2008 to December 2008 in Zhongshan Hospital, Fudan University, Shanghai, China, were included in this study. Twenty healthy male cases, aged 35–58 years, were selected as normal controls. In the exploratory study, in order to improve the matching rate of subjects, all groups were male. Whole first-morning urine samples were collected in glass cuvettes and centrifuged within 1 h at 3000 rpm for 10 min at room temperature. The supernatant was aliquotted into Eppendorf tubes with 1 mL urine in each and stored at −80 °C until use. And there were no diet or other restrictions in the sample collection [15].

2.3. Serum alanine aminotransferase (ALT) and AFP tests

A Hitachi 7181 automatic biochemistry analyzer and a Roche cfas calibrator were used for the serum ALT test, the result of which was in accordance with that tested by the International Federation of Clinical Chemistry (IFCC) recommended method [16]. Serum AFP was tested by enzyme-linked immunosorbent assay (ELISA). This assay was based on simultaneous binding of human AFP to two monoclonal antibodies. Absorption at 450 nm was counted using an ELISA microwell plate reader (Bio-Rad, USA). The test results of the two items were from the same period clinical date.

2.4. Specimen processing

Urine sample was thawed by incubation at 37 °C for 3 min and vortex-mixed for 15 s. 800 μL methanol and 100 μL L-2-chlorophenylalanine (0.05 mg mL^{-1}) were added into each 1 mL urine in an Eppendorf tube. The solution was vortex-mixed for 5 min and ultrasonicated at room temperature for 5 min. After pH carefully being adjusted to 9–10 using NaOH (0.5 mol L^{-1}), the solution was filtrated by 0.45 μm membrane. Then 100 μL of the resulting filtrate was transferred to a screw vial (2 mL) with PTFE-lined screw caps (Pyrex, UK) and evaporated to dryness under a stream of nitrogen gas. We added 100 μL BSTFA with 1% TMCS to each vial, and left the mixture to react for 1 h in a microwave oven (Haier Co. Qidao, Shandong, China) at a temperature of 100 °C [9,17–19].

2.5. GC/MS analysis

A 1 μL aliquot of derivatized sample was injected splitless into an Agilent 6980 GC system equipped with a 30.0 m × 0.25 mm i.d. fused-silica capillary column with 0.25 μm HP-5MS stationary phase (Agilent, Shanghai, China). The injector temperature was set at 250 °C. Helium was used as carrier gas at a constant flow rate of 1 mL min^{-1} through the column. The column temperature was initially kept at 80 °C for 3 min and then increased from 80 to 280 °C at 10 °C min^{-1}, where it was held for 2 min. The column effluent was introduced into the ion source of an Agilent 5973 mass selective detector (Agilent Technologies). The MS quadrupole temperature was set at 150 °C and the ion source temperature at 230 °C. Masses were acquired from *m*/*z* 50 to 800. The mass accuracy of the instrument was 0.1 atomic mass unit (amu). The acceleration voltage was turned on after a solvent delay of 180 s. GC/MSD ChemStation Software (Agilent, Shanghai, China) was used for auto-acquisition of GC total ion chromatograms (TICs) and fragmentation patterns. As each compound has a fragmentation pattern composed of a series of split molecular ions, the mass charge ratios and the abundance of which can be compared with a standard mass chromatogram in the NIST (National Institute of Standards and Technology) mass spectra library by the ChemStation Software. For each peak, the software generated a list of similarities comparing substances with each other within the NIST library. Peaks with similarity index more than 80% were assigned compound names, while those having less than 80% similarity were listed as unknown metabolites [20].

The chromatograms were subjected to noise reduction and peaks with an intensity higher than threefold of the signal-to-noise (S/N) *ratio* were recorded prior to peak area integration. The relative intensity of each peak was normalized against the internal standard in GC/MS run. All known artifact peaks, such as peaks due to column bleed and MSTFA artifact peaks, were not entered into the final data analyses. Integrated peak areas of multiple derivative peaks belonging to the same compound were summed and considered as single compound. Each sample was characterized by the same number of variables and each of these variables was represented across all observations with the same sequence. Thus, a data matrix was generated by intensities of the commensal peaks from all samples to characterize the biochemical pattern of each sample. The obtained matrix was then employed for correlation analysis and pattern recognition.

We chose three urine aliquots for each case to repeat the whole experimental process to test the producibility of sample preparation. For testing the precision and accuracy, five derivatized samples for each case were consecutively injected into the GC system.

2.6. Data processing and pattern recognition

After GC/MS analysis, each sample was represented by a GC/MS TIC, and the peak areas of compounds were integrated. The peak-area ratio of each compound to a corresponding internal standard was calculated as the response. Two sample *t*-test statistics was used for the comparison of metabolite levels to determine *the* differences between the HCC and control groups. The difference with a p-value of $\leq$0.05 was considered statistically significant.

Principal component analysis (PCA) was used to differentiate the samples, which was performed in the MATLAB software (version 7.2, MathWorks, USA) [21]. All of the data from the differentially expressed compounds were used in calculating PCA models. The score plots of the first three principal components allowed the visualization of data and compared samples between the study and control groups. Diagnostic model was constructed by the marker metabolites alone or combining AFP, using linear discrimination analysis method. The classification performance (specificity and sensitivity) was assessed by the area under the curve (AUC) of the receiver–operator characteristic (ROC) curves.

3. Results and discussion

3.1. Comparison of serum ALT levels and AFP between study and control groups

The mean ALT level of the control group was 33.5 ± 19.6 U L^{-1}, which was a little lower than 69.0 ± 39.6 U L^{-1} of the HCC group ($p < 0.01$, analysis of variance (ANOVA)). They were both within normal range, which was set between 0 to 75 U L^{-1} [16]. An elevated level of serum ALT in the HCC group represented an alteration in liver function. We expected that serum metabolome could be influenced by the altered liver function [22]. We then hypothesized that