Research Article

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# Lysophosphatidylcholine acyltransferase 1 altered phospholipid composition and regulated hepatoma progression

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**Background & Aims**: Several lipid synthesis pathways play important roles in the development and progression of hepatocellular carcinoma (HCC), although the precise molecular mechanisms remain to be elucidated. Here, we show the relationship between HCC progression and alteration of phospholipid composition regulated by lysophosphatidylcholine acyltransferase (LPCAT).

**Methods**: Molecular lipidomic screening was performed by imaging mass spectrometry (IMS) in 37 resected HCC specimens. RT-PCR and Western blotting were carried out to examine the mRNA and protein levels of LPCATs, which catalyze the conversion of lysophosphatidylcholine (LPC) into phosphatidylcholine (PC) and have substrate specificity for some kinds of fatty acids. We examined the effect of LPCAT1 overexpression or knockdown on cell proliferation, migration, and invasion in HCC cell lines.

**Results**: IMS revealed the increase of PC species with palmitoleic acid or oleic acid at the *sn-2*-position and the reduction of LPC with palmitic acid at the *sn-1*-position in HCC tissues. mRNA and protein of LPCAT1, responsible for LPC to PC conversion, were more abundant in HCCs than in the surrounding parenchyma. In cell line experiments, LPCAT1 overexpression enriched PCs observed in IMS and promoted cell proliferation, migration, and invasion. LPCAT1 knockdown did *viceversa*.

**Conclusions**: Enrichment or depletion of some specific PCs, was found in HCC by IMS. Alteration of phospholipid composition in HCC would affect tumor character. LPCAT1 modulates phospholipid composition to create favorable conditions to HCC cells. LPCAT1 is a potent target molecule to inhibit HCC progression.


Keywords: Cancer lipidomics; Imaging mass spectrometry; LPCAT1; Hepatocellular carcinoma.

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*Abbreviations:* HCC, hepatocellular carcinoma; NAFLD, non-alcoholic fatty liver disease; IMS, imaging mass spectrometry; MALDI, matrix-assisted laser desorption/ionization; LPCAT, lysophosphatidylcholine acyltransferase; PCA, principal component analysis; cPLA$_2\alpha$, cytosolic phospholipase A$_2\alpha$; iPLA$_2\beta$, $Ca^{2+}$-independent phospholipase A$_2\beta$; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine; PAF, platelet activating factor.

## Introduction

Hepatocellular carcinoma (HCC) is the seventh most frequently diagnosed cancer and the fourth most frequent cause of cancer-related death in the world [1]. The frequency of HCC has increased in the United States and in European and Asian countries [2]. In addition to HBV, HCV, alcohol, and aflatoxin, non-alcoholic fatty liver disease (NAFLD) has drawn increasing attention as a risk factor of HCC. NAFLD is the most common form of chronic liver disease in developed countries. Alteration of the lipogenic pathway plays an important role in the pathogenesis of NAFLD and also in HCC carcinogenesis and its progression [3].

In recent years, imaging mass spectrometry (IMS) using matrix-assisted laser desorption/ionization (MALDI) has emerged and developed dramatically in the field of proteomics and metabolomics [4,5]. MALDI-IMS can clarify the distribution of lipid molecules, directly from heterogeneous tissue samples, by determining the differences in the mass-to-charge ratios (*m/z*). Furthermore, tandem mass spectrometry (MS/MS analysis), which generates the fragment ion spectrum by spraying additional collision gas, enables the identification of the molecules in tissues by providing detailed information on their structures. IMS has been used to classify tumor grade or to investigate new biomarkers in cancer proteomics [6,7]. At present, there are only limited reports on molecular proteomics of HCC using IMS [8,9].

In this study, we performed IMS using HCC tissue samples as molecular lipidomic screening and revealed the alteration of phospholipid composition caused by overexpressed LPCAT1. We further investigated the role of LPCAT1 in HCC progression and

elucidated that LPCAT1 increased cell proliferation and invasion, using hepatoma cell lines.

## Materials and methods

*Clinical specimens*

Human HCC tissues, including a boundary region, were excised from resected specimens provided by our institute. A total of 37 samples were obtained from surgical specimens. The clinicopathological characteristics are listed in Table 1. Samples were snap-frozen in liquid nitrogen and stored at −80 °C. The study protocol was approved by the ethical committee of our institute, and all the patients gave informed consent for the procedures.

*Imaging mass spectrometry using clinical specimens*

The frozen tissues were sliced into 10-μm-thick serial sections; furthermore, for H&E staining, they were sliced into 5-μm-thick sections, using a cryostat (CM1950, Leica Microsystems, Germany). The samples for IMS were put onto indium-tin-oxide-coated glass slides (Bruker Daltonics, Germany) and dried at room temperature. The matrix solution was prepared by dissolving 50 mg/ml 2,5-dihydroxybenzoic acid (Bruker Daltonics) in 70% methanol. A thin matrix layer was applied to the surface of the plates as described previously [10]. Mass spectra were acquired using Ultraflex II (Bruker Daltonics). In this analysis, we collected signals between *m/z* 400 and 1000 in positive ion mode. The mechanical resolution was 100 μm × 100 μm. The number of laser irradiations was 200 shots in each spot. Image reconstruction was performed using FlexImaging 2.1 software (Bruker Daltonics). The 100 most intense peaks between HCC and normal parenchyma in a single analysis were compared by principal component analysis (PCA) utilizing ClinProTool 2.2 software (Bruker Daltonics). An alignment of mass spectra from different samples was performed using SpecAlign software (http://physchem.ox.ac.uk/~jwong/specalign/). The peak intensity value of the spectra was normalized by dividing by the total ion current, as previously described [11].

**Table 1. Patients clinicopathological features.**

| | |
|---|---|
| Sex | |
| Male | 30 |
| Female | 7 |
| Age | 68 (45-80) |
| Etiology | |
| HBV | 7 |
| HCV | 25 |
| NBNC* | 5 |
| Tumor size (maximum diameter), cm | 3.0 (1.3-10) |
| Tumor number | 2 (1-7) |
| Vascular invasion | |
| Positive | 11 |
| Negative | 26 |
| Stage (UICC) | |
| I | 15 |
| II | 19 |
| IIIA | 3 |
| Differentiation | |
| Well | 11 |
| Moderate | 24 |
| Poor | 1 |
| α-fetoprotein, ng/ml | 337 (2-8989) |
| PIVKA-II, mAU/ml | 419 (15-8830) |

NBNC, non-B, non-C hepatitis.
*NBNC contains no alcohol addict.

*Identification of biomolecules*

Tandem mass spectrometry was performed using QSTAR Elite (Applied Biosystems Inc., Foster City, CA), a hybrid quadrupole/time-of-flight mass spectrometer equipped with an orthogonal MALDI source and a pulsed Nd:YAG. The MS/MS analysis was performed directly on the tissue sections. The data acquisition conditions (i.e., the laser power, collision energy, and the number of laser irradiations) were adjusted to obtain good-quality mass spectra with high intensity and signal-to-noise ratios (S/N) in the fragmented peaks. The MS/MS data were assigned using Nature Lipidomics Gateway (http://www.lipidmaps.org/).

*Semi-quantitative RT-PCR*

Total RNA was extracted using RNeasy mini kit (QIAGEN, Valencia, CA) for tissue samples and Sepasol I (Nakalai Tesque, Japan) for cells, respectively. The extracted RNAs from tissue samples and cells were reverse-transcribed with SuperScript III (Invitrogen, Carlsbad, CA) and ReverTra Ace (TOYOBO LIFE SCIENCE, Japan), respectively. Each single-stranded cDNA of LPCATs and *GAPDH* was amplified on a GeneAmp PCR System 9700 (Applied Biosystems Inc.). PCR primer sequences and reaction conditions are listed in Supplementary Table 1. The PCR bands were visualized using gel documentation system Printgraph AE6932CP-4 (ATTO, Tokyo, Japan). The signal intensity of each band was digitized as a cumulative density of band area. This digitizing of band signal was easily done with a macro command available in Scion image software version 4.0.3.2 (Scion Corporation, Frederick, MD). Signal intensities of LPCATs of each sample were normalized to those of *GAPDH* of the same sample. We confirmed that *GAPDH* was able to be used as a reference as the difference of *GAPDH* signal between cancer and non-cancer was less than 1.2 and not statistically significant. The data were presented as the ratio of LPCATs and *GAPDH*. We loaded all the samples into one gel for quantitative comparison.

*Western blotting*

A rabbit polyclonal anti-LPCAT1 antibody (Protein Tech Group, Inc., Chicago, IL), rabbit polyclonal anti-LPCAT4 antibody (Protein Tech Group, Inc.), rabbit monoclonal anti-cytosolic phospholipase $A_2\alpha$ (cPLA$_2\alpha$) antibody (Cell Signaling Technology, Inc., Beverly, MA), rabbit polyclonal anti-$Ca^{2+}$-independent phospholipase $A_2$ β (iPLA$_2\beta$) antibody (Abcam, UK), mouse monoclonal anti-GAPDH antibody (Millipore Corporate, Billerica, MA), and mouse monoclonal anti-FLAG M2 antibody (Agilent Technology, Santa Clara, CA) were used. The immunoreactive bands were visualized using ECL plus Western Blotting Detection Reagents (GE Healthcare, UK) and an Imaging Reader LAS-3000 mini (FUJIFILM, Japan). We checked the band of LPCAT1 and 4 detected with knockdown samples. We observed that in knockdown samples, the specific band was absent. The evaluation was carried out by quantifying the signal intensity of visible bands with Scion image software.

*Cell lines*

HCC cell lines HuH7 and HepG2 cells were purchased from Human Science Research Resources Bank (Japan). They were cultured under Dulbecco's modified Eagle's medium (Invitrogen) with 10% fetal bovine serum, 100 U penicillin, and 0.1 mg/ml streptomycin at 37 °C in 5% $CO_2$.

*Small interfering RNA inhibition assay*

To knock down endogenous LPCAT1 expression in HuH7 and HepG2 cells, we used Stealth RNAi (Invitrogen). We used Stealth RNAi Negative Control Medium GC Duplex #2 (Invitrogen) as a negative control. HuH7 and HepG2 cells were seeded and transfected with Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. The HuH7 and HepG2 cells were harvested 4 days and 3 days after transfection, respectively.

*Expression plasmids*

The full-length cDNA encoding human *LPCAT1* was purchased from DNAFORM (Japan). The full-length cDNA was amplified using the following primers: sense, 5′-gggGGATCCatgaggctgcggggatgcg and antisense, 3′-gggGAATTCctaatccagcttcttgcgaaca. The amplified fragments were cloned into pCMV-Tag2 vector (Agilent Technologies, Inc.) at *Bam*HI-*Eco*RI sites. All DNA sequences were verified