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Metabolic profiling study of early and late recurrence of hepatocellular carcinoma based on liquid chromatography-mass spectrometry☆

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ABSTRACT

The objectives of this pilot study were to predict early postoperative recurrence in hepatocellular carcinoma (HCC) patients based on metabolic features and to explore the related metabolic disturbances. Liquid chromatography-mass spectrometry-based metabolic profiling was performed on the plasma of 18 late recurrent and 22 early recurrent HCC patients. Metabolic differences were found to be related to amino acid, bile acid, cholesterol, fatty acid, phospholipid and carbohydrate metabolism. Bile acids, steroids and fatty acids showed significant variation in the early recurrent HCC group compared to the late recurrence group. Decreased levels of polyunsaturated eicosapentaenoic acid, docosahexaenoic acid and linolenic acid were found to be specific metabolic features for early recurrence. With the combination of methionine, GCDCA and cholesterol sulfate, 85% of the early recurrent HCCs can be predicted correctly with the corresponding area under the curve (AUC) equal to 0.95 in the training set, and 80% of the early recurrent HCCs can be predicted correctly with the corresponding AUC equal to 0.91 in the test set.

1. Introduction

Hepatocellular carcinoma (HCC) is a common cancer [1]. Liver resection is one of the potentially curative treatment options for HCC [2] and has very low operative mortality [3], but it also has a high rate of recurrence that ranges from 30% to 50% [4]. To improve the survival of HCC patients under surgical resection, much attention has been paid to improving surgical techniques and equipment, developing effective neoadjuvant or adjuvant therapies and identifying patients at high risk of recurrence. Screening patients who are at high risk of recurrence is especially important to break through the plateau of surgical outcome. Though the developed pathological tumor-node-metastasis (TNM) staging system has been employed in clinical practice, this system is insufficient to predict recurrence in HCC patients. Different risk factors for HCC recurrence after hepatectomy have been widely evaluated [3,5–7]. Larger tumor size, liver cirrhosis, higher preoperative aspartate aminotransferase (AST) level [8], preoperative AFP level [9] and venous invasion [10] have been found to be independent risk factors in clinical statistical analysis. Studies have increasingly concentrated on identifying new genes, microRNAs or protein markers to predict early recurrence in HCC patients [2,4,11–15], but the markers correlated with aggressive behavior must still be well defined, considering the genetic heterogeneity. Metabolites, which are basic substances for biological activities, are regulated by various genes and proteins. Metabolomics is a powerful tool for the systematic investigation of the metabolic disturbances related to the occurrence, development, metastasis and recurrence of HCC [16–23].

In this study, we employed a liquid chromatography–mass spectrometry (LC–MS)-based metabolic profiling strategy to investigate the metabolic differences in plasma of early and late recurrences in HCC, explore the mechanism of early recurrence and predict patients at high risk of recurrence in HCC. According to the time point of recurrence from the date of hepatectomy, early and late recurrence were defined as less or more than two years, respectively [6,11,24,25].

2. Materials and methods

2.1. Chemicals

HPLC-grade acetonitrile (Merck, Darmstadt, Germany), laboratory-prepared Milli-Q water (Millipore, Bedford, MA) and formic acid (Sigma-Aldrich, St. Louis, MO, USA) were used

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to prepare mobile phases. For ion structure characterization, chemical standards were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Plasma specimen collection and sample preparation

Plasma specimens were collected upon initial tumor presentation from HCC patients who had attended the Cancer Center of Sun Yat-Sen University, Guangzhou, China. Then the first surgery of the enrolled HCC patients was exerted soon afterwards. Before surgical treatment, HCC patients were tested with ultrasonography, CT scan and angiography to identify the space-occupying lesion and evaluate its resectability. Curative resection was performed to obtain a large microscopic margin of 2 cm, if possible, with no residual space-occupying lesions able to be identified in the remnant liver. Patients were followed up thoroughly after hepatic resection. Liver function and α-fetoprotein (AFP) were measured one month after surgery. Patients were then followed up every three months for a year with ultrasonography and CT scans. The follow-up was then performed every six months for a second year after surgery. The last case was treated in May 2010.

The LC–MS profiling of these collected HCC plasma samples was performed before knowing the recurrence information to avoid the sample changes due to long-term storage, and the derived metabolomics data were handled until the outcome was known. To discover the metabolic differences between the early and late recurrence of HCC after curative resection, each patient was followed up for at least three years. Among the 40 cases successfully followed, there were 18 subjects with no recurrence in at least two years (2 cases recurrent two years later; 8 cases with no recurrence for at least three years; 3 cases with no recurrence for at least four years; and 5 cases with no recurrence for at least five years) and 22 patients with recurrence within two years (8 cases recurrent within three months; 6 cases recurrent from the fourth month to the sixth month; 3 cases recurrent between half a year and a year after surgery; and 5 cases recurrent one year later). From Table 1, we can observe the basic characteristics of the enrolled subjects. The two groups were basically matched in age and sex. There were significant differences in γ-glutamyl transpeptidase, alkaline phosphates and AFP, which showed significantly increased levels in the blood of the early recurrent HCC group. For the other parameters, there was no significant difference between the groups.

For each sample, four volumes of acetonitrile were added to 100 μL plasma. Then, the mixture was vortexed thoroughly and centrifuged at 15,000 × *g* for 10 min at 4 °C. A 400-μL aliquot of supernatant was lyophilized and frozen at −80 °C. Before the LC–MS analysis, the sample powder was reconstituted in 80 μL of acetonitrile/water (1:4, v/v) and was then centrifuged at 15,000 × *g* for 10 min at 4 °C. The supernatant was used for injection. Quality control (QC) samples were prepared by pooling equal aliquots of plasma from each sample and pretreated as the above real samples. The QC samples were inserted among real samples to evaluate the reliability of sample pretreatment and the LC–MS method.

2.3. LC–MS-based metabolic profiling

For LC–MS metabolic profiling, a liquid chromatograph coupled to a LTQ Orbitrap XL hybrid mass spectrometer system (Thermo Fisher) equipped with an electrospray source was employed. A 15-μL aliquot of the reconstituted solution was injected into the liquid chromatographic system. A HSS T3 column (100 mm × 2.1 mm, 1.7 μm) (Waters, Milford, MA, USA) was used for chromatographic separation. The compositions of the mobile phases A and B were, respectively, as follows: 0.1% formic acid in water (v/v) and acetonitrile for positive mode; 6.5 mM ammonium bicarbonate in water and 6.5 mM ammonium bicarbonate in 95/5 (v/v) methanol/water for negative mode. The gradient started with 2% B, increased to 40% B at 2 min, increased further to 100% B linearly within 8 min and held for another 4 min. The total run time for each injection was 20 min, including a post-equilibration period of 5 min. The column temperature and constant mobile phase velocity were set at 50 °C and 0.35 mL/min. For MS signal acquisition, the scan range was set from 80 to 500 Da in the first two-minute segment and from 100 to 1100 Da in the following segment. The MS was operated with a resolution of 60 K for the positive mode and 30 K for the negative mode. The ion source parameters set for profiling were as follows: the capillary temperature was set at 325 °C; the I-spray voltage and capillary voltage were at 4.5 kV and 49 V for the positive mode and 4 kV and 37 V for the negative mode; and the flow rates of sheath gas were 45 and 50 arbitrary units for the positive and negative modes, with the auxiliary gas set at 8 arbitrary units.

2.4. Data analysis

Peak extraction and alignment were performed using SIEVE software (V2.0, Thermo Fisher). For peak alignment, a retention time width of 0.35 min and mass width of 50 ppm were set for framing. A maximum frame number of 5000, with a peak threshold of 50,000, was set for peak filtering. For both the positive and negative modes, a large matrix for each sample with mass, retention time and peak area was exported to an Excel table. The variables were deleted if their values equal to zero in more than 20% samples in each group [26], and the variable normalization was the same as used previously [21]. Using the derived peak table, both multivariate and univariate analyses were performed to determine the metabolic differences between the early and late recurrent groups. All of the prepared variables or the variables with a significant difference ($p < 0.05$) according to Student's *t*-test analysis (SPSS 13.0, Chicago, IL, USA) were Pareto-scaled for partial least squares-discriminant analysis (PLS-DA) (Umetrics AB, Umea, Sweden). Before PLS-DA or the following hierarchical cluster analysis (HCA) (Multi Experiment Viewer (http://www.tm4.org)), differential ions from the same compound in both the positive and negative modes, such as its isotopes, fragments and adducts with similar retention times, similar eluting profiles and a tight correlation, were checked to delete redundant variables.

3. Results and discussion

3.1. Metabolic profiling of early and late recurrence of hepatocellular carcinoma

To investigate the metabolic differences in blood between the early and late recurrence of HCC after curative resection, we selected 18 late recurrent HCC subjects and 22 early recurrent HCC subjects, where the first and last cases under surgery were in December 2007 and May 2010, respectively. The plasma samples were collected and stored at −80 °C until analysis in March 2011. The effects of long-term storage can be neglected considering the lower temperature storage and the matched sample collection time points. To monitor the repeatability of sample pretreatment and stability of the LC–MS system, QC samples were prepared as real samples and analyzed during the sequence analysis. After normalization to total peak area, the RSD of each ion in the QC samples was calculated. Peak areas of ions with RSD < 30% occupied 65% and 85% of the total peak areas in the positive and negative modes. There were 5000 ions in total in each mode, indicating that the metabolic profiling data were reliable for the following statistics.