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A prognostic fingerprint in liver transplantation for hepatocellular carcinoma based on plasma metabolomics profiling

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ABSTRACT

Introduction: Tumor recurrence is a major cause of post-transplant mortality in liver transplantation for hepatocellular carcinoma (HCC). This study aimed to explore an effective noninvasive approach to accurately predict post-transplant tumor recurrence.
Materials and methods: Metabolomics profiling was performed on pre-operative plasma from 122 HCC patients undergoing liver transplantation, 52 healthy controls (HC) and 25 liver cirrhosis (LC) patients.
Results: Five prognostic metabolites were identified by univariate analysis ($P < 0.01$), including phosphatidylcholine (PC) (16:0/P-18:1), PC(18:2/OH-16:0), PC(o-16:0/20:4), nutriacholic acid and 2-oxo-4-methylthiobutanoic acid. In the HCC group, PC(o-16:0/20:4), nutriacholic acid and 2-oxo-4-methylthiobutanoic acid were decreased, while PC(18:2/OH-16:0) was elevated compared with the LC group ($e < 0.05$). PC(16:0/P-18:1) was associated with tumor size, vascular invasion, and neutrophil-lymphocyte ratio (NLR; $P < 0.05$). Moreover, PC(18:2/OH-16:0) was also related to tumor number and NLR ($P < 0.05$). Multivariate cox regression showed that PC(16:0/P-18:1), PC(18:2/OH-16:0), nutriacholic acid and alpha-fetoprotein (AFP) were independent risk factors for tumor recurrence ($P < 0.01$). A prognostic fingerprint was established as a nomogram, which divided the patients into low risk (n = 45), moderate risk (n = 48) and highrisk groups (n = 29) with discriminated prognosis ($P < 0.001$). In patients fulfilling the Hangzhou criteria, the fingerprint/nomogram could also successfully stratify the patients into two groups with different recurrence risk ($P < 0.05$).
Conclusions: The established pre-operative plasma fingerprint/nomogram is efficient in the prediction of recurrence risk, which could facilitate candidate selection in liver transplantation for HCC.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer worldwide, and the third leading cause of cancer-related mortality [1]. China has the heaviest burden of HCC as a result of the high prevalence of hepatitis B. Owing to the development of treatment techniques and anti-cancer drugs, long-term survival was improved for HCC, but the overall prognosis remains quite poor [2]. Liver transplantation is currently the most radical treatment option. The Milan criteria are the gold standard for candidate selection, and are able to ensure excellent prognosis [3]. However, Milan criteria have been frequently challenged as being too restrictive, resulting in only a small proportion of patients being eligible for transplantation. Many sets of expansion criteria have been proposed to overcome this problem, such as the UCSF criteria and the Hangzhou criteria [4,5]. Among them, the Hangzhou criteria include HCC patients with: (1) tumor burden ≤8 cm diameter in total; or (2) tumor burden >8 cm but AFP≤400 ng/ml and well-to-moderate differentiation. The Hangzhou criteria expand the candidate pool by approximately 50% and have been

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validated to have excellent prognostic capacity [6]. However, further stratification is still urgently needed for individualized medicine in liver transplantation for HCC.

Metabolomics is a high-throughput approach capable of detecting metabolic changes in different pathological or physiological status [7]. It has been frequently used to reveal metabolic alterations, and also to identify new biomarkers for various types of cancer, including HCC [8]. In this study, we enrolled 122 HCC patients undergoing liver transplantation, and performed metabolomics profiling to explore novel metabolite markers for tumor recurrence after liver transplantation. Based on the metabolites identified, we successfully established a fingerprint that predicts tumor recurrence after liver transplantation.

Materials and Methods

Chemicals

The equipment and chemicals used in the metabolomic profiling were the same as those used in a previous study conducted at our hospital [9]. In detail, we purchased acetonitrile, formic acid and leucine-enkephalin (HPLC grade) from Sigma–Aldrich (St. Louis, MO). Distilled water was acquired through a Milli-Q system (Millipore, MA). Standards for bile acids, phosphatidylcholine (PC), and lysophosphatidylcholine (LPC) were also purchased from Sigma-Aldrich, while long-chain acylcarnitines were from J&K Chemical Ltd. (Beijing, China).

Patients

According to our previous study, the recurrence rate will be around 47.5% [4]. And we generally expected to identify 1 subset (around one third patients) with a decrease of about 30% in the recurrence rate. With a power of 0.80 and two-sided alpha of 0.05, it is estimated that a total inclusion of at least 96 patients is needed (http://clincalc.com/Stats/SampleSize.aspx). This study retrospectively enrolled 122 HCC patients undergoing liver transplantation at the First Affiliated Hospital, Zhejiang University School of Medicine from April 2012 to December 2016, and included 110 males and 12 females. All the patients were pathologically diagnosed as HCC. And all the cases should be donation after cardiac death. We collected the data including demographics, body mass index, preoperative AFP level, morphological features (tumor size, number of nodules), vascular invasion, neutrophil-lymphocyte ratio, post-transplant recurrence, and patients' survival for analysis. Cases with missing data was excluded from the cohort. A cohort of 52 healthy controls (HC group) and 25 cases of liver cirrhosis (LC group) were also included.

The post-transplant patients were followed up at the outpatient clinic. Tumor recurrence was monitored by AFP, ultrasonography, chest X-ray, and computed tomography every 3 months for the first 2 years and semiannually thereafter. Imaging evidence of either intra-hepatic or extra-hepatic recurrent lesions was required for the diagnosis of recurrence. However, a simple increase in AFP was insufficient.

Compared with western countries, China has a much heavier burden of HCC. Patients with single-nodule, small-sized HCC will usually undergo hepatectomy for cure, and transplants are performed on relatively advanced large-sized HCC in China. The recurrence rate is also higher compared with western countries. To control the recurrence rate, efforts are being made to standardize candidate selection for HCC. For example, in Zhejiang province, to which our center belongs, the Hangzhou criteria as the major indication of liver transplantation for HCC is used for patients recruitment as part of the governmental regulation, and recipients exceeding the Hangzhou criteria will not receive medical insurance for transplants.

Informed consent was obtained from all patients and healthy controls, and the study protocol was also approved by the Human Ethics Committee of our hospital. None of the liver grafts were acquired from executed prisoners.

Sample preparation

The procedures of metabolomics detection were described previously [9]. The plasma samples were thawed at 4 °C, and the QC samples were prepared by pooling aliquots (10 μl) of each sample. Acetonitrile (800 μl) was added to the plasma (200 μ1) sample and vortexed for 1 min. We then incubated the mixture at room temperature for 1 min and centrifuged it at 14,000 rpm for 10 min at 4 °C. The acquired clear supernatant was transferred to UPLC vials, and was then stored at 4 °C until detection. The pretreatment of the QC samples was the same as that for the test samples.

UPLC–MS analysis of samples

We performed reversed-phase analysis on a Waters ACQUITY Ultra Performance LC system using an ACQUITY UPLC BEH C18 analytical column (i.d., 2.1 mm × 100 mm; particle size 1.7 mm; pore size, 130 A). Water/formic acid (99.9:0.1 v/v) was applied as mobile phase A, and acetonitrile/formic acid (99.9:0.1 v/v) as phase B. A linear gradient LC system (Waters, Milford MA) was then optimized as follows: the composition of mobile phase B changed from 3% to 80% in 7 min, reached 98% in 8 min, was held for 5 min, and then reached 100% in 1 min and was held for 3 min. Sample manager temperature was set at 4 °C, and the injection volume was 2 μl for analysis. The QC samples were injected at an interval of 14 samples throughout the analytical process to evaluate the repeatability of sample pre-treatment and to monitor the stability of the LC-MS system during sequence analysis.

We used a Waters Q-TOF Premier mass spectrometer in positive ion electrospray mode. The instrumental parameters were as follows: the mass scan range was 50 m/z–1000 m/z with an accumulation time of 0.2 s per spectrum; the MS acquisition rate was adjusted to 0.3 s with an inter scan delay of 0.02 s; and high-purity nitrogen was introduced as the nebulizer and drying gas. The nitrogen drying gas was kept a constant flow rate of 600 L/h, and the source temperature was 100 °C. For the positive mode, the capillary voltage was maintained at 3.0 kV and the sampling cone voltage was 40.0 V. We used argon as the collision gas. MS/MS analysis was conducted on the mass spectrometer set at different collision energies of 5 eV. The time of flight analyzer was used in V mode and tuned for maximum resolution, that is, >10,000 resolving power at 556.2771 m/z. The instrument should be previously calibrated with sodium formate. The lock mass spray for precise determination of mass was set by leucine enkephalin at 556.2771 m/z with a concentration of 0.5 ng/L in positive ion mode. All analyses were acquired using the lock spray to ensure accuracy and reproducibility.

Data processing and statistical analysis

The data generated from the UPLC/MS analysis was processed in MassLynx Application Manager Version 4.1 (Waters, Milford, MA, USA) software. These applications detected, integrated, and normalized the intensities of the peaks to the sum of the peaks within the sample and thereby generated a multivariate dataset based on retention time, m/z, and signal peak intensity. SIMCA Version 14.1 (Umetrics, Umea, Sweden) was used for principle component analysis (PCA). Pathway analysis was performed on the MetaboAnalyst platform (https://www.metaboanalyst.ca/).

Those ions with over one third cases of '0' value will be excluded from analysis. Univariate Cox regression was used to identify prognostic metabolite individually ($P < 0.01$). We used Mann-Whitney u test to compare the metabolite level between different disease groups as well as subgroups. Multivariate Cox regression was used to establish a model/nomogram predicting the risk of recurrence ($P < 0.01$). Meanwhile, Kaplan-Meier analysis was used to compare the survival between subgroups ($P < 0.05$). And if recurrence was not diagnosed, the cases were censored at the date of death or the last date of follow-up. Statistical analysis was performed using SPSS version 16.0 statistical software (SPSS Inc. Chicago, IL, USA) and R Version 3.4.0 (http://cran.r-project.org).

Results

Among the 122 HCC patients undergoing liver transplantation, 110 males and 12 females. Patients' ages ranged from 30 to 69 years old, with an average of 52.2 years. 68 patients fulfilled the Hangzhou criteria. Among the 65 patients that had tumor recurrence, 44 had intrahepatic recurrence, 40 had pulmonary metastasis, 13 had bone metastasis, 13 had peritoneal metastasis, and 9 had other types of recurrence such as brain metastasis. In addition, the time interval between liver transplant and tumor recurrence was 278 days on average.

The total ion chromatograms of a single sample from each group were acquired by the UPLC-MS platform, as presented in Fig. 1a. Using MZmine ver. 2.0 software, this pre-treatment revealed 1,242 integral peaks following extraction ion chromatography detection in all samples. PCA (R2X = 10.0%, Q2 = 17.4%) and heatmap plots are shown in Fig. 1b and c.

Univariate analysis identified 18 differential ions related to tumor recurrence after liver transplantation ($P < 0.01$). By excluding those ions with over one third cases of '0' value, five metabolites were finally identified: PC(16:0/P-18:1), PC(18:2/OH-16:0), PC(o-16:0/20:4), nutriacholic acid, and 2- oxo-4-methylthiobutanoic acid (Supplemental Table 1). Pathway analysis showed that they were related to the linoleic acid metabolism, alpha-linolenic acid metabolism, glycerophospholipid metabolism, and cysteine and methionine metabolism pathways ($P < 0.05$).

All five metabolites were significantly altered between the HC and HCC groups. However, PC(o-16:0/20:4), nutriacholic acid, and 2-oxo-4-methylthiobutanoic acid were decreased, while PC(18:2/OH-16:0) was elevated in the HCC group compared with the LC group ($P < 0.05$; Table 1). When matching the metabolite concentration with clinical features (Supplemental Table 2), we also found that PC(16:0/P-18:1) was associated with tumor size, vascular invasion, and neutrophil-lymphocyte ratio (NLR; $P < 0.05$). Moreover, PC(18:2/OH-16:0) was also related to tumor number and NLR ($P < 0.05$).

Multivariate analysis showed that PC(16:0/P-18:1), PC(18:2/OH-16:0), nutriacholic acid, and AFP were independent risk factors for tumor recurrence (Table 2). Based on these results, we established a fingerprint that predicts tumor recurrence after liver transplantation as a nomogram (Fig. 2). The AUROC level was 0.78 for tumor recurrence. According to the nomogram, the patients were divided into low risk (n = 45), moderate risk (n = 48) and high risk groups (n = 29). Prognosis was significantly different among the three groups ($P < 0.001$; Fig. 3a and b).

The distribution in regards to the criteria (the Milan criteria and the Hangzhou criteria) and nomogram were shown in Supplemental Figure 1. Those patients fulfilling the Milan criteria had decreased recurrence risk and improved post-transplant survival compared with those exceeding Milan criteria ($P < 0.001$). For the 50 patients within the Milan criteria, 32 were in the low risk group, and the outcomes were improved compared with those in the moderate or high risk group according to the nomogram

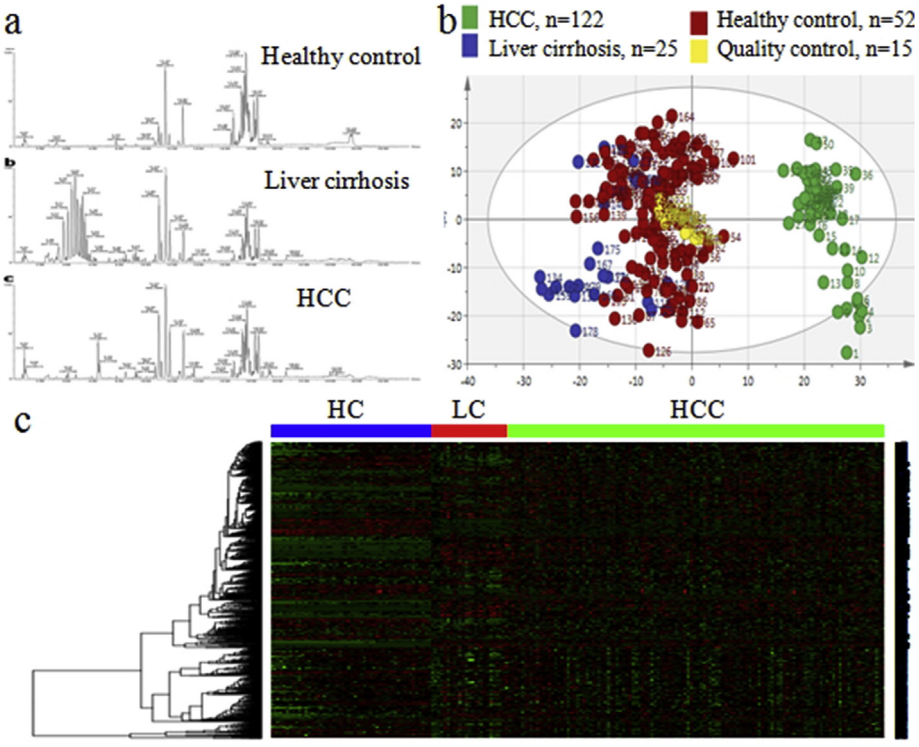

Fig. 1. Metabolomics profiling for healthy controls, patients with liver cirrhosis and those with hepatocellular carcinoma.
Legend. a. The ion chromatograms of a single sample from each patient group. b. The PCA plot for the 3 groups as well as QC samples. c. The heatmap of the 3 groups according to the normalized intensity of all ions.

Table 1
Comparison of identified metabolites among different groups.

Metabolites	HC	LC	HCC	LC vs HCC*	HC vs LC*	HC vs HCC*
PC(16:0/P-18:1)	19.1 (10.0–40.5)	11.2 (4.3–19.8)	12.4 (3.8–30.2)	0.67	<0.001	<0.001
PC(18:2/OH-16:0)	2.3 (1.1–4.1)	1.0 (0–3.2)	1.5 (0–6.5)	.011	<0.001	<0.001
PC(o-16:0/20:4)	5.6 (0–10.0)	6.1 (3.0–9.0)	4.5 (0–9.0)	<0.001	0.77	0.003
Nutriacholic acid	0 (0–3.0)	7.4 (4.0–118.0)	5.3 (2.0–113.0)	.007	<0.001	<0.001
2-oxo-4-methyl-thiobutanoic acid	0 (0–2.0)	2.5 (0–33.0)	1.8 (0–39)	0.032	<0.001	<0.001

Note. PC, Phosphatidyl cholines; HC, healthy control; LC, liver cirrhosis; HCC, hepatocellular carcinoma; *, Mann-Whitney u test.

Table 2
Independent risk factors for tumor recurrence after liver transplantation.

Variate	Hazard ratio	95% CI	p
PC(18:2/OH-16:0)	1.46	1.20–1.78	<0.001
Nutriacholic acid	1.02	1.01–1.03	0.002
PC(16:0/P-18:1)	0.93	0.89–0.98	0.004
LnAFP	1.15	1.05–1.26	0.002

Note. PC, phosphatidylcholine; CI, confidence interval; AFP, alpha-fetoprotein.

(Supplemental Figure 2, $P < 0.05$).

Those patients fulfilling the Hangzhou criteria had decreased recurrence risk and improved post-transplant survival compared with those exceeding the Hangzhou criteria ($P < 0.001$). The 1- and 3-year overall survival (OS) rates for patients fulfilling the Hangzhou criteria (n = 68) were 88.2% and 69.4%, respectively which were comparable with those fulfilling the Milan criteria (n = 46, 1- and 3-year OS, 87.0% and 73.2%, respectively). For patients exceeding the Hangzhou criteria (n = 54), the 1- and 3-year OS rates were 75.9% and 36.6%, respectively. By enrolling the Hangzhou criteria and those five identified metabolites into multivariate Cox regression, we identified the Hangzhou criteria, PC(18:2/OH-16:0), 2- oxo-4-methylthiobutanoic acid and PC(16:0/P-18:1) as independent risk factors for post-transplant tumor recurrence ($P < 0.01$, Table 3). In patients fulfilling the Hangzhou criteria (n = 68), the fingerprint/nomogram could also successfully stratify the patients into groups with different recurrence risk ($P = 0.02$; Fig. 3c and d).

Discussion

Tumor recurrence is the major cause of post-transplant mortality of HCC [10]. However, tumor recurrence after liver transplantation is difficult to predict precisely because of its heterogeneity. The traditional criteria for candidate selection attempts to stratify eligible transplant candidates. However, many patients, who should have acceptable outcomes after transplantation, are excluded by these criteria. Furthermore, there are also patients who fulfill the criteria but still suffer from tumor recurrence after transplantation.

In this study, 46 patients fulfilled the Milan criteria, while 68 patients fulfilled the Hangzhou criteria, that is an expansion of eligible HCC recipients by around 50%. Moreover, the survival of patients fulfilling the Milan and the Hangzhou criteria was comparable. Therefore, the Hangzhou criteria is reliable for candidate selection in liver transplantation for HCC. However, heterogeneity amongst patient prognosis still exists, even in patients fulfilling the Hangzhou criteria. Thus, more accurate modalities are needed for the prediction of post-transplant prognosis.

Metabolomics has frequently been used to search for candidate biomarkers, as metabolic profile variations often occur prior to radiological manifestation [11]. Moreover, metabolomics on plasma

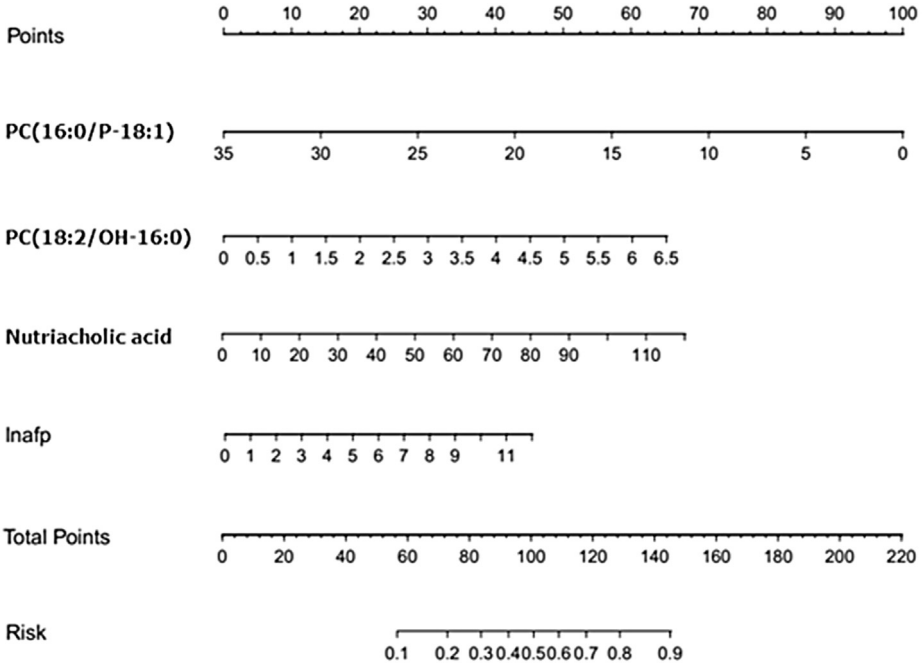

Fig. 2. The nomogram version of the metabolite fingerprint in liver transplantation for hepatocellular carcinoma.
Legend. The fingerprint was translated to a nomogram for clinical practice.

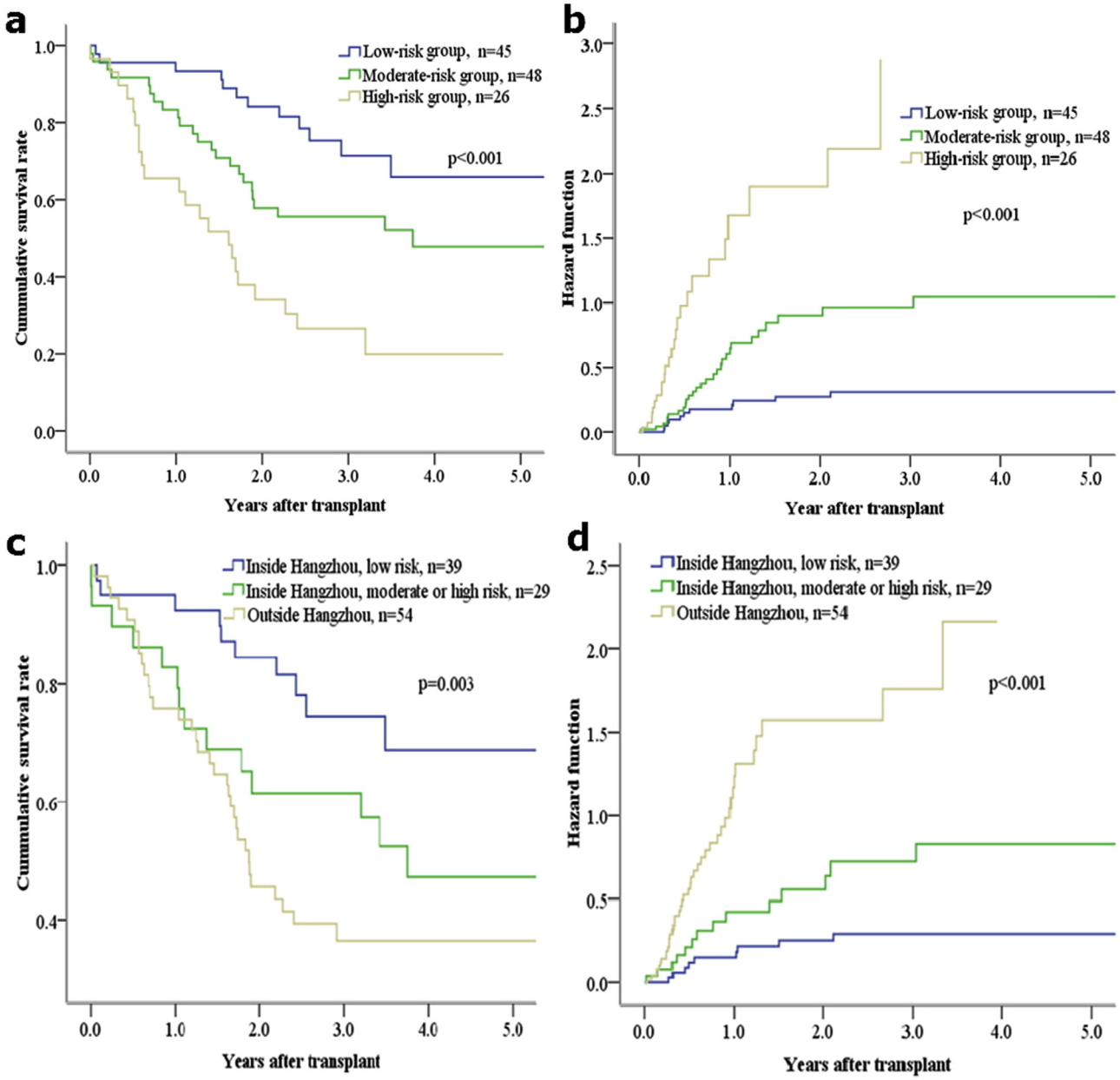

Fig. 3. The role of the metabolite fingerprint in the prediction of post-transplant tumor recurrence.
Legend. The fingerprint can stratify the patients into groups with discriminated overall survival (a) and recurrence risk (b) ($P < 0.001$). In the patients fulfilling Hangzhou criteria (n = 68), the fingerprint/nomogram can also successfully stratify the patients into groups with different overall survival (c) and recurrence risk (d) ($P < 0.05$).

Table 3
Multivariate cox regression enrolling Hangzhou criteria and identified metabolites.

Variate	Hazard ratio	95% CI	p
PC(18:2/OH-16:0)	1.35	1.12–1.64	0.002
2-Oxo-4-methylthiobutanoic acid	1.06	1.02–1.09	0.001
PC(16:0/P-18:1)	0.93	0.89–0.98	0.098
Hangzhou criteria	3.19	1.82–5.29	<0.001

Note. PC, phosphatidylcholine; CI, confidence interval.

or serum samples is noninvasive, and thus can be used to monitor disease progression in real time. By undertaking metabolomics profiling in the plasma samples of HCC patients undergoing liver transplantation, this study identified five metabolites related to tumor recurrence involved in linoleic acid metabolism, alpha-linolenic acid metabolism, glycerophospholipid metabolism, and cysteine and methionine metabolism pathways.

Among the five metabolites, three were PCs, a class of phospholipids that incorporate choline as a headgroup. Many studies have reported their association with HCC [12–15], which is known to be related to lipid metabolism reprogramming [16]. A recent study identified over-expressed LPC acyltransferase 1, a catalytic enzyme responsible for the conversion of LPC to PC in the Lands cycle of PC biosynthesis, as an enriched PC species in HCC that promoted cell proliferation [17]. Conversely, PCs have been also reported to inhibit liver carcinogenesis and tumor growth [18,19]. This contradiction was also observed in our study, that is, PC(o-16:0/20:4) is negatively associated with recurrence risk, while PC(18:2/OH-16:0) has a positive relationship. There are many different types of PCs, and their function and related mechanisms are diverse.

Metabolomics has also been recently applied in the exploration of prognostic markers. Zhou et al. compared the metabolomics profiles of plasma samples from 22 early-recurrent and 18 late-recurrent HCC patients, and identified notable changes in fatty acid and bile acid steroids in the early-recurrent HCC group [20]. Ye et al. collected urinary samples from 19 pairs of matched preoperative and postoperative HCC patients and 20 healthy volunteers. They used GC-TOF-MS-based metabolomics to investigate the physiopathological alterations in HCC after surgical resection, and identified five metabolites (aconitic acid, phenylalanine, ethanolamine, ribose, and lactic acid) that predicted early recurrence [21]. According to the proposed fingerprint/nomogram in the current study, the patients could be effectively stratified regarding recurrence risk. Moreover, when we combined the fingerprint with the Hangzhou criteria, we could further subgroup patients fulfilling the Hangzhou criteria. The Hangzhou criteria are an expansion to Milan criteria. However, expansion of the candidate pool will inevitably lead to a sacrifice in survival. Therefore, an effective stratification system for patients as a supplement to the expanded criteria will be helpful.

Nevertheless, there are limitations of this study. For new biomarkers or models, the sample size is relatively small, and external validation is needed in future studies. Also, the recurrence rate was relatively high owing to the severe burden of HCC in China and the attempts in liver transplantation beyond the Milan criteria. There will be biases when compared with the western countries, as those identified metabolites and the nomogram will not be completely suitable for their patient cohort of relative early stage HCC. Another issue is its feasibility in clinical practice. The identification of specific metabolites requires equipment that is currently not available across liver transplant centers. However, as the technique is developing fast, the new metabolites might be included in routine liver function assays or new detection modalities.

In conclusion, metabolomics profiling can identify novel prognostic metabolite markers in liver transplantation for HCC. The preoperative plasma fingerprint/nomogram established is efficient in the prediction of recurrence risk and can facilitate candidate selection in liver transplantation for HCC.

Conflicts of interests

The authors all declare that they have no conflicts of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejso.2019.07.004.

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