RESEARCH ARTICLE

Plasma Lipidomics as a Tool for Diagnosis of Extrahepatic Cholangiocarcinoma in Biliary Strictures: a Pilot Study

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Abstract

Biliary obstruction is a common clinical manifestation of various conditions, including extrahepatic cholangiocarcinoma. However, a screening test for diagnosis of extrahepatic cholangiocarcinoma in patients with biliary obstruction is not yet available. According to the rationale that the biliary system plays a major role in lipid metabolism, biliary obstruction may interfere with lipid profiles in the body. Therefore, plasma lipidomics may help indicate the presence or status of disease in biliary obstruction suspected extrahepatic cholangiocarcinoma. This study aimed to use plasma lipidomics for diagnosis of extrahepatic cholangiocarcinoma in patients with biliary obstruction. Plasma from healthy volunteers, patients with benign biliary obstruction extrahepatic cholangiocarcinoma, and other related cancers were used in this study. Plasma lipids were extracted and lipidomic analysis was performed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Lipid profiles from extrahepatic cholangiocarcinoma patients showed significant differences from both normal and benign biliary obstruction conditions, with no distinction between the latter two. Relative intensity of the selected lipid mass was able to successfully differentiate all extrahepatic cholangiocarcinoma samples from patient samples taken from healthy volunteers, patients with benign biliary obstruction, and patients with other related cancers. In conclusion, lipidomics is a non-invasive method with high sensitivity and specificity for identification of extrahepatic cholangiocarcinoma in patients with biliary obstruction.

Keywords: Extrahepatic cholangiocarcinoma - biliary obstruction - lipidomics - MALDI-TOF-MS - diagnosis

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Introduction

Biliary obstruction is a clinical manifestation that is commonly found in many abnormalities of the hepatobiliary system. Causes of biliary obstruction may be either benign or malignant conditions, which should be distinguished according to diagnostic protocols (Larghi et al., 2008). Malignancy condition of the bile duct epithelium is an important cause of biliary obstruction because of the prognosis, with the most common type being extrahepatic cholangiocarcinoma (ECC) (Soares et al., 2014). ECC is a type of cholangiocarcinoma (CCA) that presents separately from hepatic parenchyma, while intrahepatic cholangiocarcinoma (ICC), another type of CCA, occurs inside the liver (Banales et al., 2016). Tumor growth characteristics may present as mass forming, periductal-infiltrating, and intraductal tumor (Jhaveri and Hosseini-Nik, 2015). Clinical imaging, such as ultrasound, magnetic resonance imaging (MRI), computer tomography (CT) scan, and positron emission tomography scan are useful for non-invasive investigation of mass forming CCA. However, their diagnostic value is limited in early CCA, periductal-infiltrating tumor, and intraductal tumor; as such, invasive methods such as endoscopy may be required (Yao et al., 2014; Jhaveri and Hosseini-Nik, 2015). Endoscopy provides tissue sampling capability, which facilitates studies like brushed cytology, has high specificity (~100%), but lower sensitivity (averages of 35.1% and 43% from 2 meta-analysis studies) (Suzuki et al. 2014; Trikudanathan et al., 2014). Fine needle aspiration (FNA) with ultrasound guide is another procedure with the same level of specificity to brushed cytology test but higher sensitivity (~77%) (DeWitt et al., 2006). FNA, however, has inherent limitations in tumors that are not identifiable by clinical imaging. Given that endoscopy is invasive and there are no known blood tests for the indication or diagnosis of ECC in biliary obstruction in clinical use today, the development of a blood study technique for ECC is compelling.

One group of interesting molecules that may be used

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for diagnosis of ECC by blood examination is lipids. While lipid metabolism can occur in every cell in the body, the major route of lipid excretion is the biliary system (Cohen, 1999). Biliary epithelial cells do not only act as a passage for biliary excretion; they also have a synthetic and lipid excretory function (Kullak-Ublick et al., 2000). Therefore, an aberration in plasma lipids may be indicative of an abnormality of the biliary passage, such as in ECC. Moreover, different pathogeneses of the obstruction may exhibit different lipid profile patterns in plasma. Hence, lipid profile may discriminate ECC from other causes of biliary obstruction.

Lipid profiles can be determined using lipidomic methods. Lipidomics is a study in metabolomics that is used for the identification and determination of lipid components in samples. This method investigated individual lipids or lipid profiles using high-throughput methods, such as high-performance thin layer chromatography, ultra-performance liquid chromatography, gas-chromatography, liquid chromatography-electron spray ionization mass spectrometry (LC-ESI-MS), and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and compared them to either standard lipids or suitable database that matched the method and/or the instrument (Rolim et al., 2014). Lipidomics has been used to study a variety of diseases, such as metabolic diseases (Jové et al., 2014; Quehenberger and Dennis, 2011), infectious diseases (Aljohani et al., 2014; Portevin et al., 2014), cardiovascular disorders (Stegemann et al., 2014), neurological disorders (Del Boccio et al., 2011; Fonteh et al., 2006), cystic fibrosis (Ollero et al., 2011), and cancers (Zhang and Wakelam, 2014), in specific reference to pathogenesis, diagnosis, prognosis, and drug response. Published reports of lipidomic studies in cancer research have included the following types of cancer: lymphoma (Eberlin et al., 2014), thyroid cancer (Ishikawa et al., 2012), breast cancer (Hilyo et al., 2014), ovarian cancer (Arafah et al., 2014), prostate cancer (Zhao et al., 2012), colorectal cancer (Gassler et al., 2010), hepatocellular carcinoma (HCC) (Chen et al., 2013), ICC (Park et al., 2011), and a pilot study for ECC which studied in bile lipid components (Navaneethan et al., 2014).

This study is a pilot study to determine the role of plasma lipidomics in diagnosis of ECC in biliary obstruction condition. In this study, lipid profiles from ECC patient plasma by MALDI-TOF-MS method were obtained and compared with those of healthy volunteers and patients with common bile duct (CBD) stones as a representative for benign biliary obstruction conditions. In addition, plasma samples from related cancer patients, such as ICC and HCC, were also used for comparison with ECC samples. The selected lipid masses (mass-to-charge ratio or m/z) that showed a statistically significant difference between normal samples and the ECC group were used to calculate the value for diagnosis of ECC. Up-regulated selected m/z were also subjected to an online database search for matching lipids (LIPID Metabolites and Pathways Strategy, LIPID MAPS; www.lipidmaps.org) (Fahy et al., 2007). The findings may establish the efficacy of using lipid profiles for determination of ECC in biliary obstruction patients and use in clinical settings.

Materials and Methods

Patients

Blood samples from ECC and CBD stone patients who visited Siriraj Hospital were taken at the Siriraj GI Scope Center, Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University. All ECC patients were diagnosed based on evaluation of clinical presentation including either clinical imaging or tissue diagnosis or both. Plasma from EDTA blood from both patients and healthy volunteers was separated by low-speed centrifugation. Plasma from ICC and HCC patients was obtained from patients who visited Department of Surgery, Faculty of Medicine Siriraj Hospital, Mahidol University. All plasma was kept at -80°C until use. All patients were explained the consent and signed in the inform consent with agreement. This study was approved by the Siriraj Institutional Review Board (approval number Si521/2010).

Lipid extraction

Plasma lipid extraction was performed by modified Bligh and Dyer method (Bligh and Dyer, 1959). In brief, organic solvent was prepared using chloroform and methanol at a ratio of 2:1. Plasma and organic solvent were then mixed at a ratio of 1:30, respectively, and then shaken vigorously at room temperature for 10 minutes. Mixtures were then centrifuged at 5000 rpm at 4°C for 10 minutes and the lower organic phase was collected.

Sulfo-phospho-vanillin reaction was used to determine lipid concentration. The procedure for sulfo-phospho-vanillin reaction was performed as previously described (Knight et al., 1972). Briefly, 60 g/L (w/v) of vanillin (Merck, Readington Township, NJ, USA) in ethanol solution was prepared and diluted to a one-tenth concentration with distilled water. The solution was then mixed with 15 M phosphoric acid at a ratio of 1:4, respectively. The resulting phospho-vanillin solution was then stored in a dark bottle at room temperature until use. Soy bean oil was used for standard curve preparation. Lipid solution was mixed with 96% (v/v) sulfuric acid at a ratio of 1:10, respectively, boiled for 10 minutes, and then quickly chilled on ice for 5 minutes. Twenty-two μL of lipid-sulfuric acid solution was then mixed with 1 mL of phospho-vanillin solution and then incubated at 37°C for 15 minutes. Lipid concentrations were measured by absorbance at 540 nm using spectrophotometer. Finally, lipid solution concentrations were adjusted to 1 μg/μL using chloroform/methanol solution.

MALDI-TOF lipidomics

Matrix was prepared by mixing 2,5 dihydroxybenzoic acid (Sigma-Aldrich, St. Louis, MO, USA) saturated in acetone solution with acetonitrile at a ratio of 1:9, respectively. The mixture was then dissolved in 0.1% w/v trifluroacetic acid solution at a ratio of 1:4, respectively. Matrix with a volume of 1 μL was spotted on MTP AnchorChip 600/384 (Bruker Daltonics, Billerica, MA, USA) and air-dried. Then, an equal volume of samples was spotted over layer on each dried matrix and air-dried. Analysis was performed by linear mode of Ultraflex III®

MALDI-TOF/TOF (Bruker Daltonics) machine with laser power setting at 33-38 and range of mass setting at 500-3000 m/z. Each sample test was performed in octuplicate. Raw data were generated by Bruker Daltonics flexAnalysis® (Bruker Daltonics) software for baseline subtraction and spectral calibration.

Data analysis and statistical analysis

Wilcoxon signed-ranks test was used for comparing lipid profiles among groups and Mann-Whitney rank sum test was used for comparing serum chemistry data between benign and ECC groups. This method was also used to compare among each m/z. Fisher Exact test was used to determine diagnostic value of serum tumor marker between benign and ECC groups. These tests were performed using PASW Statistics 18® software (SPSS Inc, Chicago, IL, USA). A P-value of less than 0.05 was considered to be statistically significant. Discrimination of each sample by Principal component analysis (PCA) was performed by TANAGRA software version 1.4.50 (University of Lyon, Lyon, France) and the diagram was generated by PASW Statistics 18® software. Hierarchical clustering was generated by Cluster 3.0 software and visualized by Java TreeView software version 1.1.6r4 (de Hoon et al., 2004; Saldanha, 2004).

Results

Demographic data

Blood plasma from 6 healthy volunteers was collected and used for normal samples. Lipid profiles of benign biliary obstruction condition were determined from the plasma of 6 CBD stone patients. ECC plasma was collected from 14 ECC patients. For other related cancers, plasma from 1 HCC and 2 ICC patients were collected. Demographic data, sample data, and pre-operation blood chemistry data were shown in Table 1. Almost all ECC patients presented with mass at bile duct, as determined by either CT scan or MRI, with mass diameter up to 6 cm. Tumor invasion and metastasis were also observed in almost all ECC, ICC, and HCC patients. Blood chemistry comparison between benign and ECC groups showed significant increase in alkaline phosphatase (ALP) (P-value = 0.012) and direct bilirubin (DB) (P-value < 0.001) in ECC groups (Figure 1). Fisher Exact could not determine ECC from benign patients by both CEA (P-value = 0.429) and CA19-9 (P-value = 0.273).

Overall lipid profiles and comparison

All m/z represented the average mass of the similar m/z from each sample. After discarding m/z peaks presented

Table 1. Demographic Data and Pre-Operative Blood Chemistry Data of All Patients

Group	Code	Age	Gender	Tumor size[1] (cm)	Metastasis[2]	AST 0-37 U/L	ALT 0-40 U/L	ALB 3.5-5.5 g/dL	ALP 39-117 U/L	DB 0-0.50 mg/dL	CEA 0-3.4 ng/mL	CA19-9 0-39 U/mL
Normal	N1	33	M	-	-	-	-	-	-	-	-	-
	N2	30	M	-	-	-	-	-	-	-	-	-
	N3	29	M	-	-	-	-	-	-	-	-	-
	N4	31	F	-	-	-	-	-	-	-	-	-
	N5	27	F	-	-	-	-	-	-	-	-	-
	N6	27	F	-	-	-	-	-	-	-	-	-
Benign	B1	77	M	-	-	17	12	3.7	89	0.24	ND	ND
	B2	58	M	-	-	283	646	4.6	160	3.16	ND	ND
	B3	64	F	-	-	65	79	4.2	372	0.88	2.48	2.48
	B4	89	F	-	-	25	13	3.8	129	0.34	ND	40.76
	B5	68	F	-	-	150	49	2.4	819	5.97	2.67	>1000
	B6	82	F	-	-	8	27	4.1	94	0.01	ND	ND
ECC	E1	63	M	-	+	87	78	2.3	306	21.1	ND	ND
	E2	76	F	4.3	+	110	143	2.7	1,080	18.1	ND	ND
	E3	69	F	2.2	+	101	86	4	381	2.48	3.37	59.71
	E4	46	F	-	+	122	72	3.7	1,372	6.96	ND	>1000
	E5	64	M	1	+	143	102	3.1	987	12	ND	79
	E6	63	M	5.6	+	48	47	3.8	254	18.2	47	>1000
	E7	40	M	3.3	+	126	108	3.5	949	22	ND	ND
	E8	89	M	-	+	109	120	3	787	6.38	ND	ND
	E9	57	M	3.1	+	214	200	4.8	969	7.3	ND	ND
	E10	35	M	-	+	75	100	3.4	218	8.5	ND	>1000
	E11	61	M	3.8	+	140	147	3.8	672	3.51	8.23	39.02
	E12	74	F	-	+	97	39	2.3	651	11.8	65.6	>1000
	E13	55	F	6	+	185	110	4.4	912	9	ND	ND
	E14	53	M	5.1	-	100	47	2.9	287	15.1	2.81	103
HCC	H1	68	M	9.5	+	43	22	3.2	236	0.1	2.47	1.57
ICC	I1	69	M	3.3	+	43	27	3.2	168	0	2.47	<0.60
	I2	68	F	10.5	+	45	32	3.2	507	0.38	9.97	>1000

[1]Tumor size determined from the widest dimension of tumor by either imaging or surgery; [2]Metastasis determined from imaging, surgery, or clinical presentation. The normal values of all blood chemistry used at Siriraj Hospital are indicated. AST = aspartate transaminase, ALT = alanine transaminase, ALB = albumin, ALP = alkaline phosphatase, DB = direct bilirubin, M = male, F = Female, ND = not determined

in bare matrix spots which should be background, a total of 125 peaks were identified. Average intensity values from experiments performed in octuplicate were used for analysis. Each m/z intensity was normalized to percentage of total intensity for all m/z in the sample. Hierarchical clustering of all samples showed that each ECC sample was located within a group (Figure 2A). Three-axis PCA for all samples was performed and revealed separation among the normal group, the benign group, and the ECC group (Figure 2B).

Normalized intensities for each m/z from all samples are shown as lipidomic spectra, with average values from the m/z of each group having been calculated and compared using Wilcoxon signed-ranks test (Figure 2C). The results described a statistically significant difference between the ECC group and both the normal group and the benign group, with P-values of 0.008 and 0.006, respectively. In addition, there was a statistically significant difference between the ICC group and both the normal and the benign group, with P-values of 0.01 and 0.037, respectively. There was, however, no statistically significant difference between the normal group and the benign group. Moreover, there was no statistically significant difference among the 3 malignant groups in this study.

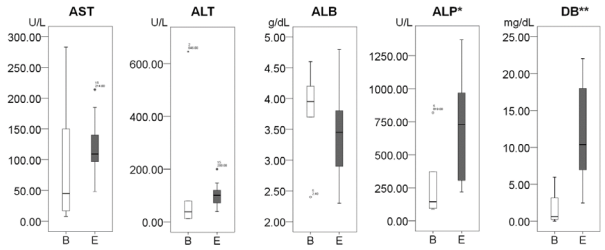

Figure 1. Boxplot for Comparison of Serum Chemistry Data Between Benign and ECC Groups. Mann-Whitney rank sum test was used for this comparison. The symbol * indicated statistical significance with P-value < 0.05 and ** indicated P-value < 0.001. Abbreviations: AST = aspartate transaminase, ALT = alanine transaminase, ALB = albumin, ALP = alkaline phosphatase, DB = direct bilirubin, B = benign group and E = ECC group

Diagnostic efficacy of lipidomics determination

Each m/z from ECC samples was compared to that of normal samples by Mann-Whitney rank sum test. There were 9 m/z from ECC samples that showed statistically significant difference (2 up-regulation: m/z 630.6 and 634.5 Da; and, 7 down-regulation: 572.0, 599.8, 601.9, 611.0, 654.6, 667.5, and 671.6), as compared to those

Table 2. Statistically Significant m/z Differences between ECC and Normal Samples

m/z	Adducted	Groups	Lipid species
630.6	M+H	GL	LMGL02010083, LMGL02010084, LMGL02010085, LMGL02010318, LMGL02010447, LMGL02010470, LMGL02010507, LMGL02010530
		GP	LMGP10010076, LMGP10010096, LMGP10010115, LMGP10010166, LMGP10010204, LMGP10010249, LMGP10010278, LMGP10010339, LMGP10030008, LMGP10030031
		PR	LMPR01070007
		SP	LMSP03020003, LMSP03020030, LMSP03020047
		ST	LMST05050015
	M+Na	FA	LMFA01020326, LMFA07010008
		GL	LMGL02010007, LMGL02010029, LMGL02010030, LMGL02010041, LMGL02010329, LMGL02010372, LMGL02010418, LMGL02010437, LMGL02010460, LMGL02010522
		GP	LMGP01050057, LMGP02010058, LMGP02010243, LMGP02010313, LMGP02011251
		SP	LMSP00000016, LMSP02010017, LMSP02010045, LMSP02010080, LMSP02010091
		ST	LMST01020022, LMST01080077
634.5	M+H	FA	LMFA11000249
		GL	LMGL02010074, LMGL02010075, LMGL02010076, LMGL02010088, LMGL02010446, LMGL02010469, LMGL02010483, LMGL02010513, LMGL02010529
		GP	LMGP02010362, LMGP02010384, LMGP02010407, LMGP02010424, LMGP02010475, LMGP02010514, LMGP02030008, LMGP10010055, LMGP10010074, LMGP10010094, LMGP10010225, LMGP10010306, LMGP10010872, LMGP10010912, LMGP10010919
		SP	LMSP00000008, LMSP00000013, LMSP02010030
		ST	LMST01020061, LMST01031011, LMST05050004
	M+Na	GL	LMGL02010025, LMGL02010036, LMGL02010079, LMGL02010086, LMGL02010328, LMGL02010371, LMGL02010394, LMGL02010401, LMGL02010424, LMGL02010436, LMGL02010482, LMGL02010505
		GP	LMGP06050018, LMGP06070001, LMGP10010054, LMGP10010423
		PR	LMPR01070008, LMPR01070066
		SP	LMSP02020031, LMSP02020036, LMSP02030007, LMSP02030010
		ST	LMST01010329, LMST01020027, LMST05010042

FA = fatty acyls, GL = glycerolipids, GP = glycerophospholipids, PR = prenol lipids, SP = sphingolipids, ST = sterol lipids

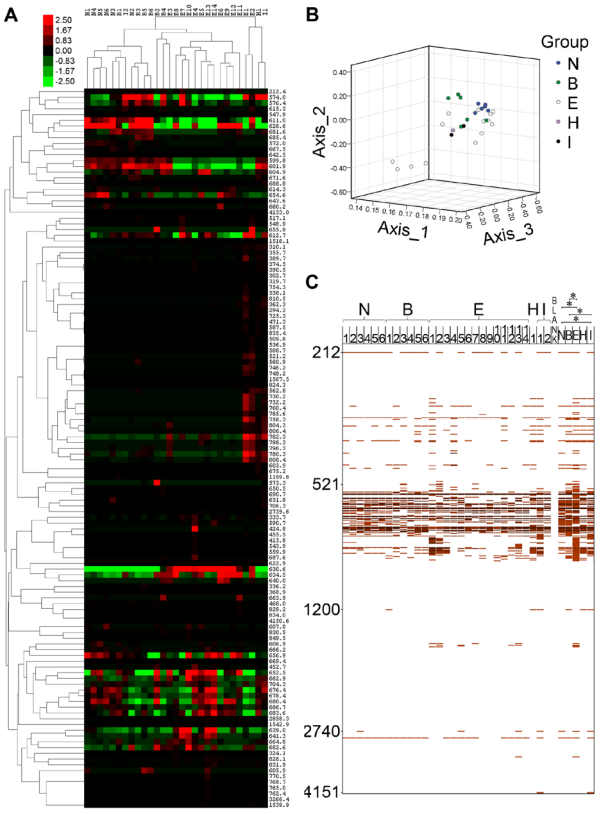

Figure 2. Overall Lipid Profiles and Comparisons. (A) Hierarchical clustering; (B) Three-axis PCA; (C) Normalized intensities of all m/z from each sample as lipidomic spectra. Last 5 lanes were generated from average values from m/z of each group and were calculated and compared using Wilcoxon signed-ranks test. The symbol * indicated statistical significance with P-value < 0.05. Abbreviations: N = normal, B = benign biliary obstruction, E = ECC, H = HCC, and I = ICC.

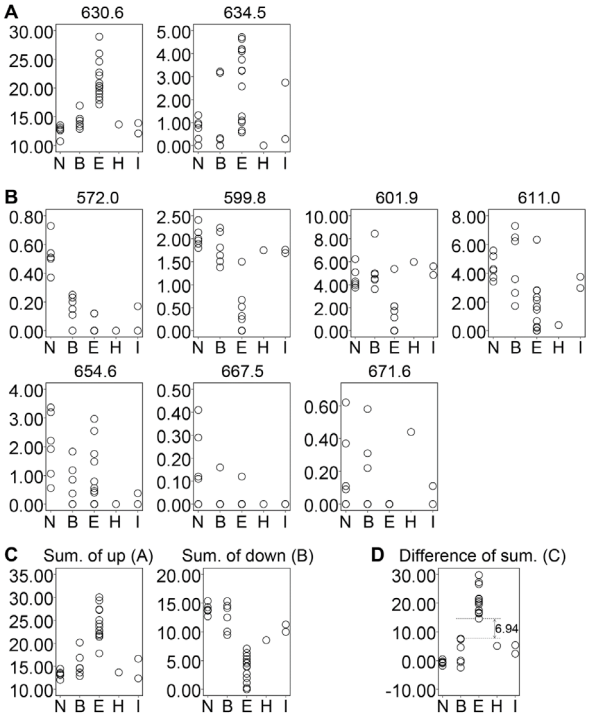

Figure 3. Scatter Plots of Relative Intensity from Statistically Significant m/z. Y-axis of all diagrams showed relative intensity. (A) Scatter plots of relative intensity of up-regulated m/z: 630.6 and 634.5; (B) Scatter plots of relative intensity of down-regulated m/z: 572.0, 599.8, 601.9, 611.0, 654.6, 667.5, and 671.6; (C) Summation of relative intensities of up-regulated m/z (left) and down-regulated m/z (right); (D) Differences between relative intensity of up-regulated and down-regulated m/z. Abbreviations: N = normal, B = benign biliary obstruction, E = ECC, H = HCC, and I = ICC

of normal samples. Scatter plots of relative intensities from each significant m/z are shown in Figure 3A-B. Summation of relative intensities of up-regulation and down-regulation m/z of each sample was performed and plotted (Figure 3C). Differences between both values were also calculated and plotted (Figure 3D). Gap distance between the lowest in ECC group and highest in other groups (benign biliary obstruction) is shown as 6.94 (Figure 3D).

Lipid identification from database.

Up-regulated selected m/z were identified in a search of the lipid database (LIPID MAPS) in the form of single proton adduct (M+H) or single sodium adduct (M+Na) and shown in Table 2.

Discussion

ECC has one of the worst prognoses for cancers that normally present in biliary obstruction (Soares et al., 2014). Reliable screening and diagnosis are required for appropriate management of benign biliary obstruction or ECC. Lipidomics is projected as a new method for evaluation and diagnosis of various cancers including ECC (Navaneethan et al., 2014). MALDI-MS is a powerful tool, as it has been reported the ability for quantitative experiments of both low molecular weight molecules and macromolecules (Duncan et al., 2008; Wang et al., 2011). This study was the determination of lipid profiles by relative expression and MALDI-TOF was chosen to determine the efficacy of lipidomics as a tool to effectively discriminate ECC from normal samples, benign biliary obstruction (CBD stone), and other related malignant conditions (HCC and ICC). Lipid profiles from ECC plasma analyzed by clustering method, PCA method, and Wilcoxon signed-ranks test indicated that plasma lipid profile by MALDI-TOF can be used to discriminate ECC from normal and benign biliary obstruction samples. This finding corresponds to the previous report in ICC and ovarian cancer, both of which used MALDI/MS for analysis (Gassler et al., 2010; Park et al., 2011). This result supports the potential value of plasma lipidomics for ECC diagnosis.

Selected m/z by Mann-Whitney rank sum test analysis of normalized intensities from each m/z of the ECC group, compared to those of the normal group, clearly identified ECC patients among all samples in this study. From individual m/z, only m/z 630.6 Da showed the complete separation of all ECC samples from other samples. While the summation of relative intensity of the 2 up-regulated m/z did not show the complete separation of ECC from other groups, the summation of relative intensity of all 7 down-regulated m/z could discriminate all ECC samples from the others. In addition, the differences between both

values showed that the gap between the minimum value in the ECC group and the maximum value in the other groups (presented in benign biliary obstruction) was as wide as 6.94, which can be used for diagnosis of ECC by plasma lipidomics study. Moreover, for comparison between ECC and other related malignancies (ICC and HCC), there was no statistical significance among these cancers by overall lipid profile. However, calculation of these 9 m/z facilitated discrimination between ECC and both ICC and HCC in this study. This phenomenon requires further study and larger samples to prove its diagnostic efficacy. The idea of using plasma or serum lipids as biomarkers for diagnosis has been previously demonstrated in prostate cancer and HCC using LC-ESI-MS (Chen et al., 2013; Zhou et al., 2012). Both of these reports showed separation of normal control lipid profiles from cancers by PCA method, which corresponds to this study. One issue of concern is that all ECC patients in this study presented with a tumor mass identifiable by clinical imaging at either the primary site or metastasis or both. To determine the sensitivity and specificity of lipidomics for diagnosis of ECC, further study with samples lacking a clearly presented mass or metastasis will be required. However, based on our knowledge of the literature, this is the first report to introduce plasma lipid profiles for the diagnosis of ECC in biliary obstruction condition. At present, this may be the most effective non-invasive method for ECC diagnosis.

From a previous study, the biliary lipid profile of ECC was compared with benign biliary obstruction conditions (Navaneethan et al., 2014). This publication described the increase of 1-palmitoyl-2-succinoyl-sn-glycero-3-phosphatidylcholine (molecular weight 596.4 Da) and 1-palmitoyl-2-(9-oxononanoyl)-sn-glycero-3-phosphatidylcholine (molecular weight 649.4 Da) in bile from ECC patients and used them for diagnosis of ECC in biliary obstruction condition cases, with a sensitivity of up to 100% and specificity of up to 83.3% when using the combination of both lipids. Up-regulation of plasma phosphatidylcholines was also mentioned in HCC, thyroid, and prostate cancer (Ishikawa et al., 2012; Zhou et al., 2012; Chen et al., 2013). However, there are various species of phosphatidylcholines, with some increasing and some decreasing. Our results from plasma lipids showed 2 up-regulation m/z in ECC samples (630.6 and 634.5 Da), as compared to normal samples. These m/z were also significantly increased in ECC, when compared to the benign biliary obstruction group (data not shown). In comparison with the LIPID MAPS database, both m/z were mostly compatible with glycerolipids, phosphoglycerolipids, and sphingolipids. However, at present, the lipid database is not as comprehensive as the genome and protein databases. As a result, more analysis is required by other methods, such as tandem mass spectrometry for standard lipids to facilitate more accurate identification of up-regulated lipid species in ECC. Known up-regulated lipids might be useful in the future development of blood tumor biomarkers that can be measured by simple directed methods. The most interesting was m/z 630.6, which demonstrated the highest level of efficacy in identifying ECC in this study. The m/z 630.6 also demonstrated the highest intensity, when compared to other significant lipid species, in specific reference to its abundance in plasma.

Blood chemistry from benign and ECC patients in this study showed a statistically significant difference in biliary obstruction markers: ALP and DB. Previous reports have also shown significant increases in blood ALP in ECC patients, compared to benign biliary condition (Navaneethan et al., 2014). ALP has also been reported as a prognostic marker for ICC (Hunsawong et al., 2012; Jiang et al., 2011). Our previous report exhibited the association between ALP and DB with serum estrogen level in male ICC patients and correlated with prognosis (Hunsawong et al., 2012). In addition, ALP has been identified as an independent prognostic score in the Fudan score for ICC patients (Jiang et al., 2011). However, there have been some reports of non-malignant conditions that may potentially affect the level of either ALP or DB in patient sera, such as portal cavernous transformation, which may result in "pseudo-cholangiocarcinoma" sign (Bayraktar et al., 1995). Almost all ECC patients have biliary obstruction; therefore, the use of either ALP or DB may not be suitable for diagnosis, but can be used as a prognostic factor in clinical practice. In addition, in this study, serum tumor marker CEA and CA19-9 could not be used to determine ECC from benign patients. This might be from the small number of patients in this study because there tumor markers would not be evaluated in each patient. However, routinely these tumor markers were used in clinical practice with acceptable sensitivity and specificity (Lumachi et al., 2014).

In conclusion, plasma lipid profiles can be used for diagnosis of ECC in biliary obstruction. This can be applied in clinical practice with a minimally invasive blood draw. Additional studies should be conducted that include larger clinical samples, ECC cases without clinical presentation of tumor mass, other benign conditions, and other types of cancer. In addition, studies focusing on the identification of specific lipid species for use as individual biomarkers for ECC diagnosis should be conducted. This is a pilot study therefore extended study with more sample is in further progress. The early correct diagnosis can provide the appropriate treatment such as radiofrequent abrasion, for example (Wu et al., 2015).

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