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Bile Metabolites and Risk of Carcinogenesis in Patients With Pancreaticobiliary Maljunction: A Pilot Study

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Abstract. *Background/Aim: Pancreaticobiliary maljunction (PBM), a disease with reflux of pancreatic and bile juice in the pancreaticobiliary tract, is a high-risk factor for biliary tract cancer. The aim of this study was to investigate the mechanism of carcinogenesis in PBM using a metabolomics analysis of bile sampled during surgery. Patients and Methods: Three patients with PBM without biliary tract cancer, four patients with extrahepatic bile duct cancer (EHBC), and three controls with benign disease were enrolled. Metabolomics analysis of bile samples was performed using capillary electrophoresis-mass spectrometry and liquid chromatography-mass spectrometry to discriminate the amino acid and lipidomic profiles. Results: The principal component analysis in the capillary electrophoresis-mass spectrometry and liquid chromatography-mass spectrometry revealed similar metabolites in patients with PBM and those with EHBC; furthermore, there was a clear difference between patients with PBM or EHBC compared to controls. The amino acid profiles revealed the following 20 potential carcinogenic candidates for PBM: isoleucine, phenylalanine, tyrosine, leucine, tryptophan, arginine, lysine, valine, asparagine, methionine, aspartic acid, serine, threonine, histidine, glutamine, alanine, proline, glutamic acid, and pyruvic acid. The lipidomic profiles revealed the following 11 carcinogenic candidates: lysophosphatidylcholine, lysophosphatidylethanolamine, phosphatidyl glycerol, lysophosphatidyl glycerol, triacylglycerol, diacylglycerol, ceramide, sphyngomyeline, fatty acid, hyperforin, and vitamin D. Among these characteristic metabolites, the branched-chain amino acids, methionine and lysophosphatidylcholine are known to be related to carcinogenesis. Conclusion: The bile metabolites were extremely similar in patients with PBM and those with EHBC. Furthermore, amino acid and lipid metabolism was markedly different in patients with PBM or EHBC compared to healthy controls.*

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Pancreaticobiliary maljunction (PBM) is a congenital abnormality in which the pancreatic duct and bile duct are anatomically joined outside the duodenal wall. This anatomical anomaly causes reciprocal reflux of pancreatic juice and bile, leading to various pathological conditions, especially PBM is a high-risk factor for biliary tract cancer (1). The reflux of pancreatic juice and bile in PBM generates carcinogens such as activated pancreatic enzymes and secondary bile acid, which repeatedly damage and repair the biliary mucosa, eventually resulting in the contribution to the mutations of various genes. This causes histological changes, called the "hyperplasia-dysplasia-carcinoma sequence", such as epithelial hyperplasia, epithelial metaplasia, and epithelial dysplasia, ultimately resulting in biliary carcinogenesis in turn (2, 3).

Many researchers have tried to elucidate the carcinogenic process from many different angles. The biliary epithelium is damaged by toxic substances, such as phospholipase A2 and lysolecithin, and the repair process involves multiple changes in oncogenes and tumor-suppressor genes, leading to carcinogenesis *via* a multistep interaction. In PBM, the biliary epithelium has a characteristically high incidence and degree of hyperplasia. Patients with PBM have mutations of the K-ras gene in cancerous and non-cancerous tissues of the biliary tract (4-6). These cancerous and noncancerous lesions also have p53 gene mutations and overexpression of the *p53* protein (6, 7). However, the detailed oncogenic mechanism of biliary tract cancer in patients with PBM is still unclear.

The advent of biliary tract cancer associated with PBM is expected to be accompanied by metabolic alterations reflected by changes in the expression of genes, microRNA and so on. Metabolomics includes a comprehensive and

unbiased clarity of small molecule complement in biological fluids, tissues, organs, or organisms (8-11). Because metabolomics teaches us information on disease incidence and progression, this technique has widely application in the elucidation of pathophysiology of various diseases (12-15). Therefore, this approach facilitates the elucidation of the mechanism of carcinogenesis in the biliary tract of patients with PBM. However, bile metabolomics have never been investigated in patients with PBM.

This study is the first to report the metabolome analysis of a wide range of free amino acids and lipids in bile samples obtained from patients with PBM, patients with extrahepatic bile duct cancer (EHBC), and healthy controls. In the present study, we attempted to clarify the biliary tract carcinogenic mechanisms in PBM.

Patients and Methods

Patients. The study population comprised three patients with PBM without biliary tract cancer and four patients with EHBC who underwent total excision of the extrahepatic biliary tract with biliary reconstruction, and three healthy controls with benign disease who underwent cholecystectomy. The study was approved by the Ethics Committee of the Tokushima University Hospital and the corresponding regulatory agencies, and all experiments were carried out in accordance with approved guidelines. All patients provided their written informed consent for surgery and study participation. All bile samples were collected from the gallbladder just after laparotomy and immediately preserved at –80˚C before preparation and analysis of sample.

Sample preparation. The bile samples were thawed at 4˚C prior to analysis. The samples were centrifuged by 10,000 relative centrifugal force at 4˚C for 5 min and prepared. A pooled quality control sample was prepared by mixing the same amount (10 μl) of each sample. A mixture standard was also used to observe and check the stability of the analysis system.

Metabolite extraction procedure. A 20 μl sample of bile was 2.5-fold diluted with Milli-Q water and mixed with 450 μl of methanol containing internal standards (20 μM). Milli-Q water (200 μl) and chloroform (500 μl) were then added, and the solution was mixed and centrifuged at 2,300 g at 4˚C for 5 min. A 375 μl sample of the aqueous layer was filtered through a 5-kDa cut-off filter (EMD Millipore, Billerica, MA, USA) to remove macromolecules. The filtrate was lyophilized and dissolved in 50 μl of Milli-Q water containing reference compounds before mass spectrometry (MS) analysis.

Measurement of amino acid metabolites. Bile metabolic profiling and the mixture of 110 standard metabolites (50 μM each, HMT, Tsuruoka, Japan) were analyzed using a capillary electrophoresis electrospray ionization time-of-flight MS system (Agilent 7100 CE - 6230 TOFMS, Agilent Technologies, Palo Alto, CA, USA) in cationic and anionic modes, with a mass range of 50-1,000. Metabolites in the sample were analyzed using a fused silica capillary (50 μm i.d. × 80 cm length) with the electrolyte buffered solutions (HMT), and the applied voltage or cationic or anionic mode was set to 27 or 30 kV, respectively.

The peaks detected by capillary electrophoresis electrospray ionization time-of-flight MS were extracted using MassHunter integration software (Agilent Technologies, Palo Alto, CA, USA) to obtain peak information, including mass-to-charge ratios (m/z), migration time (MT), and peak area. The peaks were determined with estimated metabolites from the standard metabolites, based on their MT and m/z values. The permissible level for the peak annotation was set at ±0.2 min for MT and ±10 parts per million for m/z. In addition, peak areas were normalized to those of the internal standards. Principal component analysis (PCA) was carried out using multivariate analysis software (Mass Profiler Professional, Agilent Technologies).

Measurement of lipid metabolites. The lipophilic fraction of the bile samples was isolated using the Bligh and Dyer method (16). The fractions were analyzed by the Agilent liquid chromatography-time-of-flight MS system 6200 with the ZORBAX Eclipse Plus C18 column (Agilent Technologies) at a flow rate of 1 ml/min using water:methanol (40:60) as the initial mobile phase. After sample injection, the percentage of methanol was increased to a water:methanol ratio of 0:100 at 10 to 30 min, and was continued for 20 min. The equipped ion source of MS was the Agilent Jetstream electrospray ionization source (Dual AJS ESI, Agilent Technologies). The electrospray ionization source was operated in positive mode with spray voltages of 3,500 V for the capillary entrance and 500 V for the nozzle, nitrogen sheath gas temperature of 250˚C at a flow rate of 12 l/min, nitrogen drying gas temperature of 150˚C at a flow rate of 10 l/min, and nitrogen nebulizer at 45 psig. Purified ubiquinone-8 from *Escherichia coli* (Avanti Polar Lipids, Alabaster, AL, USA) was used as the internal standard for the calculation of the recovery rate during sample purification. The m/z values, retention times, and ion counts of metabolites were extracted from the total ion chromatogram using the Molecular Future Extraction method of MassHunter software (Agilent Technologies). The metabolites that significantly differed between controls and patients (fold change >2.0, $p<0.05$ by one-way analysis of variance) were selected by a multivariate analysis using Mass Profiler Professional software (Agilent Technologies). The selected metabolites were identified using the METLIN Personal Metabolite Database (Agilent Technologies).

Statistical analysis. Statistical testing was performed using STATISTICA 10.0 software and the Metabo Analyst 3.0 web portal (www.metaboanalystca). Both uni- and multivariate statistical analyses were performed to identify the metabolites which had the best discrimination ability. The Shapiro-Wilk test was used to test for normality. Levene's test and the Brown-Forsythe test were used in order to check the equivalence of variances. The differences in free amino acid and lipidomic profiles between the patients with PBM or EHBC and the controls were evaluated using the *t*-test. In all tests, $p<0.05$ was considered statistically significant. The differences between the three groups in the amino acid and lipid concentrations were evaluated by using a one-way analysis of variance.

Results

Pattern recognition analysis in amino acid profiling. PCA was initially conducted to generate an outline of the amino acid metabolic variabilities between patients with PBM, patients with EHBC, and healthy controls. Figure 1A shows

a clear separation between patients with PBM or EHBC and healthy controls in the score plot of first two principal components, indicating that changes in some endogenous amino acid metabolites were related to disease processes.

Differences in metabolites between patients with PBM or EHBC and healthy controls in amino acid profiling. In amino acid profiling, bile metabolites that met the following criteria were regarded as potential carcinogenic candidates involved in carcinogenesis in PBM: metabolite levels with both a variable importance in the projection of >1 and a significant difference ($p<0.05$, *t*-test) between the levels of metabolites of patients with PBM or EHBC and healthy controls. Compared to healthy controls, patients with PBM had significantly higher bile metabolite levels of isoleucine, phenylalanine, tyrosine, leucine, tryptophan, arginine, lysine, valine, asparagine, methionine, aspartic acid, serine, threonine, histidine, glutamine, alanine, proline, glutamic acid, and pyruvic acid ($p<0.05$), and significantly lower levels of glycerol 3-phosphate, gluconic acid, creatine, and creatinine ($p<0.05$) (Table I). In addition, compared to healthy controls, patients with EHBC had significantly higher bile metabolite levels of leucine, phenylalanine, isoleucine, tyrosine, valine, methionine, citrulline, lysine, aspartic acid, tryptophan, alanine, threonine, asparagine, serine, lactic acid, arginine, proline, ornithine, histidine, glutamic acid, glutamine, pyruvic acid, and 2-hydroxybutyric acid ($p<0.05$), and significantly lower levels of creatine and cytidine ($p<0.05$) (Table I).

We sorted the metabolites that were elevated in the bile of both patients with PBM and those with EHBC compared to healthy controls, and identified 20 metabolites as potential carcinogenic biomarkers for PBM. Figure 1B shows the Venn diagram used to visualize these metabolites. Both patients with PBM and those with EHBC had increased levels of isoleucine, phenylalanine, tyrosine, leucine, tryptophan, arginine, lysine, valine, asparagine, methionine, aspartic acid, serine, threonine, histidine, glutamine, alanine, proline, glutamic acid, and pyruvic acid compared to healthy controls (Table I).

Pattern recognition analysis in lipidomic profiling. PCA was performed to produce an overview of the lipid metabolic alterations between patients with PBM, patients with EHBC, and healthy controls. Figure 2A shows that there was a satisfactory distinction between patients with PBM or EHBC and healthy controls, suggesting that changes in some endogenous lipid metabolites were associated with disease progression.

Differences in metabolites between patients with PBM or EHBC and healthy controls in lipidomic profiling. In lipidomic profiling, bile metabolites that met the same statistical criteria as in the amino acid analysis described above were considered as potential carcinogenic biomarker candidates for PBM. Eleven metabolites were elevated in the bile of both patients with PBM and patients with EHBC compared to healthy controls, and were identified as potential carcinogenic biomarkers for patients with PBM. Figure 2B shows the Venn diagram that was used to visualize these metabolites. Both patients with PBM and those with EHBC had increased levels of lysophosphatidylcholine (LPC), lysophosphatidylethanolamine, phosphatidyl glycerol, lysophosphatidyl glycerol, triacylglycerol (TAG), diacylglycerol, ceramide, sphyngomyeline, fatty acid, hyperforin, and vitamin D compared to healthy controls (Table II).

Discussion

PBM is a known risk factor for biliary tract cancer. According to a Japanese nationwide survey, the cancer development rate of patients with PBM is 200-times higher than that of usual carcinogenesis of the bile duct in Japan (17, 18). However, the underlying molecular mechanisms of cancer development in PBM remain elusive.

Our amino acid profiling results showed that the levels of circulating leucine, isoleucine, and valine were higher in patients with PBM or EHBC than in healthy controls (Table I). Branched-chain amino acids (BCAA) have been reported to be involved in several kinds of cancers. These BCAA include valine, leucine, and isoleucine and they regulate various signaling pathways such as cell growth, protein and lipid synthesis and autophagy (19). Catabolism of BCAA occurs mainly in skeletal muscle and BCAA has high activity of aminotransferase. This catabolism of BCAA is an important process in amino acid synthesis (20). It is widely accepted that cancer cells mainly use glycolysis for energy production rather than oxidative phosphorylation, regardless of the availability of oxygen (Warburg effect) (21). Furthermore, the unique properties of cancer cells and the expression of hypoxia-inducible transcription factors activate the genes encoding glycolytic enzymes, glucose transporters (22, 23), and amino acid transporters (24) for growth and proliferation; therefore, glucose transporters and amino acid transporters are highly expressed in cancer cells. Thus, the increase of leucine, isoleucine, and valine in patients with PBM and patients with EHBC may be explained by the necessities for energy and proliferation of both the host and the tumor. Similarly, other studies have reported that BCAA are the most commonly detected amino acids in gastric cancer (25), serum leucine and isoleucine levels are increased in patients with lung cancer (26), fecal leucine, isoleucine, and valine levels are elevated in patients with colorectal cancer (27), and BCAA play an important role in tumor growth and survival by regulating the bioenergetics and biosynthesis of cells through the tricarboxylic acid cycle (28).

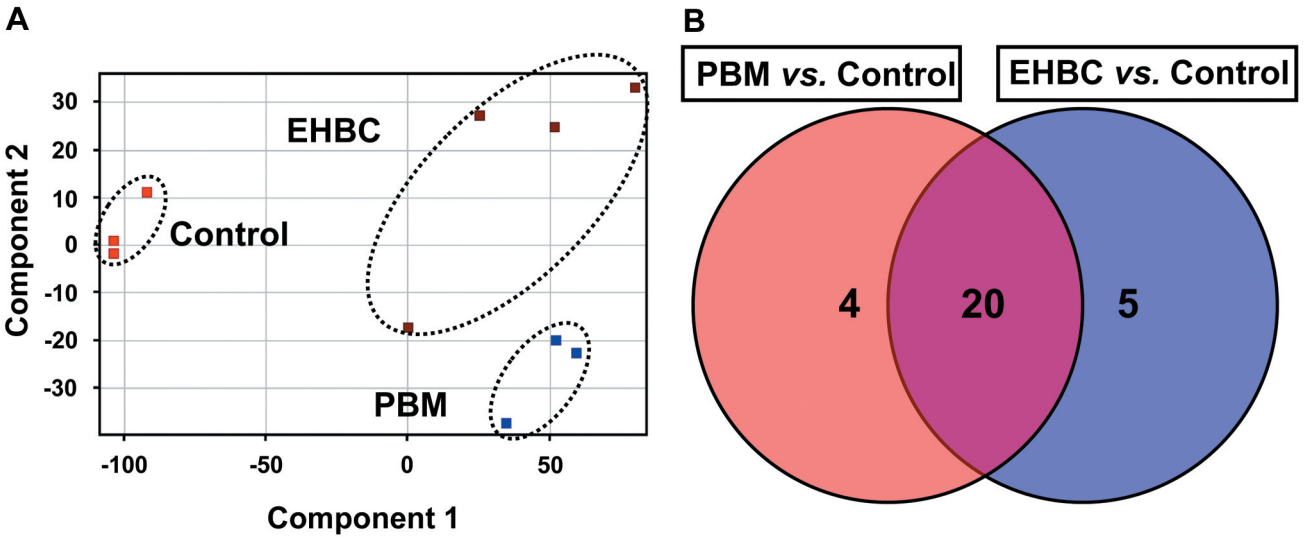

Figure 1. *(A) Principal component analysis to determine the bile metabolomics status in amino acid profiling. The PBM, EHBC, and control groups are indicated in blue, brown, and red, respectively. (B) Venn diagram for the potential carcinogenic biomarker candidates of PBM. Amino acids up-regulated by more than two-fold in the PBM, EHBC, and control groups are indicated. PBM: Pancreaticobiliary maljunction; EHBC: extrahepatic bile duct cancer.*

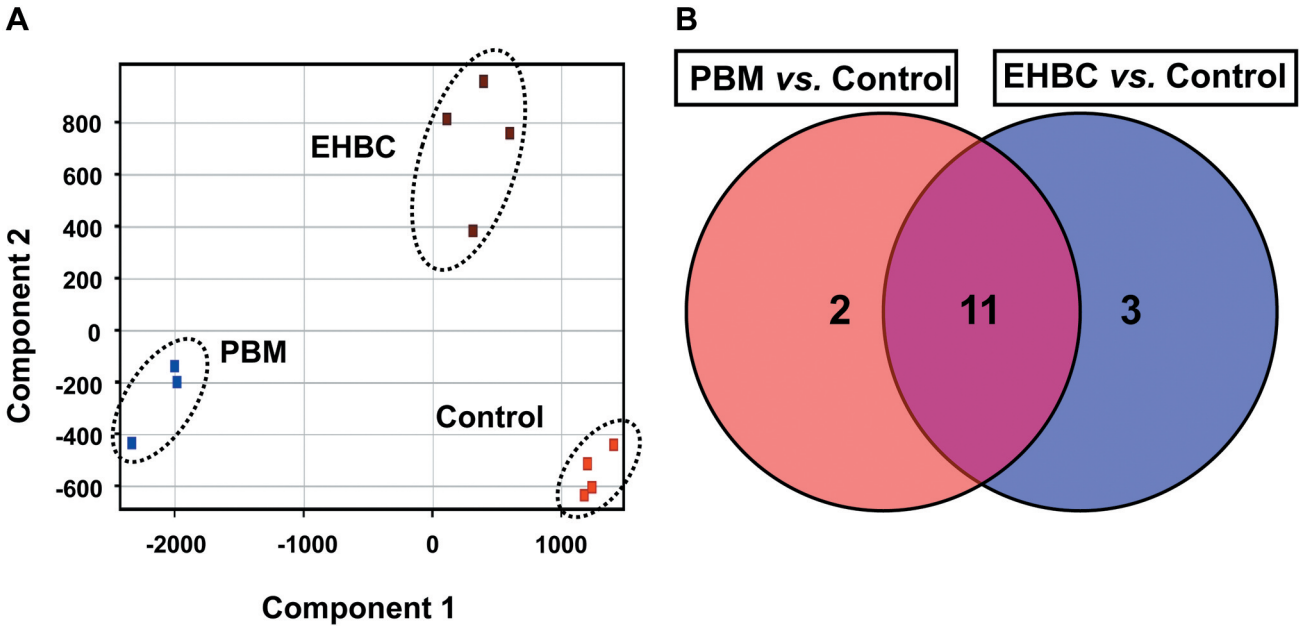

Figure 2. *(A) Principal component analysis to determine the bile metabolomics status in lipidomic profiling. The PBM, EHBC, and control groups are indicated in blue, brown and red, respectively. (B) Venn diagram for the potential carcinogenic biomarker candidates of PBM. Amino acids up-regulated by more than two-fold between the PBM, EHBC, and control groups are indicated. PBM: Pancreaticobiliary maljunction; EHBC: extrahepatic bile duct cancer.*

In the present study, an increased level of phenylalanine was also a prominent feature in the bile amino acid profile of patients with PBM and patients with EHBC. Phenylalanine is an essential amino acid, and is a precursor of tyrosine and neurotransmitters. Tyrosine is an important precursor of melanin, catecholamine neurotransmitters and thyroid hormones, but it also facilitates lipid metabolism. Gu *et al.* (29) reported increased serum levels of

Table I. *Differences in the concentrations of free amino acids in the bile samples of the PBM, EHBC, and control groups.*

PBM *vs*. Control		EHBC *vs*. Control	
Compound	Fold	Compound	Fold
Isoleucine	73.1	Leucine	39.6
Phenylalanine	66.6	Phenylalanine	38.6
Tyrosine	60.6	Isoleucine	29.6
Leucine	55.3	Tyrosine	25.4
Tryptophan	43.0	Valine	23.8
Arginine	39.9	Methionine	22.7
Lysine	37.1	Citrulline	19.1
Valine	35.0	Lysine	17.1
Asparagine	33.7	Aspartic acid	15.4
Methionine	26.0	Tryptophan	13.1
Aspartic acid	19.7	Alanine	12.6
Serine	18.3	Threonine	12.2
Threonine	17.8	Asparagine	11.9
Histidine	14.7	Serine	11.3
Glutamine	12.0	Lactic acid	11.3
Alanine	11.8	Arginine	10.7
Cysteine	7.9	Proline	8.9
Proline	4.9	Ornithine	8.2
Glutamic acid	4.3	Histidine	8.0
pyruvic acid	3.2	glutamic acid	7.4
Glycerol	–2.3	Glutamine	5.2
3-phosphate Gluconic acid	–2.5	Pyruvic acid	4.6
Creatine	–3.6	2-hydroxybutyric acid	3.7
Creatinine	–4.3	Creatine	–2.4
		Cytidine	–2.7

PBM: Pancreaticobiliary maljunction; EHBC: extrahepatic bile duct cancer.

Table II. *Differences in the concentrations of free lipids in the bile samples of the PBM, EHBC, and control groups.*

PBM *vs*. Control		EHBC *vs*. Control	
Compound	Fold	Compound	Fold
Lysophosphatidylcholine	422,482	Sphyngomyeline	57,450
Triacylglycerol	141,506	Lysophosphatidylethanolamine	23,071
Sphyngomyeline	82,070	Triacylglycerol	18,734
Lysophosphatidylinositol	53,590	Ceramide	18,506
Ceramide	53,330	Phosphatidylglycerol	15,623
Hyperforin	46,983	Phosphatidylethanolamine	15,498
Vitamin D	40,977	Lysophosphatidylcholine	12,302
Phosphatidylglycerol	22,298	Phosphatidylcholine	12,216
Lysophosphatidylglycerol	10,858	Lysophosphatidylglycerol	7,453
Diacylglycerol	8,969	Diacylglycerol	5,054
Lysophosphatidylethanolamine	7,839	Vitamin D	3,636
Fatty acid	6,468	Fatty acid	1,140
Phosphatidyl acid	5,732	Hyperforin	884
Phosphatidyl Serine	–44,546	Phosphatidyl acid	–614
Phosphatidylethanolamine	–130,200	Phosphatidyl Serine	–2,504
Phosphatidylcholine	–148,601	Lysophosphatidylinositol	–29,297

PBM: Pancreaticobiliary maljunction; EHBC: extrahepatic bile duct cancer.

phenylalanine and tyrosine in rats with high-grade gastric dysplasia; moreover, similarly to the high-grade gastric dysplasia stage, the gastric cancer stage showed phenylalanine and tyrosine biosynthesis. Dependence on tyrosine and phenylalanine regulates the cell behavior of melanoma cells (30), and the use of diet restricted in phenylalanine leads to the inhibition of growth and metastasis of several malignancies (31, 32). This study showed that phenylalanine may be associated with the carcinogenesis of biliary tract in patients with PBM.

The other characteristic feature in the metabolome analysis of patients with PBM and patients with EHBC was an increased concentration of methionine. Methionine is an essential amino acid that has many important roles in mammalian metabolism, including protein synthesis, DNA methylation, so-called epigenetic regulation, and polyamine synthesis. Many researchers have tried to obtain therapeutic effects utilizing the methionine dependence of tumors in vitro and *in vivo*. A number of malignant cell lines, such as colon, bladder, lung, kidney, breast, glioblastoma, and melanoma are methionine-dependent (33-36). Methionine-deprived total parenteral nutrition also reduces the tumor volume in rats with hepatoma (37, 38). Methionine-free diet or total parenteral nutrition deprived of methionine causes the tumor regression in a variety of animals. Therefore, the elevation of methionine seen in patients with PBM and patients with EHBC in the present study may be due to increased protein synthesis and methylation of DNA.

In our lipidomic profiling, LPC was markedly elevated in patients with PBM or EHBC compared to healthy controls. LPC is derived from phosphatidylcholine through the enzymatic action of phospholipase A2. Many studies have shown that LPC acts as an important lipid mediator and is associated with the pathogenesis of several diseases, including diabetes, ovarian cancer, rheumatoid arthritis, bronchial asthma, atherosclerosis, and psoriasis (39-43). In addition, recent studies have shown that LPC not only exhibits cytotoxicity *via* significant induction of apoptosis in cholangiocytes, but also provokes cholangiocyte senescence; this leads to the up-regulation of genes encoding a series of senescence-associated secretory phenotype components, which may alter the surrounding environment and neighboring cells, leading to carcinogenesis (44, 45).

A high level of TAG was also a significant feature in the lipidomic profile of the bile of patients with PBM or EHBC. Recent reports suggest that inflammation, such as that caused by lipopolysaccharide, increases intracellular TAG and reactive oxygen species production and leads to impairment of mitochondrial function, and thus predisposes the body to metabolic disorders and carcinogenesis (46, 47). Therefore, the elevation of LPC and TAG seen in patients with PBM or EHBC in the present study may suggest that LPC and TAG contribute to the development of cholangiocarcinoma.

In this study, we performed metabolomic analysis to compare amino acid and lipidomic profiles of bile obtained from patients with PBM, EHBC, and benign diseases. The involvement of amino acid and lipidomic profiles using metabolomics for the carcinogenesis of pancreaticobiliary maljunction has not been reported thus far. This is the first report of a metabolome analysis focusing on bile, and this paper is a valuable report with high originality. The idea of this study, that metabolic comparison of bile is performed between these patients, is interesting itself. Furthermore, we found that both amino acid and lipidomic profiles contained similar characteristics in PBM and EHBC patients and different from benign disease. This study can actually identify carcinogenesis-related metabolites, and the results of this study may contribute to the elucidation of the carcinogenesis mechanism for this disease in the future. These are the strength of this research which has a strong scientific interest. However, our study had some limitations. Firstly, one limitation was that the sample size was small. Therefore, it is necessary to perform validation studies. In our ongoing work, we are collecting samples from a larger patient population. Another limitation was that the capillary electrophoresis- and liquid chromatography-based targeted metabolomics approach limited the candidate markers, focusing only on amino acid and partial lipidomic metabolic pathways. Therefore, we are currently building up nucleic acid-, sugar-, fatty acid-, and another lipid-based metabolome analysis to validate the feasibility of more comprehensive metabolomics approaches and identify additional unknown carcinogens. Although this was a preliminary study and further research is necessary, our results provide a basis for the future investigation of the detailed biliary tract carcinogenic mechanism in PBM. As amino acid and lipid metabolism is involved in complicated networks of interactions in carcinogenesis, further research is required to link an accurate association between the altered amino acid and lipid metabolisms and cancer initiation and tumor progression.

In conclusion, we performed amino acid and lipidomic metabolomic profiling of bile obtained from patients with PBM, patients with EHBC, and healthy controls. The bile metabolites were extremely similar between patients with PBM and those with EHBC, and the amino acid and lipidomic metabolism of patients with PBM or EHBC was found markedly altered compared to healthy controls. Therefore, patients with PBM were considered to be regularly exposed to a carcinogenic environment, just like patients with EHBC.

Conflicts of Interest

The Authors declare no conflicts of interest for this article.

Authors' Contributions

HM participated in the research design, performance of the research, data analysis and writing manuscripts. YM participated in the research design. KM, AC and AT was responsible for data collection and analysis. YM, SY, YS and HI participated in performance of the research. MS participated in the critical comments and administrative support. All Authors discussed the results and contributed to the final article.

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