Metabolic characterization of hepatocellular carcinoma using non-targeted tissue metabolomics

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Running Title: Metabolic modifications of HCC based on tissue metabolomics

Precis: A comprehensive metabolic study of hepatocellular carcinoma defines two
novel candidate metabolic biomarkers for this disease

**Abbreviations**

Hepatocellular carcinoma (HCC), hepatitis B viral (HBV), hepatitis C viral (HCV), liquid chromatogram-mass spectrometry (LC-MS), hepatocellular carcinoma tissue (HCT), adjacent noncancerous tissue (ANT), distal noncancerous tissue (DNT), ultra high performance liquid chromatography (UHPLC), mass spectrometry (MS), electrospray ionization (ESI), ammonium bicarbonate (NH$_4$HCO$_3$), quality control (QC), partial least squares discriminate analysis (PLS-DA), base peak chromatogram (BPC), human metabolome database (HMDB), kyoto encyclopedia of genes and genomes (KEGG), tricarboxylic acid (TCA), metabolomics pathway analysis (MetPA), reactive oxygen species (ROS), valine (Val), leucine (Leu), isoleucine (Ile), phenylalanine (Phe), tryptophane (Trp), methionine (Met), proline (Pro), serine (Ser), threonine (Thr), aspartate (Asp), gamma-aminobutyric acid (GABA), glutathione (GSH), glutathione disulfide (GSSG), adenosine monophosphate (AMP), glycerol 3-phosphate (G3P), glycerol 2-phosphate (G2P), phosphorylcholine (PC), phosphoethanolamine (PE), lysophosphorylcholine (LPC), lysophosphoethanolamine (LPE), glycercylphosphorylethanolamine (GPEA), glycerophosphocholine (GPCho), 5'-methylthioadenosine (MTA), S-adenosylhomocysteine (SAH), succinyladenosine (SAdo), succinic acid semialdehyde (SSADH), glycursonodeoxycholic acid (GUDCA), glycocholic acid (GCA), glycodeloxycholic acid (GDCA), glycochenodeoxycholic acid (GCDDA), glycochenodeoxycholate-3-sulfate (GCDDCS), amino acids (AA), branched-chain amino acids (BCAA), aromatic amino acids (ArAA), free fatty acids (FFA), saturated fatty acids (SFA), monounsaturated fatty
acids (MFA), polyunsaturated fatty acids (PUFA), fructose 1,6-bisphosphate (F1,6bisP), glucose 6-phosphate (G6P), receiver operating characteristic (ROC), area under the curve (AUC), alpha-fetoprotein (AFP)
Abstract

Hepatocellular carcinoma (HCC) has a poor prognosis due to its rapid development and early metastasis. In this report we characterized the metabolic features of HCC using a non-targeted metabolic profiling strategy based on liquid chromatography-mass spectrometry. Fifty pairs of liver cancer and matched normal tissues were collected from HCC patients, including tumor tissues, adjacent noncancerous tissues and distal noncancerous tissues, and 105 metabolites were filtered and identified from the tissue metabolome. The principal metabolic alternations in HCC tumors included elevated glycolysis, gluconeogenesis and β-oxidation with reduced tricarboxylic acid cycle and Δ-12 desaturase. Furthermore, increased levels of glutathione and other anti-oxidative molecules, along with decreased levels of inflammatory-related polyunsaturated fatty acids and phospholipase A2 were observed. Differential metabolite levels in tissues were tested in 298 serum specimens from patients with chronic hepatitis, cirrhosis and HCC. Betaine and propionylcarnitine were confirmed to confer good diagnostic potential to distinguish HCC from chronic hepatitis and cirrhosis. External validation of cirrhosis and HCC serum specimens further showed that this combination biomarker is useful for HCC diagnosis with a supplementary role to AFP.

Key words: metabolomics, metabolic profiling analysis, hepatocellular carcinoma, liver tissue, metabolic disorder
Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. In China, chronic hepatitis B virus infection is the primary risk factor for HCC (1, 2), and the majority of HCC cases develop from hepatitis infections and subsequent cirrhosis. Rapid development and early metastasis are the typical characteristics of HCC, which always results in a poor prognosis. The large population infected with HBV makes the prevention of HCC a formidable task. Therefore, investigating the hepatocarcinogenesis mechanism is very important for decreasing the incidence and mortality of HCC.

The abnormal metabolism of cancer has been considered an important characteristic of tumors, which could clarify the pathogenesis and provide potential therapeutic targets for clinical treatments (3). According to the Warburg effect, the deregulated energy metabolism of cancer cells may also modify many related metabolic pathways that influence various biological processes, such as cell proliferation and apoptosis. As a common characteristic of cancer cells (4, 5), modified metabolism has been the focus of cancer research.

Metabolomics is a top-down platform in the field of systems biology, which focuses on the dynamic changes of small molecules in response to the disturbance of the organism (6). Non-targeted metabolomic approaches are being widely used for the discovery of new biomarkers and investigation of the carcinogenesis mechanism, and these approaches have also been used to investigate chronic liver diseases (CLD) and HCC (7, 8). Some serum or urine metabolites, such as sphingosines (7), bile acids, glycine, trimethylamine-N-oxide and dipeptides (8, 9), have been reported to be differential metabolites or candidate biomarkers for CLD or HCC. Serum and urine are commonly considered to be a pool of metabolites that reflect systemic metabolic
deregulation in patients, and the markers in these biofluids could reflect the characteristics of the system during the course of diseases.

Tissue metabolomics is a useful tool for studying the abnormal metabolisms of diseases, and it can provide information about the metabolic modifications and the upstream regulative mechanism in diseases (10, 11). More importantly, the systemic metabolic characteristics of tissues could provide opportunities for exploring novel diagnostic markers or therapeutic targets for clinical applications (12). Tissue metabolomics is conducted using a pairwise comparison of different parts of tissue from each patient, which can remove individual differences, such as age, sex, region, etc. The differences between the tumor cells and their surrounding host cells may reflect the interactions of the tumor and the host, which are important clues for studying the invasion and metastasis of tumors (13).

In this study, 50 sets of matched HCC tissues, including hepatocellular carcinoma tissue (HCT), adjacent noncancerous tissue (ANT) and distal noncancerous tissue (DNT), were collected. The metabolic characteristics of the tumor tissues and the impact of the tumors on the surrounding host cells were explored through metabolomics strategy based on liquid chromatography-mass spectrometry (LC-MS). Based on the defined differential metabolites, metabolic pathways and correlation networks were investigated and their potential for use in clinical diagnostics was investigated.

**Experimental procedures**

**Chemicals**

Acetonitrile and formic acid (HPLC grade) were purchased from Merck (Germany). Methanol (HPLC grade) was purchased from Tedia (USA). Distilled
water was filtered through a Milli-Q system from EMD Millipore Corporation (Billerica, MA, USA). Glycerophosphocholine, betaine, carnitine, hypoxanthine, acetyl carnitine, methionine, niacinamide, propionyl carnitine, 5'-methylthioadenosine, glycourso deoxycholic acid, glycocholic acid, glycodoxycholic acid, glutathione disulfide, fructose-1,6-bisphosphate, glucose-6-phosphate, palmitoyl ethanolamide, malic acid, fumaric acid, N-acetyl-L-alanine, gamma-aminobutyric acid, aspartic acid, taurine, serine, threonine, succinate, uric acid, 3-hydroxybutyric acid, xanthine, xanthosine, valine, ethylmalonic acid, proline, glutamine, S-adenosylhomocysteine, N-acetyl-L-methionine, leucine, phenylalanine, isoleucine, tryptophan, glycochenodeoxycholic acid, free fatty acid (FFA) C14:0, FFA C18:3, FFA C18:2 and FFA C18:1 were purchased from Sigma-Aldrich (Louis, MO, USA). Lysophosphorylcholine (LPC) C18:2, LPC C16:0 and LPC C18:0 were purchased from Avanti Polar Lipids (Louis, MO, USA). Ammonium bicarbonate (NH₄HCO₃, HPLC grade) was purchased from Fluka (Germany).

Clinical sample collection and pretreatment

Written informed consents were obtained from the patients, and the study was approved by the ethics committee of the Eastern Hepatobiliary Surgery Institute. Tissues were collected from 50 HCC patients (male/female, 39/11) that ranged in age from 30 to 70 years old. Of these, 38 of the patients were HBsAg-positive, and 1 patient was anti-HCV-positive. In each patient, 3 types of tissues were resected during the operation, including HCTs, ANTs and DNTs. The ANTs were collected from less than 2 cm into the solid tumor border. The DNTs were collected from the distal edge of the resected tissues during the operation, which were more than 2 cm from the solid tumor border. The lesions were analyzed using histopathology studies. Detailed
clinical information about the collected samples is presented in Table 1. The tissue samples were directly placed into liquid nitrogen after surgical resection and stored at -80 °C until analysis.

To investigate the diagnostic potentials of the differential metabolites discovered from the tissues, a total of 298 fasting serum samples, including 81 chronic hepatitis, 78 cirrhosis and 139 HCC samples, were collected and the detailed clinical characteristics are described in Table S1 of supplementary materials. Another batch of serum samples from 25 cirrhosis and 22 HCC patients was employed for external validation (Table S2 in supplementary materials). The serum samples were stored at -80 °C until analysis.

Tissue pretreatment: A piece of the tissue (100 mg) was mixed with 1 ml of cold methanol/water (4:1, v:v) and then homogenized using a high-speed blender. After ultrasonication, the sample was placed on ice for 20 min and then deproteinized by centrifugation at 4 °C (15,000 rpm, 10 min). Finally, 800 μL of the supernatant was freeze-dried at -52 °C and dissolved in 100 μL of methanol/water (4:1, v:v) before analysis.

Serum pretreatment: 400 μL acetonitrile was added to 100 μL serum and the mixture was vortexed for 1 min. Then, at 4 °C the mixture was centrifuged for 10 min at a rotation speed of 12,000 rpm. After that, 400 μL supernatant was transferred and lyophilized in a freezedryer. Finally, it was dissolved with 100 μL water/acetonitrile (4:1, v:v) solution and 10 μL sample injection for LC-MS analysis. The serum pretreatment for CE-MS analysis was described in supplementary materials.

**Metabolic profiling analysis**

The tissue metabolic profiling analysis was performed on a Thermo Fisher Accela
ultra-high-performance liquid chromatography (UHPLC) coupled to a linear ion trap quadrupole (LTQ) Orbitrap hybrid mass spectrometry (MS) system (Thermo Fisher, USA). A 2.1×100 mm BEH 1.7 µm C8 column (Waters, Ireland) was used. The column oven was set at 60 °C, and the sample manager temperature was maintained at 12 °C. The eluents A (water containing 0.1% formic acid) and B (acetonitrile) were employed in the electrospray ionization positive (ESI+) mode, whereas eluents C (water containing 5 mM NH₄HCO₃) and D (95% CH₃OH and 5% water containing 5 mM NH₄HCO₃) were used in the ESI negative (ESI-) mode. The flow rate was 0.35 mL.min⁻¹ with a linear gradient elution over 30 min. From the start to 1 min, B for the ESI+ mode (or D for ESI-) was held at 5%, linearly increased to 30% during the next 9 min, linearly increased to 90% during an addition 10 min, and then increased to 100% in 2 min and kept constant for 4 min. Subsequently, B (or D) was returned to 95% in 0.1 min and held for an additional 3.9 min before returning to the initial conditions. The sample sequence was random. The MS spray voltages were 4.5 kV in the ESI+ mode and 3.0 kV in the ESI- mode. The capillary temperature was set at 300°C with the sheath gas at 35 arbitrary units and the aux gas at 5 arbitrary units. The tube lens was set to 100 V and the mass scan range was set from 100 to 1000 m/z. The resolution of the Orbitrap was set at 60,000. The MS/MS data were collected with the collision energy between 10 to 35 eV.

The same LC-MS system and mobile phases were used for serum metabolic profiling with a 2.1×100 mm HSS 1.8 µm T3 column (Waters, Ireland). Instrument parameters of liquid chromatography-mass spectrometry were similar to our previous work (14). The column temperature was maintained at 35 °C and the sample manager was set to 4 °C. The analysis time for each serum sample was 22 min. Resolution of the Orbitrap was set at 60,000 to ensure the mass errors less than 2 ppm. Both betaine
and propionylcarnitine are polar metabolites, and CE-MS system is appropriate for polar compounds analysis. Thus, the quantitative analysis in the external validation from HCC to cirrhosis patients was performed by using capillary electrophoresis-mass spectrometry (CE-MS) system, and the detailed method was described in supplementary materials.

To ensure data quality for metabolic profiling, pooled quality control (QC) samples were prepared by mixing all of the samples. Before analyzing the sample sequence, 5 QC samples were run. During analysis of the sample sequence, one QC sample was run after every 10 injections, which is similar to our previous method (15).

**Data processing and analysis**

First, the raw data were acquired and aligned using the SIEVE software package (V1.2, Thermo Fisher) based on the $m/z$ value and the sample retention time. Before chemometrics analysis, all of the detected ions in each sample from one sample class were normalized to the sum of the peak area defined as 10,000 (16). After the missing values for each sample class were treated using the 80% rule (17), the statistical significance was calculated using the Students’ t-test ($p<0.05$) as implemented in the Statistical Package for the Social Sciences (SPSS) version 16 for Windows software package.

Ions from both ESI+ and ESI- with statistical significance were merged and imported into the SIMCA-P program (version 11.0, Umetrics, Umea, Sweden) for multivariate analysis. Principal component analysis (PCA) and partial least squares discriminate analysis (PLS-DA) were applied with unit variance (UV) scaling (16). The parameters of the models, such as the $R^2_X$, $R^2_Y$, $Q^2_Y$ and the $R^2_{Y-}$,
Q^2-Y-intercepts, were analyzed to ensure the quality of the multivariate models and to avoid the risk of over-fitting. The normalized amount of each metabolite was plotted in a histogram using the Origin software package (version 8.0). Hierarchical cluster analysis (HCA) was performed using the MeV software package (version 4.5.1), and the correlation network was constructed using the Cytoscape software package. Receiver operating characteristic curve (ROC) and binary logistic regression were applied to the serum data using SPSS software.

Results

A total of 50 patients with HCC were enrolled in this study. The HCC diagnosis was confirmed by histopathology studies after surgery. Twenty-three patients had a level of serum alpha-fetoprotein (AFP) that was less than the cut-off value (20 μg/L). The Edmonson stages of the patients (9 were not determined) ranged from stage II to stage IV. There was no statistical significance observed between male and female patients (Student’s t-test, p>0.05) in the employed clinical tests. Steatotic liver tissue was not observed in any of the samples.

Metabolic profiling of liver tissues

In this study, a non-targeted metabolomics strategy was applied. The details of the metabolic profiling method, including sample preparation, metabolite extraction and LC-MS analysis, were reported in our previous study (18). Fig. S1 in supplementary materials presents the typical base peak chromatograms (BPC) of the HCT, ANT and DNT groups. The metabolic profiles of the ANT and DNT groups were similar, but they were substantially different from that of the HCT group in both ESI+ and ESI- modes.
The method validations, including linearity, precision, stability and recovery, were also performed (18), which indicates that this tissue metabolomics method is reliable. Furthermore, the PCA score plot (Fig. S2a, supplementary materials) exhibited a clear cluster of the pooled QC samples ($R^2_X=0.647$, $Q^2 =0.404$), indicating that the sample analysis sequence had a satisfactory stability and repeatability.

After the peak alignment and the removal of the missing values (17), a total of 880 ions significantly changed (Student’s $t$-test, $p<0.05$) between the HCT and DNT group, 942 ions between the HCT and ANT group and only 46 ions between the ANT and DNT group were maintained (Fig. S2d).

For further analysis of the metabolic differences between the DNT and HCT group, all of the significant ions from the ESI+ and ESI- modes were merged and imported into the SIMCA-P software package. Subsequently, PLS-DA was applied to the classification of cancer and noncancerous tissues. As illustrated by the PLS-DA score plot (Fig. S2b), the noncancerous tissues were clearly separated from the HCC tissues. The cumulative $R^2_Y$ and $Q^2$ were 0.804 and 0.657 respectively, when 2 components were calculated. No over-fitting was observed according to the results of the chance permutation ($R^2_Y$-intercept was 0.420 and $Q^2$-intercept was -0.153). However, there was no obvious separation trend between the ANT and DNT groups.

**Differential metabolites between HCT and DNT**

PLS-DA S-plot (Fig. S2c) can be used to define the differential metabolites for distinguishing the DNT group from the HCT group, 390 ions with a variable importance in the project (VIP) >1 were selected for subsequent chemical structure identification (19). Based on our previously published strategy (20), the following
steps were used for the identification of chemical structures in this study. First, the quasi-molecular ions were confirmed. Secondly, the exact masses of the monoisotopic molecular weights were used to search the online databases, such as the Human Metabolome Database (http://www.hmdb.ca/), Metlin (http://metlin.scripps.edu/) and the Mass Bank (http://www.massbank.jp/) (21). Then, the MS/MS spectra were also analyzed to verify the structure of the identified metabolites. Subsequently, 44 metabolites in ESI+ mode and 65 in ESI- mode were identified, and some of them were further confirmed using authentic standard samples. The names of the compounds and the related enzymes are presented in Table S3 of the supplementary materials.

The relative average normalized quantities of the identified differential metabolites in the HCT and ANT groups compared to those in the DNT group were plotted in a heat map (Fig. 1) using the MeV software package. The metabolites were clustered according to their Pearson correlation coefficients. Overall, the changes in these important metabolites in the HCT samples were quite different from those in the noncancerous tissues.

Based on the knowledge of these differential metabolites and an online database of metabolic pathways (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/), a map of the HCC-related metabolic pathways was constructed (Fig. 2). Several metabolic pathways were modified by the tumor cell, for example, an increase in glycolysis metabolites (6-phosphogluconic acid) and a decrease in TCA cycle metabolites, including succinate, fumarate, malate and succinic acid semialdehyde, were observed. Most of the amino acids and their related metabolites significantly increased in the HCT group. The levels of two glucogenic amino acids (serine and threonine), which can be transformed into glucose for energy,
significantly increased in the HCT group. Glutamine, glutathione, branched-chain amino acids (BCAA), including valine, leucine and isoleucine, and the aromatic amino acids (ArAA), such as tryptophan, phenylalanine and tyrosine, were also increased in the HCT group. The increased catabolism may be responsible for the increase in total amino acids. These modifications to the host metabolism represented the influence of the cancer cells on energy metabolism, which have also been confirmed in colon and stomach tumor tissues (13).

The change in the free fatty acids was not the same as that of the amino acids. The levels of saturated fatty acids (SFA) and monounsaturated fatty acids (MFA) increased, whereas the polyunsaturated fatty acids (PUFA) levels decreased (Fig. 3a). The ratio of FFA C18:2 to C18:1 also decreased in the HCT group (Fig. 3b), which suggested inhibition of Δ-12 desaturase. Interestingly, the ratio of acetyl carnitine to carnitine (C2/C0) was upregulated in the HCT group, whereas the ratio of propionyl carnitine to carnitine (C3/C0) was downregulated (Fig. 3c). The ratio of C2/C0 indicated that the β-oxidation of even-numbered fatty acids was upregulated in the HCT group. The ratio of C3/C0 conflicted with BCAA (Fig. 3d), which also reflected an increase in lipid oxidation for the production of energy in the tumor tissue (22). A decreased level of lysophosphatidycholine (LPC) was observed in the cancer tissues. However, phosphorylcholine (PC) and phosphoethanolamine (PE) levels were significantly greater in the cancer tissues (Fig. 2).

**Correlation network of differential metabolites in tumor tissue**

To investigate the latent relationships of the differential metabolites, a correlation network diagram was constructed using the Cytoscape software (Fig. 4). All of the differential metabolites (880 ions) were included in the diagram to obtain a global
view of tumor metabolism. We observed that the metabolites could be divided into two primary groups: upregulated and downregulated. These groups were bridged by lipids. Fatty acids, including SFA, MFA and PUFA, were located in the center of the metabolic network. Glycolysis-related metabolites and some amino acids were upregulated in the tumor. Lipids, especially phospholipids, were the most correlated metabolites in the downregulated group. There was a decreased amount of bile acids in the tumor tissues. Because the metabolism of phospholipids and bile acids, the deregulation of these metabolites may reflect low differentiation levels of the tumor cells. Unlike long chain acylcarnitines, the quantity of short chain acylcarnitines decreased in the cancer tissues. The correlation of the metabolites in the TCA cycle and the short chain acylcarnitines supported the hypothesis that energy metabolism shows low efficacy in tumor tissues.

Investigation of the diagnostic potential of differential metabolites found in tissues

The tissue metabolome can more directly reflect metabolic deregulation than the body fluid metabolome, but the biomarkers for clinical diagnosis should return from the tissue to the biofluids. Therefore, we also investigated the serum diagnostic potentials of these significantly different metabolites. Totally 298 serum samples, including 81 chronic hepatitis, 78 cirrhosis and 139 HCC, were enrolled.

Considering the complementary role of novel biomarkers to AFP in clinical diagnosis, we focused more on the metabolites which were not related with AFP level. The results of the Pearson correlation analysis ($|C_{ij}| < 0.15$) indicated that eight important metabolites were irrelevant to serum AFP level, five of them including betaine, propionylcarnitine, proline, methionine and isoleucyl-L-proline were found to be greatly different (Student’s $t$-test, $p < 0.001$) between nonmalignant liver diseases...
and HCC patients (Table S5 in the supplementary materials). After the binary logistic regression analysis of the data, betaine and propionylcarnitine were selected as the optimal combination for HCC diagnosis. As shown in Fig. 5a, both betaine and propionylcarnitine were markedly decreased in the HCC group. According to the data in patients with HCC versus nonmalignant liver diseases, the ROC curve of betaine plus propionylcarnitine yielded an area under the curve (AUC) of 0.982, whereas the AUC of AFP was only 0.697 (Fig. 5b). Two-thirds of the samples (54 hepatitis, 53 cirrhosis and 93 HCC) were randomly selected from each group as the training set, and the remaining one-third of the samples (27 hepatitis, 25 cirrhosis and 46 HCC) constituted the test set for validation. In the training set, the diagnostic accuracy of the chronic liver diseases and HCC patients achieved 91.6% and 96.8%, respectively. Meanwhile, the accuracy rate of the chronic liver diseases and HCC patients also reached up to 94.2% and 93.5% in the test set (Fig. 5c). Noticeably, the combination of betaine and propionylcarnitine had a diagnostic accuracy of 92% in the AFP false-negative (AFP < 20 μg/L) HCC patients. More valuably, these two metabolites were also effective for excluding AFP false-positive (AFP > 200 μg/L) nonmalignant patients, which attained a diagnostic accuracy of 100% both for hepatitis and cirrhosis (Fig. 5d). The above results demonstrated that the capacity of betaine and propionylcarnitine combination as a novel HCC serum biomarker, especially its supplementary role to AFP.

To further externally validate the above experiment results, another batch of serum samples was enrolled from 25 cirrhosis and 22 HCC patients. Quantitative analysis of betaine and propionylcarnitine in the external validation serum samples was carried out on CE-MS system. As shown in Fig. S3a, the levels of betaine and
propionylcarnitine in the HCC group were significantly lower than those in the cirrhosis group, which were consistent with the previous 298 serum samples study. The diagnostic accuracies of betaine plus propionylcarnitine for cirrhosis and HCC were 80.0% and 86.3%, respectively (Fig. S3b).

**Discussion**

Cancer metabolism has recently become a subject of considerable interest for both the pharmaceutical industry and clinical research. However, a systematic understanding of cancer metabolism is still a challenge. The primary goal in this study is to understand the metabolic features in HCC-related tissues by investigating the metabolic differences among the HCT, ANT and DNT groups using a non-targeted tissue metabolomics method.

*Metabolic features of tumor tissue*

Based on the current results, the glycolysis metabolites in tumor tissues, such as 6-phosphogluconic acid, increased while the metabolites in the TCA cycle decreased (Fig. 2). These data revealed the rapid expenditure of glucose through the glycolysis pathway with a low level of aerobic oxidation through the TCA cycle. Another important feature of the tumor cell can also be observed from the correlation network (Fig. 4), all of the enriched metabolites in tumor tissues are related to energy supply, such as glycolysis-related metabolites and some AA, SFA and MFA. These results revealed the high energy requirements of the tumor cells for proliferation. Glucose is rapidly consumed in tumor cells through glycolysis. Furthermore, gluconeogenesis and β-oxidation in the tumor cells are upregulated for energy supply. In contrast, the TCA cycle is downregulated, and the short chain and middle chain acylcarnitines are
also decreased, which demonstrates decreased consumption of carboxylic acids in the mitochondria. This result is consistent with the “Warburg effect” (23, 24). Moreover, the increased consumption of glucose may upregulate gluconeogenesis from lipids and proteins. Because of the increased catabolism, the quantity of amino acids (AA) and free fatty acids (FFA) consequently increases. Therefore, modified metabolism of FFA and AA was observed in the tumor tissues. FFA β-oxidation is a multi-step process in which fatty acids break down in various tissues to produce energy. As shown in Fig. 4, lipids, including phospholipids and FFA, are the most correlated metabolites. First, the tumor cells modified their lipid metabolism to satisfy the large energy demand during cellular proliferation, which could be observed in the tumor tissue via the increased amounts of SFA and MFA. Furthermore, oxidative stress was increased by the effect of lipid peroxidation (25). Therefore, the suppressed functional enzyme Δ-12 desaturase (Fig. 3a) might be the mechanism through which tumor cells maintain high levels of SFA and MFA. The expression of the desaturase enzyme confirmed that β-oxidation was accelerated in the HCC tumor tissues compared to noncancerous tissues.

Because oxidative damage is the primary driver for cell apoptosis, the level of anti-oxidative metabolites would be accordingly elevated. We observed increased levels of some metabolites that could protect the tumor cells from oxidative damage in the tumor tissue. The striking elevation of 6-phosphogluconic acid suggested an increased flux of metabolic fuels into the pentose-phosphate pathway (PPP), perhaps to create NADPH to manage the perceived increase in oxidative stress in the HCC (26). Glutamine is a major nitrogen carrier and a carbon substrate for anabolic processes in cancer cells, and it may protect cancer cells from oxidative stress and apoptosis (27, 28). Similarly, glutathione and glutathione disulfide, which are the
major redox couple in animal cells (29), were also increased. The increase of
 glutathione reflected the alteration of the redox state, which is one of the key
 performance indicators in pathologic conditions, especially in cancer (30).

Chronic inflammation is an important factor of hepatocarcinogenesis (4), and it
could influence tumor lipid metabolism. The metabolism of cholesterol and
phospholipids is a primary function of normal liver cells, and the downregulated
synthesis of bile acids may lead to the malabsorption of lipids. The significant
increases in taurine and 4-hydroxythreonine and decreases in urobilinogen and
glycine-conjugated bile compounds in HCC suggested that the co-metabolism of gut
microbes was altered in HCC, and potential changes in the enterohepatic circulation
may also have occurred (31). Moreover, bile acids are closely correlated with lipids,
which may cause potential regulatory effects on nuclear receptors (32). The abnormal
metabolism of phospholipids may influence many biological processes, e.g.,
inflammation. PUFA, especially arachidonic acid, is the precursor for diverse
inflammation molecules (33). Phospholipids from the plasma membrane are the
primary source of arachidonic acid. A decreased level of LPC was observed in the
cancer tissues. However, the levels of PC and PE were significantly greater in the
cancer tissues (Fig. 2). PC could be converted into LPC and arachidonic acid by the
catalysis of phospholipase A2 (PLA2). To escape possible damage from the immune
system, the tumor cell may reduce the generation of endogenic inflammation
molecules by downregulating the related enzymes, such as PLA2 (34). According to
the current results, it can be hypothesized that PLA2 or Δ-12 desaturase levels are
altered in tumor cells in an attempt to modify the metabolism of lipids, thereby
supporting the proliferation of tumor cells as well as their escape from immune attack
and apoptotic cell death.
In addition to energy metabolism, amino acids also serve a variety of biological functions. For example, several N-acetyl amino acids (Ala, Met, and Trp) consistent with the observed increase of acetyl carnitine were elevated in HCC (Fig. 3c). These observations might suggest elevated acetyl coenzyme A concentrations in HCC (35). Furthermore, increased levels of BCAA and ArAA were observed in the tumor tissues (Fig. 3d and e). The increase of BCAA occurred in concert with increases in aromatic amino acids (Trp, Tyr, and Phe), which implied that the enhancement of large neutral amino acids (LNAA) was facilitated by L-type amino acid transporter-1 (LAT1) (36).

The ratio between Phe and Tyr can be used as an indicator of phenylalanine hydroxylase (PAH) (37). The decrease of the Tyr/Phe ratio in the HCT group (Fig. 3f) suggests the inhibition of PAH in tumor tissues. There were also some other metabolic features of HCC that reflected the upregulation of cellular proliferation. For example, the level of hypoxanthine increased in the HCT group, whereas that of xanthine declined sharply, which may suggest decreased activity of xanthine dehydrogenase/oxidase (38) and active cellular proliferation.

As indicated by the current results, the rapid expenditure of energy causes the cancer cells to modify the metabolic pathway to provide a sufficient amount of energy. Moreover, the cancer cells also try to avoid oxidative damage and chronic inflammation, which may reduce the occurrence of cellular apoptosis.

**Diagnostic potentials of differential metabolites**

The metabolic changes observed in biofluids might be caused by differences among individuals, including diet, sex, age, etc. In this study, the significantly changed metabolites were defined by a pairwise comparison of different parts of the liver tissue from individuals, and the differences of other non-disease factors were
removed. The relationship of differential metabolites with the clinical information of samples, including HBsAg, tumor diameter, AFP, ALT and AST level, was investigated carefully (Table S4 in supplementary materials). Thirty-eight cases of the HCC patients were HBsAg-positive, and twelve patients were HBsAg-negative (Table 1). Citramalic acid in tumor tissue was significantly decreased in the HBsAg-positive patients compared to the HBsAg-negative ones (Fig. S4 in supplementary materials). This finding may explain the suppression of TCA cycle under inflammatory status.

The differential metabolites found in tissues can be used as candidates of biomarkers to investigate their diagnostic potential by using serum as the sample. According to the results from Pearson correlation analysis, betaine and propionylcarnitine were found to be irrelevant to serum AFP level, they were defined to investigate the potential for HCC diagnosis (Fig. 5). The result indicated that betaine plus propionylcarnitine was efficient for distinguishing HCC from nonmalignant liver diseases with a 0.982 AUC value of ROC curve, which was much better than that of AFP (0.697, Fig. 5b). The changes of betaine and propionylcarnitine in the external validation had the same change trend as the first batch of serum samples. And good validation result further confirmed that the combination of these two metabolites was effective for HCC diagnosis.

**Conclusions**

In summary, the metabolic profiling analysis of liver tissues provided a holistic view of the metabolic features of HCC. The differential metabolites in the tumor tissues were filtered and identified, and the results demonstrated that the metabolism in the tumor was modified to promote cellular proliferation or escape from apoptosis. The rapid consumption of energy by the tumor cells upregulated glycolysis and
downregulated the TCA cycle, which are consistent with the “Warburg effect”. Gluconeogenesis and β-oxidation are also upregulated for energy supply, which could be observed from the enriched quantities of amino acids and fatty acids. Based on the correlation network, modified metabolism of lipids is the most important feature of the HCC tumor, which may be important to protect the tumor cell from oxidative damage. This protective effect included increased levels of anti-oxidative metabolites and decreased levels of inflammation related metabolites of PUFA, which is supposed to be related to the upstream enzymes, such as PLA2. This mechanism may be important for the tumor cell to break the balance of proliferation and apoptosis.

The diagnosis potential of the differential metabolites found in tissues, further studied in serum samples and validated in another group of serum samples demonstrated that the combination of betaine and propionylcarnitine has a better ROC curve than AFP, it is useful for both AFP false-positive and false-negative patients in distinguishing HCC from hepatitis and cirrhosis. These two metabolites have potential as HCC biomarkers in clinical diagnosis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgement

We want to thank Jun Zeng for the measurement of metabolites by using CE-MS.

Grant Support

The study has been supported by the State Key Science & Technology Project for Infectious Diseases (2012ZX10002-009, 2008ZX10002-019), the key foundation (No.
and the creative research group project (81221061, 21021004) from National Natural Science Foundation of China.

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Table 1

Clinical characteristics of HCC patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Male, n=39</th>
<th>Female, n=11</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFP (μg/L)-Below 20/Above 20</td>
<td>17/22</td>
<td>6/5</td>
<td>0.708</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>51.9 (30 ~ 70)</td>
<td>51.2 (38-65)</td>
<td>0.845</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>116.8 (20.0~417.0)</td>
<td>88.0 (22.0 ~ 495.0)</td>
<td>0.437</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>47.3 (18.4 ~ 322.2)</td>
<td>37.6 (16.2 ~ 64.1)</td>
<td>0.527</td>
</tr>
<tr>
<td>Cirrhosis/No cirrhosis</td>
<td>23/16</td>
<td>0/11</td>
<td></td>
</tr>
<tr>
<td>HBsAg-Positive/Negative</td>
<td>30/9</td>
<td>8/3</td>
<td></td>
</tr>
<tr>
<td>anti-HCV-Positive /Negative</td>
<td>1/38</td>
<td>0/11</td>
<td></td>
</tr>
<tr>
<td>Tumor diameter (cm)</td>
<td>7.1 (2 ~ 18)</td>
<td>7.1 (3.6 ~ 11)</td>
<td>0.996</td>
</tr>
<tr>
<td>Edmonson stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>8</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>21</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Edmonson stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II+III</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>III+IV</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Not determined</td>
<td>6</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

Age, ALT, AST and tumor diameter in both male and female were expressed as median (range).
Figure legends

Figure 1. Heat map of the 105 significantly changed metabolites among the DNT, ANT and HCT groups. The colors from green to red indicate the relative contents of metabolites in the ANT or HCT compared to those in the DNT group.

Figure 2. Metabolic network of the significantly changed metabolites. The normalized contents are shown under the chemical name. Blue and red bar chart: normalized content in the DNT group and HCT group, respectively. All the p values were calculated using Student’s t-test. * , p<0.05; ** , p<0.01; *** , p<0.001. Un-significantly changed metabolites are shown only by name, and the names with a dashed line frame represent the undetected metabolites. Abbreviations: Phosphorylcholine (PC), o-phosphoethanolamine (PE), glycercylvphosphorylethanolamine (GPEA), glycerophosphocholine (GPCho), 5’-methylthioadenosine (MTA), s-adenosylhomocysteine (SAH), succinyladenosine (SAdo), succinic acid semialdehyde (SSADH), glycoursoxycholic acid (GUDCA), glycocholic acid (GCA), glycureoxycholic acid (GDCA), glycochenoreoxycholic acid (GCDCA), glycochenoreoxycholate-3-sulfate (GCDCS), fructose-1,6-bisphosphate (F1,6bisP), and glucose-6-phosphate (G6P).

Figure 3. Metabolic changes of free fatty acids, carnitines and amino acids in the tumor tissues. (a) FFA metabolism; (b) Ratio of FFA C18:2 to FFA C18:1; (c) Ratios of carnitines; (d) BCAA (leucine, isoleucine and valine ); (e) ArAA (phenylalanine and tyrosine); (f) Activity of phenylalaninehydroxylase (PAH) according to the ratio of tyrosine to phenylalanine. Light and dark chart: relative amount in the DNT group.
and HCT group, respectively. * $p<0.05$;  * * $p<0.01$;  * * * $p<0.001$.

**Figure 4. Metabolic correlation networks of the differential metabolites and related pathways.** Highly correlated metabolites ($|C_{ij}|>0.6$) are connected with a line. Red: upregulated in tumor tissue; green: downregulated in tumor tissue. Abbreviations: Polyunsaturated fatty acid (PUFA), monounsaturated fatty acid (MUFA), saturated fatty acid (SFA), and short chain acylcarnitine (SC-AC).

**Figure 5. Diagnostic potential for HCC by betaine plus propionylcarnitine from serum.** (a) Relative amount in hepatitis, cirrhosis and HCC patients; (b) ROC curves for distinguishing HCC from nonmalignant liver diseases by AFP and the combination of two metabolites, respectively; (c) Diagnostic accuracy for nonmalignant liver diseases and HCC in the training set and test set by using betaine plus propionylcarnitine; (d) Diagnostic accuracy in different concentration of AFP serum samples among hepatitis, cirrhosis and HCC patients. * * * $p<0.001$. 
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Cancer Res  Published OnlineFirst July 1, 2013.

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