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Serum metabolites may be useful markers to assess vascular invasion and identify normal alpha-fetoprotein in hepatocellular carcinoma undergoing liver resection: a pilot study

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Abstract

Purpose: Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver with a dismal prognosis. Vascular invasion, among others, is the most robust indicator of postoperative recurrence and overall survival after liver resection for HCC. Few studies to date have attempted to search for effective markers to predict vascular invasion before the operation. The current study would examine the plasma metabolic profiling via ^{1}H-NMR of HCC patients undergoing liver resection and aim to search for potential biomarkers in the early detection of HCC with normal alpha-fetoprotein (AFP) and the diagnosis of vascular invasion preoperatively.

Materials and methods: HCC patients scheduled to receive liver resections for their HCC were recruited and divided into two separate groups, investigation cohort and validation cohort. Their preoperative blood samples were collected and subjected to a comprehensive metabolomic profiling using ^{1}H-nuclear magnetic resonance spectroscopy (NMR).

Results: There were 35 HCC patients in the investigation group and 22 patients in the validation group. Chronic hepatitis B remained the most common etiology of HCC, followed by chronic HCV infection. The two study cohorts were essentially comparable in terms of major clinicopathological variables. After ^{1}H-nuclear NMR analysis, we found in the investigation cohort that HCC with normal alpha-fetoprotein (AFP < 15 ng/mL) had significantly higher serum level of O-acetylcarnitine than those with higher AFP (AFP ≥ 15 ng/mL, $P = 0.025$). In addition, HCC with microscopic vascular invasion (VI) had significantly higher preoperative serum level of formate than HCC without microscopic VI ($P = 0.023$). These findings were similar in the validation cohort.

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Conclusion: A comprehensive metabolomic profiling of HCC demonstrated that serum metabolites may be utilized to assist the early diagnosis of AFP-negative HCC patients and recognition of microvascular invasion in order to facilitate preoperative surgical planning and postoperative follow-up. Further, larger scale prospective studies are warranted to consolidate our findings.

Keywords: Metabolomics, Metabolites, Vascular invasion, Formate, Hepatocellular carcinoma, Hepatoma

Introduction

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver with an estimated incidence of mortality at 700,000 per year worldwide [1]. In Taiwan, HCC is the second most common cause of cancer death, with more than 8000 deaths annually [2]. Surgical resections remain the most effective therapy in selected patients; however, the extent and feasibility of such are still complicated by the patients' coexisting liver diseases, such as chronic hepatitis B or C and alcoholic liver disease. Less than 40% of non-cirrhotic HCC patients received liver resections, and the rate was even much lower in patients with chronic liver disease or overt cirrhosis [3]. Regardless a significant improvement in the surveillance program, diagnostic modality, surgical techniques, and postoperative care, the recurrence rate of HCC after liver resection, liver transplantation, or radiofrequency ablation (RFA) still remains as high as 60% [4]. Given the low operative and high recurrence rate of HCC, it is therefore of paramount importance to search for potential markers in the prediction of tumor recurrence or long-term survival after liver resection to ensure optimal surveillance or opportune adjuvant treatment postoperatively.

Risk factors for HCC recurrence after surgery have been identified, including lymph node metastasis (LNM) [5], vascular invasion, poor tumor differentiation, large tumor size, and multinodularity [6, 7], among which, vascular invasion (VI), being the most robust indicator of postoperative recurrence and overall survival [8]. VI can be subdivided into macroscopic VI when larger or major vessels are invaded and detectable by preoperative radiologic study and microscopic VI when small blood vessels are invaded and identified only under microscopic examination. In the presence of preoperative VI, more aggressive surgical treatment such as wider safety margin or anatomical liver resection can be performed. Moreover, when VI is highly suspected, perioperative transarterial chemoembolization (TACE) can be applied. That said, microscopic VI is barely detectable by conventional radiologic studies. Few studies to date have attempted to search for effective markers in the prediction of preoperative microscopic VI. An overexpression of abnormal spindle-like microcephaly associated (ASPM) and upregulation of cytohesin-3 have been found to be associated with VI and early tumor recurrence [9, 10]. Nevertheless, these molecular markers are barely detectable in percutaneous biopsy or before the operation. For real-world clinical practicing, a reliable predictive marker readily available from blood samples may be the best resort.

Metabolomics, a new science that simultaneously detects many metabolites in biofluids and tissues, has been employed in the fields of oncology and various other diseases for the evaluation of their altered levels in a pathological state. Recently, metabolomics has also been employed in the field of HCC in search for diagnostic as well as predictive biomarkers for HCC. HCC patients in different pathological status were found to possess different plasma profiling [11]. For example, Di Poto et al. had demonstrated that a panel consisting of 11 serum metabolites and three clinical factors (AFP, Child–Pugh score, and etiologic factors) had a higher area under the ROC curve compared to AFP alone for the diagnosis of HCC [12, 13]. Luo et al. identified a biomarker panel (phenylalanyl-tryptophan and glycocholate) that had a better diagnostic value compared to AFP for distinguishing HCC patients from a high-risk cohort of cirrhotic patients [14]. Pathways of lysophospholipid, sphingolipid, bile acid, amino acid, and reactive oxygen species metabolism were found to be consistently associated with the development of HCC [15]. Moreover, increased uptake of glucose, enhanced lactate production, and altered lipid metabolism have been defined to be characteristic metabolic signatures in HCC [16]. As for the prediction of outcome, by using metabolomic platforms, ethanolamine, lactic acid, acotinic acid, phenylalanine, and ribose were found useful in discriminating patients with early recurrence from those without recurrence [17], and metabolomic fingerprints rather than tumor size appeared to show an impact on poor prognosis [18]. Lately, Zhou et al. [19] identified that a combination of serum methionine, GCDCA, and cholesterol sulfate levels could predict early HCC recurrence with high accuracy. Despite these remarkable results, few studies to date have tried to search for potential metabolites predictive of microscopic VI in HCC. In this study, we present plasma metabolic profiling via ^{1}H-nuclear magnetic resonance spectroscopy (^{1}H-NMR) of HCC patients undergoing liver resection and aim to search for

potential biomarkers in the early detection of HCC with normal alpha-fetoprotein (AFP) and the diagnosis of VI preoperatively.

Materials and methods

Patient selection

Under the approval of the Institutional Review Boards of Chang Gung Memorial Hospital (CGMH), primary HCC patients scheduled to receive curative liver resection for their tumors were included into our study. After informed consent was completed, 10 ml of blood was drawn both before the operation and 1 month after the operation. The patients were asked to stay fasted prior to blood tests. The serum was extracted by centrifugation and was stored into aliquots and frozen at − 80 °C until further batch analysis by hydrogen-1 nuclear magnetic resonance (^{1}H-NMR) in the Metabolomic Core Laboratory at CGMH and Chang Gung University (CGU). Patient demographic data including age, gender, cigarette smoking, alcohol consumption, hepatitis B virus (HBV) infection, anti-hepatitis C virus antibody (anti-HCV) level, serum bilirubin, albumin, prothrombin levels, international normalized ratio (INR), serum alanine aminotransferase (ALT) activity, serum aspartate aminotransferase (AST) activity, serum gamma-glutamyltransferase (GGT) activity, platelet count, and alpha-fetal protein (AFP) level were collected. Their pathological factors and oncological outcome including tumor lymph node metastasis status, tumor encapsulation, histological grade, VI, daughter nodules, resection margin, fatty change, liver cirrhosis, tumor recurrence, and long-term survival were also recorded. Early recurrence is defined as the occurrence of tumor recurrence within 2 years of the curative operation. Overall survival (OS) is defined by the time elapsing from the date of diagnosis to either the date of death or the date of the last contact. Cases with surgical mortality, defined as death within 1 month of surgery were excluded from the survival analyses. A separate cohort was also recruited for validation.

NMR spectroscopy analysis

General procedure

The procedures briefly described below were aimed to obtain high-quality information for metabolic study, including (a) chemical shifts (value, TMS, or TSP as 0.0), (b) spin-spin coupling and coupling constants (J value), (c) relaxation (may not necessary all times), and (d) signal intensity (for quantitation). The standardized procedure currently used in the Metabolomic Core Laboratory, CGU, is a modified protocol of that described by Beckonert et al. [20].

NMR measurement

Measurement of the ^{1}H-NMR spectra for aqueous extracts A NOESY pulse sequence with water irradiation during the relaxation delay and during the mixing time were used (RD-90°-t-90°-tm-90°-ACQ) (RD is the relaxation delay, t is a short delay typically of about 3 s, 90° represents a 90° RF pulse, tm is the mixing time, and ACQ was the data acquisition period. The mixing time is tm = 100–150 ms). Other parameters are as follows: spectral width = 20 ppm, number of time domain data points = 32,768, relaxation delay, RD = 2.0 s, acquisition time = 1.36 s, number of scans = 64 for urine and 128 for plasma, serum, and tissue extracts. This resulted in a total acquisition time of about 8–12 min per sample (128 scans) [20]. The pulse sequence was suitable for NMR signal quantitation.

Lipophilic metabolites (lipidomics) The ^{1}H-NMR spectra of lipid extracts did not require water suppression. A routine 90° pulse sequence was sufficient in measuring fully relaxed spectra (for quantitation purpose). In this core laboratory, a routine collect 64 transients (NS) into 32,768 data points was carried out. A relaxation delay = 2 s, spectral width = 20 ppm, and acquisition time = 1.36 s can be used [20]. For quantitation, signal intensity was used. A mixture of lipid metabolites containing known amounts of free cholesterol and cholesterol oleate, phospholipids, and TG were used as reference sample. The crude lipid extracts (Folch method) of a mixture of plasma samples were used for calibration.

NMR data management The conversion of FID signals to spectral data was carried out using the softwares of the available software package of NMR instruments. Assignment of the chemical shifts to specific metabolites will base on spectral data in the literature or by the information based on the measurement of authentic metabolites in our chemical library. An NMR data base (not complete) of LMW metabolites (pH adjusted) was available. Several non-commercial and commercial application softwares such as *MetaboAnalyst* [21] were used for metabolite identification. The NMR spectrum was normalized by internal standard (TSP, 3-(Trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt). The missing value of the identified metabolites was defined as zero.

Statistical analysis

The statistical analysis was performed with IBM SPSS Statistics 21 (IBM Corporation, Software Group, Somers, NY, USA). Fisher's exact test and Pearson's $\chi 2$ test were used to analyze categorical data. Student's t test was used to analyze quantitative variables. Statistical significance is defined as $P < 0.05$.

Table 1 Patient demographics and tumor characteristics of the investigation cohort (n = 35)

	No (%)
Age (year) (mean 62.7 ± 1.9)	
≦ 65	20 (57.1)
> 65	15 (42.9)
Gender	
Male	27 (77.1)
Female	8 (22.9)
Smoking	
Yes	11 (31.4)
No	24 (68.6)
Alcohol	
Yes	6 (17.1)
No	29 (82.9)
HBsAg	
Positive	23 (65.7)
Negative	12 (34.3)
Anti-HCV Ab	
Positive	8 (22.9)
Negative	27 (77.1)
Cirrhosis	
Yes	14 (40.0)
No	21 (60.0)
ICG-15 (%)	
≦ 10	20 (57.1)
> 10	15 (42.9)
AFP (ng/ml)	
≦ 15	14 (40.0)
> 15	21 (60.0)
AFP (ng/ml)	
≦ 200	27 (77.1)
> 200	8 (22.9)
Surgical procedure	
Major	9 (25.7)
Minor	26 (74.3)
Size (cm)	
≦ 5	24 (68.6)
> 5	11 (31.4)
Encapsulation	
Yes	28 (80.0)
No	7 (20.0)
Vascular invasion	
Yes	14 (40.0)
No	21 (60.0)
Tumor rupture	
Yes	3 (8.6)
No	32 (91.4)
Daughter nodules	
Yes	4 (11.4)
No	31 (88.6)
Resection margin	
Positive	0 (0)
Negative	35 (100)
Edmondson grade	
I/II	4(11.8)/10(28.6)
III/IV	18(52.9)/3(8.8)
Early recurrence[a]	
Yes	4 (11.4)
No	31 (88.6)

[a]Tumor recurrence within 2 years after surgery

Results

Patient characteristics

A total of 35 patients were enrolled to the study as the investigation group. Their blood samples were collected the day before and 1 month after the operation. As shown in Table 1, of the 35 patients in the investigation cohort, the majority were males, with an average of 62.7 ± 1.9 years of age. Twenty-three patients (65.7%) had HBV infection and 8 (22.9%) had HCV infection. The majority of the investigation group had serum AFP levels greater than 15 ng/mL, with an average of 16,096 ± 12, 568 ng/mL (Table 2). Nine patients received major surgical procedures, and about 70% of tumors were less than 5 cm in diameter. Among the investigation group, 20% was non-encapsulated HCC, 40% had VI, 11.4% had daughter nodules, 8.6% was ruptured tumor, and 40% had pathologically proven cirrhosis. Using Edmondson and Steiner grade, the distribution of grade I to IV HCC in the investigation cohort was 11.8%, 28.6%, 52.9%, and 8.8%, respectively. Another 22 patients were recruited as the validation cohort with similar clinicopathological characteristics as the investigation cohort, except all 22 patients in the validation cohort were encapsulated HCC (Table 2).

NMR plasma profiles in HCC patients in relation to serum AFP levels

As demonstrated in Fig. 1, heatmap showed the clustering of metabolites that highly correlated with the status of HCC in the absence of AFP elevation. Also, ^{1}H-NMR plasma profiles using OPLS-DA, in which red area denoted HCC patients with normal AFP levels (less than 15 ng/mL) while green area denoted

Table 2 Comparison of clinicopathological characteristics between investigation cohort (n = 35) and validation cohort (n = 22)

	Categorical variables (***n*** (%))				Continuous variables (mean ± SE)		
	Investigation	Validation	***P*** value		Investigation	Validation	***P*** value
Male gender	27 (77.1)	19 (86.4)	0.502	**Age (year)**	62.7 ± 1.9	60.2 ± 2.4	0.429
HBV infection	23 (65.7)	11 (50.0)	0.277	**BMI (Kg/m^2)**	23.9 ± 0.5	25.6 ± 0.7	0.063
HCV infection	8 (22.9)	9 (40.9)	0.234	**ICG-15 (%)**	9.53 ± 0.88	11.44 ± 1.64	0.316
AFP > 15 ng/mL	21 (60.0)	10 (45.5)	0.413	**AFP (ng/mL)**	16096 ± 12568	17938 ± 17777	0.931
Major procedure	9 (25.7)	6 (27.3)	1.000	**CEA (ng/mL)**	6.07 ± 4.07	1.57 ± 0.20	0.361
Encapsulation	28 (80.0)	22 (100.0)	0.036	**CA 19-9 (U/mL)**	26.6 ± 3.7	22.1 ± 3.9	0.421
Vascular invasion	14 (40.0)	10 (45.5)	0.785	**Size (cm)**	4.59 ± 0.61	4.05 ± 0.68	0.557
Daughter nodules	4 (11.4)	2 (9.1)	1.000				
Tumor rupture	3 (8.6)	1 (4.5)	1.000				
Cirrhosis	14 (40.0)	13 (59.1)	0.184				
Edmondson and Steiner Grade (I/II/III/IV)	4(11.8)/10(28.6)/ 18(52.9)/3(8.8)	3(13.6)/13(59.1)/ 5(22.7)/1(4.5)	0.073				

HCC patients with abnormal AFP levels (greater or equal to 15 ng/mL), have shown a separation between the patients with normal (< 15 ng/mL) and elevated AFP (≥ 15 ng/mL).

Further analysis on the identifications of metabolites associated with AFP levels revealed a few metabolites possibly responsible for the group separation, including glycine, O-acetylcarnitine, histidine, and ornithine. Of particular interest, the level of O-acetylcarnitine was much higher in the normal AFP-HCC group (N-HCC) than abnormal AFP-HCC group (A-HCC) with statistical significance (0.600 ± 0.08 vs. 0.374 ± 0.11, P value

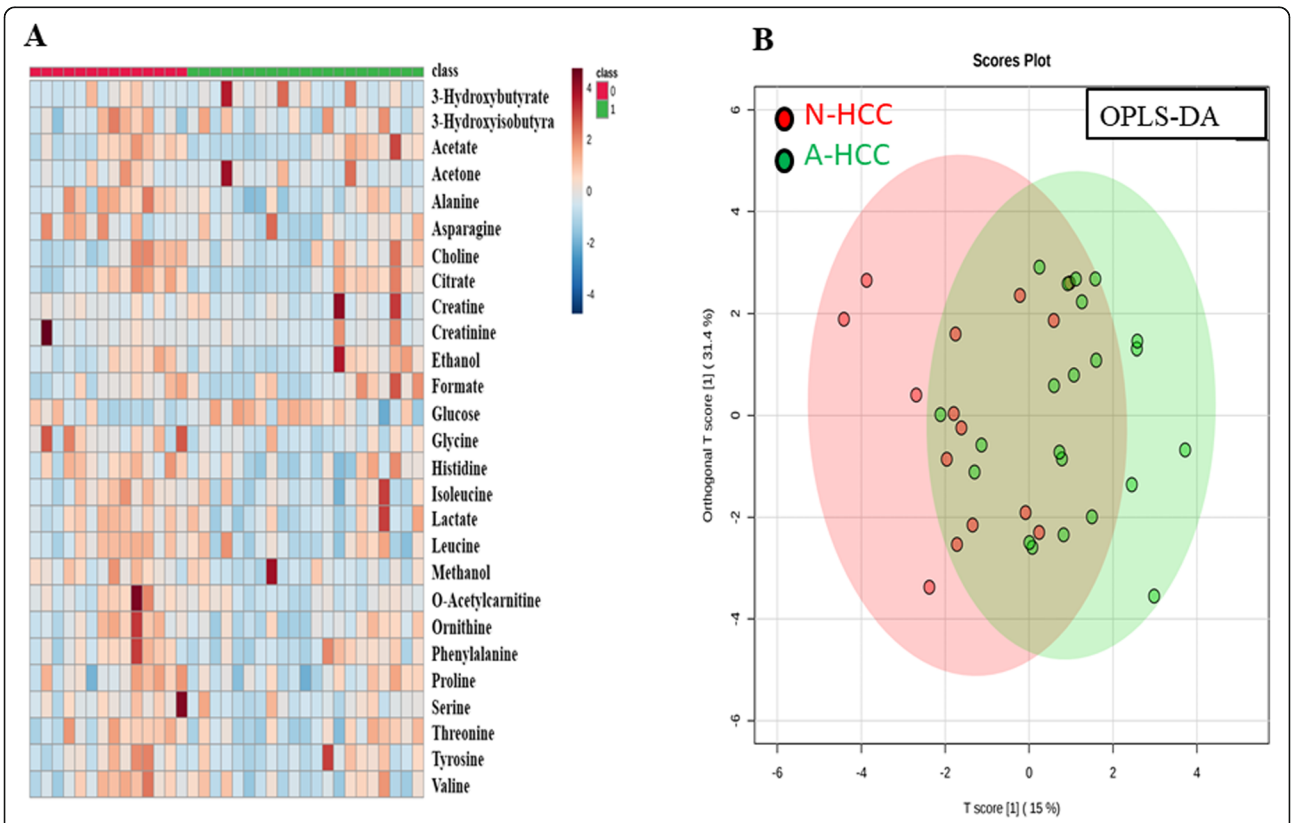

Fig. 1 Metabolome analysis by NMR in HCC. **a** Heatmap analysis in HCC with or without normal AFP. **b** ^{1}H-NMR plasma profiles using OPLS-DA. A separation is seen between the two groups. Red area denotes HCC patients with normal AFP levels (less than 15 ng/mL, N-HCC) while green area denotes HCC patients with abnormal AFP levels (greater or equal to 15 ng/mL, A-HCC)

Table 3 Nuclear magnetic resonance (NMR) observed serum metabolites with intensity differences associated with different AFP for hepatocellular carcinoma

Metabolites	AFP < 15 ng/ml	AFP ≥ 15 ng/ml	*P* value[a]
Investigation cohort (*n* = 35)			
Glycine	26.128 ± 2.83	18.627 ± 1.09	0.006
O-Acetylcarnitine	0.600 ± 0.08	0.374 ± 0.11	0.025
Histidine	6.597 ± 0.36	5.532 ± 0.37	0.062
Ornithine	9.263 ± 1.05	7.065 ± 0.58	0.073
Validation cohort (*n* = 22)			
Glycine	26.810 ± 1.33	27.195 ± 2.19	0.752
O-Acetylcarnitine	0.958 ± 0.09	0.687 ± 0.08	0.032
Histidine	7.303 ± 0.32	6.070 ± 0.55	0.153
Ornithine	10.504 ± 0.86	9.061 ± 0.75	0.215

Metabolites were expressed as mean ± SE in arbitrary units
[a]Student's *t* test

0.025). Similar trend was observed in the validation group in which the level of O-acetylcarnitine was 0.958 ± 0.09 vs. 0.687 ± 0.08 in N-HCC and A-HCC, respectively, as shown in Table 3.

The diagnostic ability of O-acetylcarnitine in discriminating N-HCC from the entire HCC cohort was demonstrated by the ROC curves in Fig. 2. In terms of N-HCC, O-acetylcarnitine has shown an area under the curve (AUC) of the receiver-operating characteristics (ROC) or AUROC of 0.753 in the investigation group and an AUROC of 0.718 in the validation group.

NMR plasma profiles in HCC patients in relation to VI

As VI is one important prognostic factor in HCC and can potentially alter the extent of surgical treatment, ^{1}H-NMR was conducted in an attempt to distinguish the status of microvascular invasion in HCC patients preoperatively. In Fig. 3, heatmap and OPLS-DA demonstrated a separation of metabolites contributing to the microvascular invasion. Red and green denoted the absence and the presence of microvascular invasion. From this model, few metabolites with high variable importance projection (VIP) scores were selected. In Fig. 4, formate appeared to contribute to the separation with the highest VIP score [22]. The level of formate was significantly higher in HCC with VI than HCC without VI (37.592 ± 7.83 vs. 19.697 ± 3.23, *P* value 0.023) (Table 4). Similar trend was observed in the validation group in which the level of formate was 18.776 ± 0.99 vs. 14.807 ± 1.48 in HCC with and without VI, respectively.

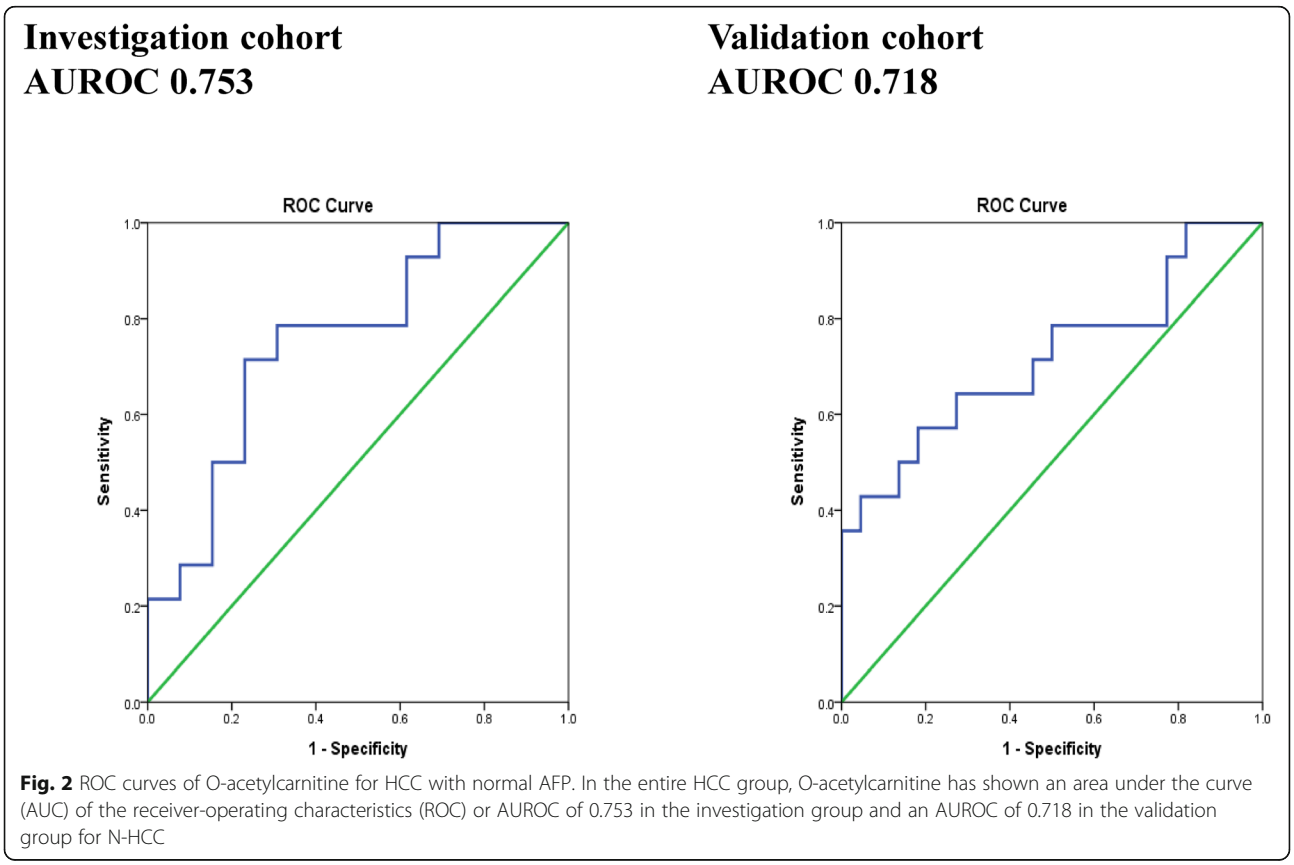

Fig. 2 ROC curves of O-acetylcarnitine for HCC with normal AFP. In the entire HCC group, O-acetylcarnitine has shown an area under the curve (AUC) of the receiver-operating characteristics (ROC) or AUROC of 0.753 in the investigation group and an AUROC of 0.718 in the validation group for N-HCC

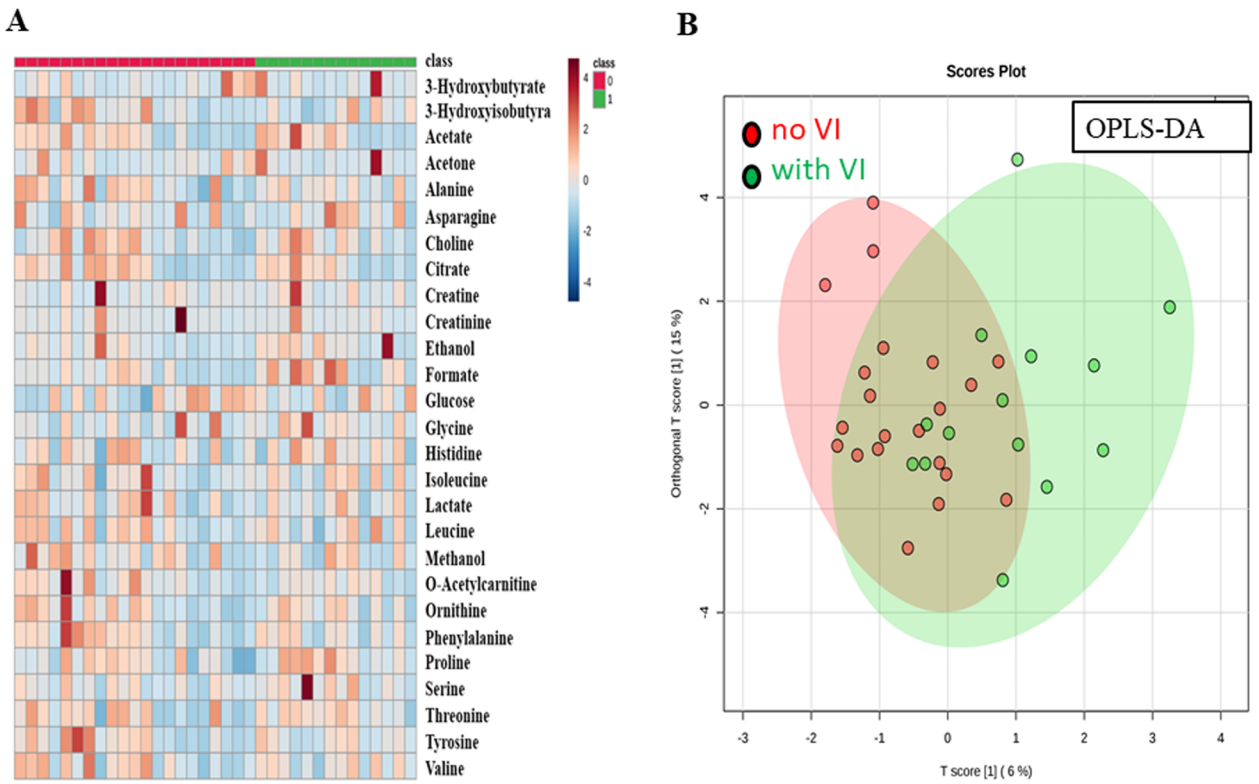

Fig. 3 Metabolome analysis by NMR in HCC in association with vascular invasion. **a** Heatmap analysis with or without microscopic vascular invasion. **b** ^{1}H-NMR plasma profiles using OPLS-DA. A separation is seen between the two groups. Red area denotes HCC patients without vascular invasion while green area denotes HCC patients with vascular invasion

Discriminative ability of clinical parameters and biomarker for VI

As previously demonstrated, formate was highly selective for the presence of microvascular invasion in HCC patients. Tumor size, serum AFP, and ICG-15 were compared between HCC patients with and without microscopic VI. As shown in Table 4 and Fig. 5, even though tumor size, AFP, and ICG-15 individually may not be adequate in distinguishing the presence of VI, formate appeared to display an acceptable AUROC of 0.612 in the investigation group and 0.727 in the validation group. In combination of formate, AFP, and tumor size, the AUROC in predicting microscopic VI raised to 0.806 in the investigation group and 0.745 in the validation group (Fig. 6).

Discussions

HCC is one of the most common malignant tumors, with increasing incidence and high mortality rate, in the world [23, 24]. Clinically, HCC is diagnosed by tissue-based histopathological findings, characteristic radiologic features, or blood-based assays such as AFP, and a majority of HCC are diagnosed at an advanced stage. It appears that the sensitivity and specificity of such clinically used methodology may not be adequate in identifying early stage HCC and thus significantly reducing the reliability. Recently, metabolomics has gained its popularity in the field of surgical oncology as homeostasis perturbations related to cancer development may be detected with the new platform. As an extreme complex organ responsible for glycolysis and metabolisms of carbohydrates, amino acids, and fatty acids, literature has revealed that metabolomics based on NMR could be used to identify biomarkers in varying stages of liver diseases [25] in hope for early tumor detection.

AFP level is the most frequently used tumor marker for HCC; however, in the absence of AFP elevation, the diagnosis can be difficult. Metabolomic analysis comparing HCC and healthy individuals has demonstrated that an increased level of acetylcarnitine is associated with HCC and highly correlated with tumor grade [26–28]. In consistency with the literature, we also found elevated serum acetylcarnitine, more so in these normal AFP HCC patients than those with abnormal AFP. Among HCC patients, acetylcarnitine showed a discriminative accuracy of > 70% for those

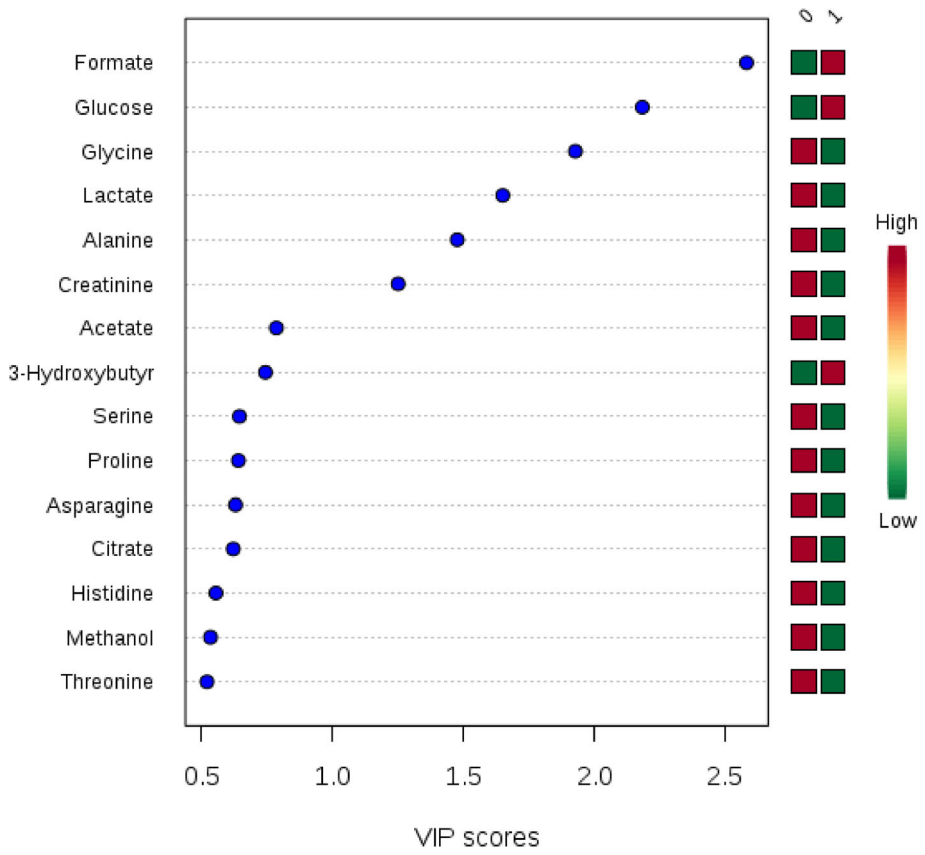

Fig. 4 PLS-DA variable importance projection (VIP) scores. The metabolites responsible for the classification of VI or not would be identified using the variable importance in projection (VIP) scores. Metabolites with high VIP are more important in providing class separation, while those with small VIP provide less contribution [22]. Formate had the highest VIP score in terms of VI. Red or green on the right of Fig. 4 indicated the low or high concentration of metabolites by comparing the concentration of each metabolite in patients with (1) or without (0) vascular invasion

Table 4 Clinical variables and nuclear magnetic resonance (NMR) observed serum metabolites with intensity differences associated with vascular invasion (VI) for hepatocellular carcinoma

Metabolites	Without VI	With VI	*P* value[a]
Investigation cohort (*n* = 35)			
Tumor size (cm)	3.47 ± 0.49	6.29 ± 1.22	0.047
AFP (ng/ml)	6541.91 ± 6486.76	30428.30 ± 30156.35	0.360
ICG-15 (%)	9.80 ± 1.10	9.11 ± 1.54	0.711
Formate	19.697 ± 3.23	37.592 ± 7.83	0.023
Validation cohort (*n* = 22)			
Tumor size (cm)	3.80 ± 0.98	4.33 ± 0.95	0.710
AFP (ng/ml)	145.82 ± 87.38	39289.31 ± 39109.13	0.343
ICG-15 (%)	13.18 ± 2.42	9.35 ± 2.10	0.256
Formate[b]	14.807 ± 1.48	18.776 ± 0.99	0.042

Data were expressed as mean ± SE
[a]Student's *t* test
[b]Expressed in arbitrary units

with normal AFP (AFP < 15 mg/L), suggesting that acetylcarnitine may be served as a sensitive and specific biomarker, in complementary to AFP, for monitoring the development of HCC. Acetylcarnitine is an acetylated form of L-carnitine mainly used for energy production by transporting activated long chain fatty acids from the cytosol into mitochondria. Carnitine is naturally produced by the body. As advanced stage of HCC often predisposes patients to cancer cachexia, low serum levels of acetylcarnitine are mainly the result of reduced synthesis of carnitine in HCC patients. Further studies are warranted to elucidate the roles of acetylcarnitine in the diagnosis and follow-up of HCC patients.

In clinical practice, the presence of VI may influence the surgeons' decision in terms of the extent of resection. VI is found to be associated with the presence of ascites in patients with HBV-related cirrhotic HCC [29]. Other evidence has also shown that derived neutrophil

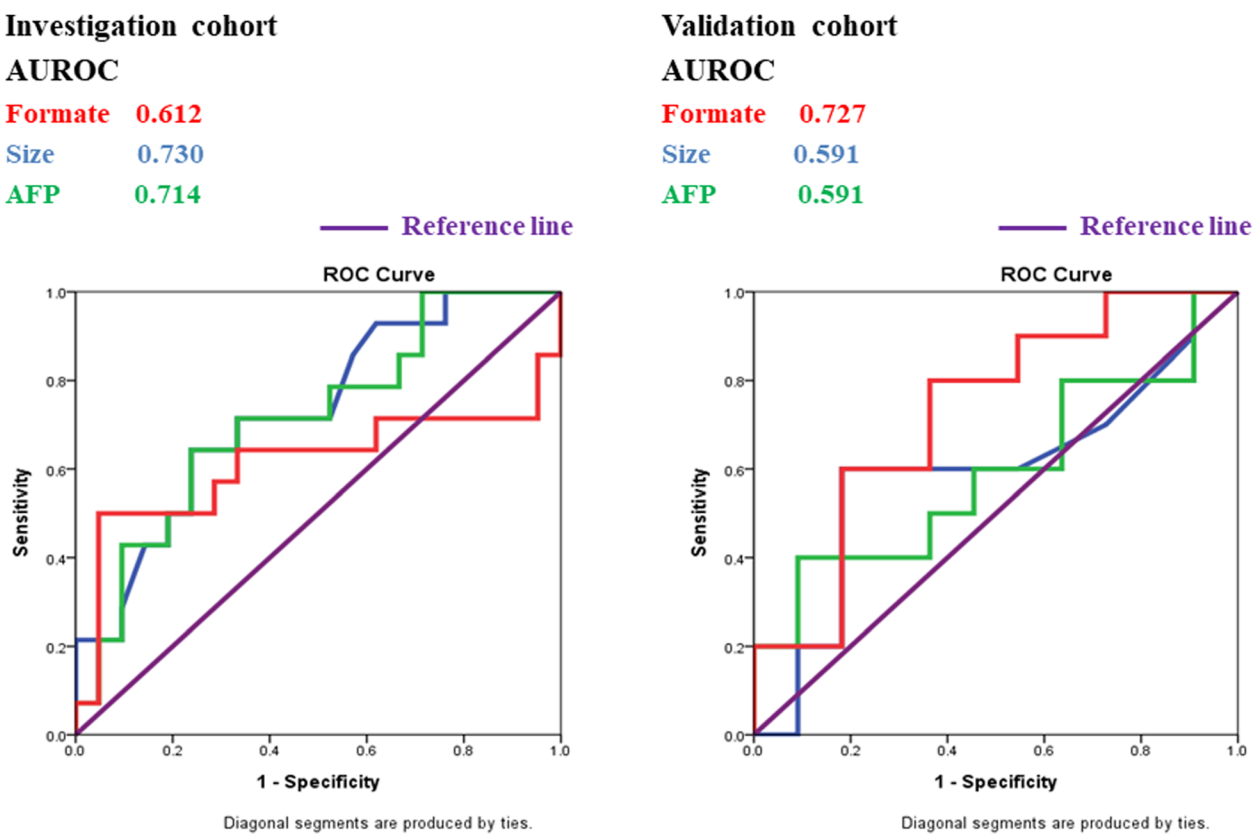

Fig. 5 ROC curves of formate, tumor size, and AFP in diagnosing vascular invasion in HCC. Formate has shown an AUROC of 0.612 in the investigation group and an AUROC of 0.727 in the validation group in terms of predicting VI. Tumor size and AFP levels, on the other hand, have AUROC of 0.730 and 0.714 in the investigation group, and 0.591 and 0.591 in the validation group, respectively, for predicting VI

to lymphocyte ratio (dNLR), lymphocyte-to-monocyte ratio (LMR), age, and tumor size were independent predictors of micro-vascular invasion [30]. Kurokawa T et al. [31] discovered that next generation des-r-carboxy prothrombin (NX-DCP) may be used to predict the presence of VI in HCC. However, these researches are mostly correlation studies, and they failed to explain the mechanism underlying the association between the markers and VI. Moreover, most of them did not have validation cohorts to confirm their findings in the investigation cohort. As a result, it is of urgent need to develop a reliable biomarker to predict and explain VI prior to liver resection.

Several recent studies have demonstrated that formate is a useful biomarker in HCC [32–34]; however, what we have shown is that formate itself is a highly specific and selective biomarker for the prediction of VI and a combination of formate, tumor size and AFP further improves its performance. Our finding is supported by the study done by Meiser et al. that an increased formate overflow is a hallmark of oxidative cancers and that high formate levels promote tumor invasion [35]. Furthermore, they also discovered that inhibition of formate production by genetic interference could reduce cancer cell invasion. Their study is remarkable and further confirmed our finding that formate may be an effective serum marker to predict VI in HCC. Moreover, from the study mentioned above, the influence of formate is potentially targetable and may be utilized to develop treatment strategies against HCC with VI.

The results of the current study have shown that these metabolic changes are a result of carcinogenesis related to energy metabolism, lipid metabolism, and tricarboxylic acid (TCA) cycle. Given the small sample size and multiple comparisons, interpretation of the results must be cautious, even with validation data. As liver is a complex organ also responsible for lipid and amino acid metabolism, the use of high throughput liquid chromatography and mass spectrometry such as liquid chromatography mass spectrometry (LC-MS) with larger sample size is required.

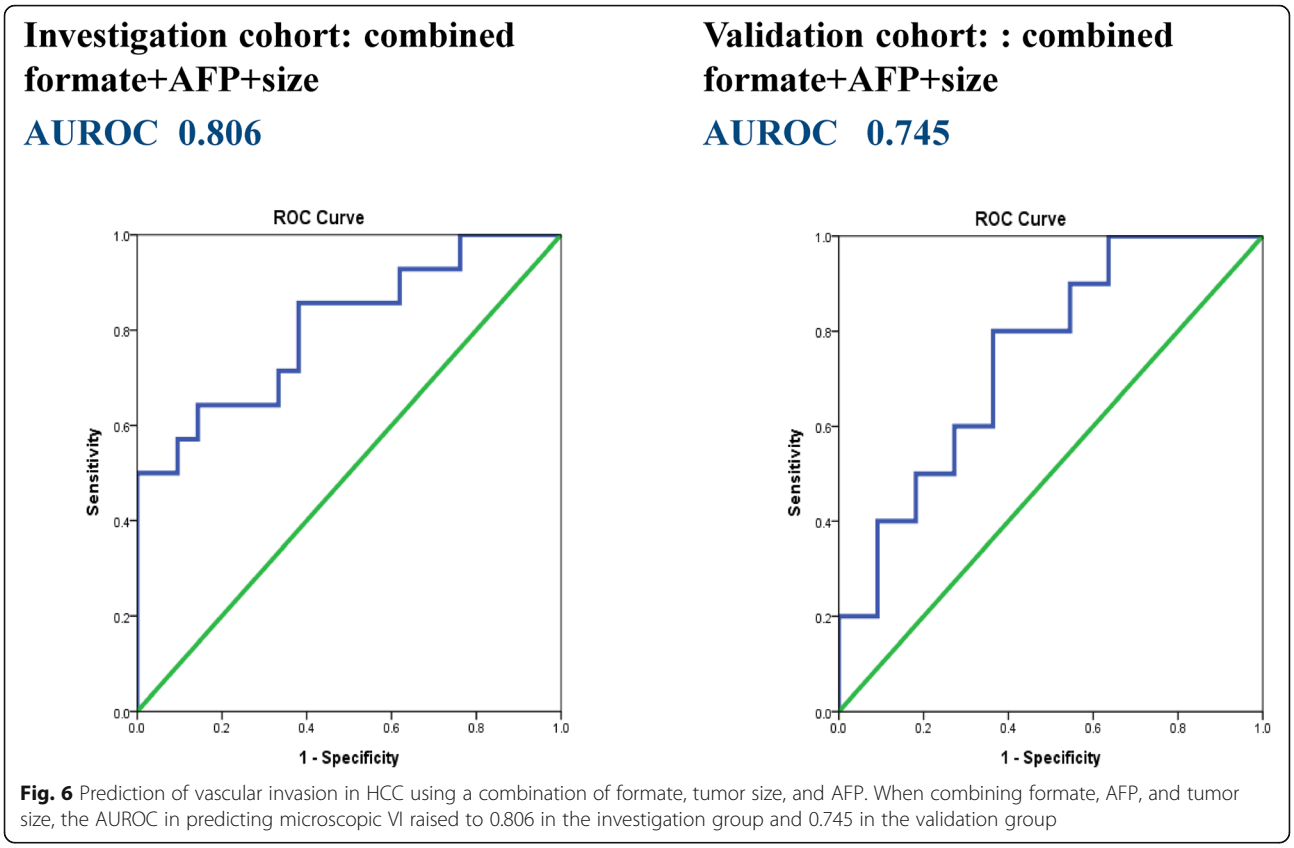

Fig. 6 Prediction of vascular invasion in HCC using a combination of formate, tumor size, and AFP. When combining formate, AFP, and tumor size, the AUROC in predicting microscopic VI raised to 0.806 in the investigation group and 0.745 in the validation group

Conclusion

In conclusion, metabolomic profiling can identify novel biomarkers that assist in the early diagnosis of AFP-negative HCC patients and recognition of microvascular invasion in order to facilitate preoperative surgical planning and postoperative follow-up. Further, larger scale prospective cohort studies are warranted to consolidate our findings.

Abbreviations

ACQ: Acquisition period; A-HCC: HCC with elevated or abnormal AFP levels (greater or equal to 15 ng/mL); AFP: Alpha-fetoprotein; ALT: Alanine aminotransferase; AJCC: American Joint Committee on Cancer; ASPM: Abnormal spindle-like microcephaly associated; AST: Aspartate aminotransferase; AUC: Area under the curve; AUROC: Area under the curve of the receiver-operating characteristics; BMI: Body mass index; CA 19-9: Cancer antigen 19-9; CEA: Carcinoembryonic antigen; CGMH: Chang Gung Memorial Hospital; CGU: Chang Gung University; CI: Confidence intervals; CT: Computed tomography; DM: Diabetes mellitus; ECOG: Eastern Cooperative Oncology Group; GGT: Gamma-glutamyltransferase; GCDCA: Glycochenodeoxycholic acid; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; ^{1}H-NMR: Hydrogen-1 nuclear magnetic resonance; HR: Hazard ratios; ICG-15: Indocyanine green retention test at 15 min; INR: International normalized ratio; LC-MS: Liquid chromatography mass spectrometry; LNM: Lymph node metastasis; MRI: Magnetic resonance imaging; N-HCC: HCC with normal AFP levels (less than 15 ng/mL); NX-DCP: Next generation des-r-carboxy prothrombin; OPLS-DA: Orthogonal partial least squares-discriminant analysis; OS: Overall survival; RD: Relaxation delay; RFA: Radiofrequency ablation; ROC: Receiver-operating characteristics; TACE: Transarterial chemoembolization; TCA: Tricarboxylic acid; tm: Mixing time; TMS: Tetramethylsilane; TSP: 3-(trimethylsilyl)-propionate, sodium salt; VI: Vascular invasion; VIP: Variable importance projection

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Authors' contributions

Conceptualization, Chao-Wei Lee, Gi-gin Lin, and Ming-Chin Yu; Data curation, Chao-Wei Lee, Jo-Chu Chiu, and Meng-Han Chiang; Formal analysis, Chao-Wei Lee, Jo-Chu Chiu, and Meng-Han Chiang; Funding acquisition, Chao-Wei Lee and Ming-Chin Yu; Investigation, Chang-Mu Sung, Yi-Chung Hsieh, and Cheng-Yu Lin; Methodology, Hsin-I Tsai, Chang-Mu Sung, and Tony Kuo; Software, Yi-Chung Hsieh; Supervision, Gi-gin Lin and Ming-Chin Yu; Validation, Hsin-I Tsai, Tony Kuo, and Cheng-Yu Lin; Writing—original draft, Hsin-I Tsai and Chao-Wei Lee; Writing—review and editing, Chang-Mu Sung, Gi-gin Lin, and Ming-Chin Yu.

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Availability of data and materials
All data generated or analyzed during the study are included in this published article. Raw data may be requested from the authors with the permission of the institution.

Ethics approval and consent to participate
This study was approved by the Institutional Review Boards (CGMH IRB No 201600940B0C501 and 201602025B0) of Chang Gung Memorial Hospital (CGMH). Written informed consents were obtained from all subjects who entered the study.

Consent for publication
Not applicable

Competing interests
Hsin-I Tsai, Chao-Wei Lee, Jo-Chu Chiu, Meng-Han Chiang, Chang-Mu Sung, Yi-Chung Hsieh, Tony Kuo, Cheng-Yu Lin, Gi-gin Lin, and Ming-Chin Yu have no conflicts of interest or financial ties to disclose.

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