Identification of bile biomarkers of biliary tract cancer through a liquid chromatography/mass spectrometry-based metabolomic method

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Abstract. The incidence and mortality rate of biliary tract cancer have been increasing worldwide; however, its diagnosis and prognosis have not improved in recent years. A novel approach, termed 'metabolomics', may have the potential to be developed as an effective diagnostic tool. The present study prospectively obtained bile samples from 115 individuals, including 32 patients with biliary tract cancer, 61 patients with benign biliary tract diseases and 22 normal controls. A liquid chromatography/mass spectrometry (LC/MS)-based approach was used to investigate the differences in bile samples between the three groups, followed by multivariate statistical analysis, which included partial least squares projection to latent structures with discriminant analysis (PLS-DA) and orthogonal projection to latent structures with discriminant analysis (OPLS-DA). The metabolomic 2D score plot and 3D plot revealed clear separation between the cancer, benign and normal control groups by PLS-DA. To further address the significant difficulties in clinically differentiating between biliary tract cancer and benign biliary tract diseases, OPLS-DA was performed to distinguish between the two disease groups and to select potential biomarkers. The cancer and benign groups were well differentiated. The metabolic analysis revealed significantly lower levels of lysophosphatidylcholine, phenylalanine, 2-octenoylcarnitine, tryptophan and significantly higher levels of taurine- and glycine-conjugated bile acids in the bile from patients with biliary tract cancer compared with those in the bile from patients with benign biliary tract disease. The present study suggested that an LC/MS-based metabolomic investigation provides a potent and promising approach for discriminating biliary tract cancer from benign biliary tract diseases and the identified specific metabolites may offer potential as novel biomarkers for the early detection of biliary tract cancer.

Introduction

Biliary tract cancer includes types of cancer which originate from epithelial cells of the intrahepatic or extrahepatic bile ducts and anatomically comprise intrahepatic cholangiocarcinoma, perihilar cholangiocarcinoma, gallbladder cancer and distal cholangiocarcinoma (1). Though biliary tract cancer is an uncommon type of tumor, the incidence and mortality of biliary tract cancer has been increasing worldwide (2-4). Patients with biliary tract cancer are usually asymptomatic or may present with nonspecific clinical features, including fever, fatigue, anorexia, mild abdominal pain, weight loss and occasional jaundice late in the course of the disease (1). Patients with biliary tract cancer have a high mortality rate and poor prognosis, with a five-year survival rate of <5% due to its late clinical presentation (5).

The majority of tumors are at an advanced stage at the time of diagnosis, which is one of the reasons for the high mortality rate and poor prognosis of biliary tract cancer (6). Confirmation of the diagnosis of biliary tract cancer is often difficult and the diagnosis is based on a combination of serum tumor markers, radiological imaging and histological verification; however, each of these approaches has its own drawbacks (7-11). Tissue diagnosis has been accepted as the 'gold standard'. Percutaneous fine-needle aspiration biopsy, brush and scrape biopsy and cytological examination of bile have all been used to establish a tissue diagnosis; however, the sensitivity in detecting a malignancy is low and the possibility that a benign result is unreliable

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requires consideration (12,13). Tissue diagnosis cannot generally be performed due to the location and size of the tumor (7). Percutaneous fine-needle aspiration biopsy cannot be used in a number of cases when the tumors are located in the hepatic hilum and accompanied by important arteries and veins (8). Although serum tumor markers, including carbohydrate antigen 19-9 (CA19-9) and carcinoembryonic antigen (CEA) have been widely used to assist in the diagnosis of biliary tract cancer, they have poor sensitivity (50-60%) and these serum tumor markers can also be elevated in other types of malignancy and certain benign conditions (9-11).

A novel approach, termed 'metabolomics' or 'metabonomics', deals with the diverse properties of low molecular metabolites in the body. This novel approach has been demonstrated to be an effective tool for biomarker screening, disease diagnosis and prognosis as well as in the characterization of the metabolic network (14-16). Metabolomics can be applied to any bio-fluid, including serum, urine, saliva or bile and has been identified as a promising and reliable novel diagnostic approach for several types of tumor, including breast cancer, prostate cancer, hepatocellular carcinoma and pancreatic carcinoma (17-20). However, few studies have investigated the application of metabolomics in biliary tract cancer or chlangiocarcinoma.

In the present study, bile was obtained from 115 individuals and the metabolite patterns of these bile samples were examined using a liquid chromatography/mass spectrometry (LC/MS)-based metabolomic method. Potential biomarkers were identified for distinguishing between patients with biliary tract cancer, patients with benign biliary tract disease and healthy individuals at an early stage to improve the diagnosis and prognosis of biliary tract cancer.

Materials and methods

Patient recruitment and sample collection. Informed consent was obtained from each individual and the present study was approved by the Ethics Committee of The First Affiliated Hospital of Zhejiang University (Hangzhou, China). A total of 115 individuals were enrolled in the present study, which were divided into three groups: 32 patients with biliary tract cancer (male/female, 5:3), 61 patients with benign biliary tract diseases (male/female, 1:1) and 22 normal controls (male/female, 10:1). The 22 normal control individuals were all liver donors for transplant at the First Affiliated Hospital of Zhejiang University. Bile samples were prospectively obtained from patients with biliary tract cancer or benign biliary tract diseases either during surgery or endoscopic retrograde cholangiopancreatography (ERCP) and from normal control individuals during the donor liver transplantation surgery. The collected bile samples were then immediately frozen at -80°C.

Patient characteristics. The 115 enrolled individuals consisted of 32 patients with biliary tract cancer, 61 patients with benign biliary tract diseases and 22 normal controls. The 32 patients in the biliary tract cancer group included 13 cases of intrahepatic cholangiocarcinoma, 4 cases of Klatskin tumor, 2 cases of gallbladder cancer, 11 cases of common bile duct cancer and 2 cases of a combination

of gallbladder cancer and common bile duct cancer. The 61 patients in the benign biliary tract disease group included 40 cases of biliary calculi, 12 cases of benign biliary tract stricture and 9 cases of choledochal cyst. The patient characteristics of the three groups differed due to the epidemiology of the different diseases. The average age was 59 years in the cancer group, 58 years in the benign biliary tract disease group and 26 years in the normal control group. The ratio of males to females was 5:3 in the cancer group, ~1:1 in the benign biliary tract diseases group and almost 10:1 in the normal control group.

Bile specimen pretreatment. Prior to LC/MS analysis, 2,200 μl acetonitrile Sigma-Aldrich (St.Louis, MO, USA) was added to bile samples (200 μl) and vortex was performed for 2 min at room temperature. The sample mixture was left to stand for 5 min and centrifuged at 18,000 x g for 10 min at 4°C. Subsequently, 1,500 μl supernatant was used in the LC/MS analysis.

LC separation. Chromatographic separations were performed on an ACQUITY Ultra Performance LC system using an ACQUITY UPLC BEH C18 analytical column (internal dimension, 2.1x100 mm; particle size, 1.7 μm; pore size, 130 Å; Waters MS Technologies, Manchester, UK). A mixture of water and formic acid (99.9:0.1, v/v; Sigma-Aldrich) was used as mobile phase A and acetonitrile with formic acid (99.9:0.1, v/v) as mobile phase B at a flow rate of 300 μl/min. The linear gradient LC system was optimized as follows: The composition of mobile phase B was increased from 3 to 30% over 2 min, to 60% over 8 min, 90% over 1 min and 100% over 6 min, then leveled for 8 min. The column was maintained at 50°C and the sample manager was set at 4°C. A 2 μl injection of each sample was made into the column.

MS assay. MS was performed using a Q-Tof Premier Mass Spectrometer (Waters MS Technologies) operating in positive ion mode. The nebulization gas was set to 600 l/h at a temperature of 350°C, the cone gas was set to 0 l/h and the source temperature was set to 120°C. The capillary voltage was set at 2.8 kV and the sampling cone voltage was set at 40 V (Waters MS Technologies). The Q-Tof Premier acquisition rate was set to 0.5 sec, with a 0.1 sec inter-scan delay. The instrument was used with the first resolving quadrupole in a wide pass mode with the collision cell operating with two alternating collision energies, 5 eV and 50 eV. The data were collected into two separate data channels, with the instrument spending 0.5 sec on data acquisition for each channel with a 0.05 sec inter-channel delay. The instrument was previously calibrated with sodium formate, the lock mass spray for precise mass determination was set using leucine enkephalin at a mass to charge ratio (m/z) of 554.2615 (0.5 ng/l) in the positive ion mode, which increased the maximum relative error up to 10 ppm. Data were collected in centroid mode with a lock spray frequency of 5 sec and the average from 10 scans was obtained. All analyses were acquired using lock spray to ensure accuracy and reproducibility.

Data processing and statistical analysis. The raw data files obtained from the LC/MS runs were analyzed using
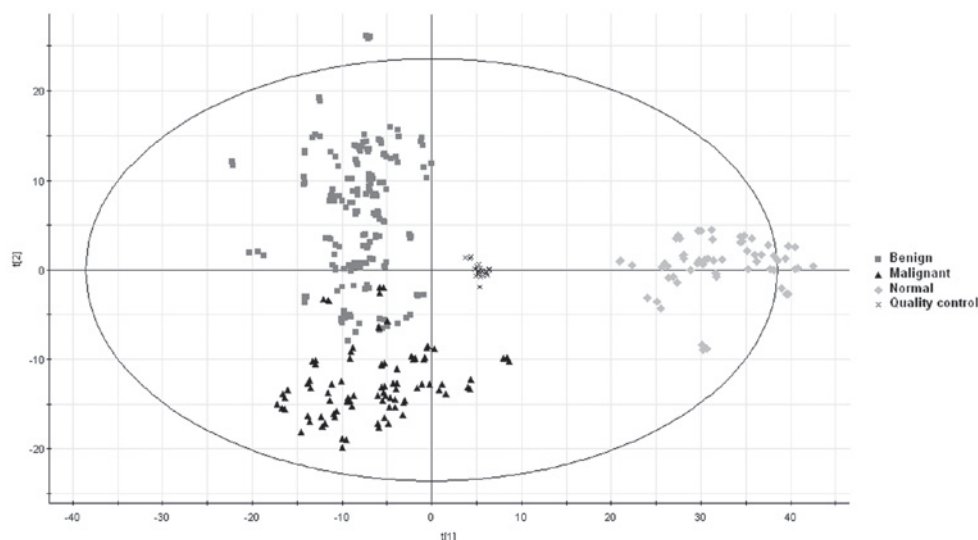
Figure 1. Metabolomic 2D partial least squares projection to latent structures with discriminant analysis score plot. The aggregation of diagonal crosses demonstrated a high-grade quality control, the disease groups (biliary tract cancer and benign biliary tract diseases) and the normal control group were clearly separated, with clear separation between the biliary tract cancer group and benign biliary tract disease group. The majority of the cancer and benign samples appeared clustered in their respective regions with only a few overlaps between them. Benign, bile samples from patients with benign biliary tract diseases; Malignant, bile samples from patients with biliary tract cancer; Normal, bile samples from normal control individuals. t, component in metabolic study.

MassLynx v4.1 and MarkerLynx v4.1 software (Waters MS Technologies). MarkerLynx extracted components from the exact mass chromatograms and listed the detected peaks as their mass, retention time and associated intensities. Chromatographic peaks in the raw data files were detected by extracting nominal mass chromatograms and tracking the apex of the peaks in the chromatograms. The spectra from each of the detected peaks were listed as the retention time, exact mass pairs and associated intensities, which were saved as either normalized or absolute intensities. Following extraction of all the data, it was aligned within user-defined mass (0.05 Da) and retention time windows (0.2 min). The resulting multivariate dataset, consisting of the peak number based on the retention time, m/z, sample name and the normalized peak intensity, was exported and analyzed by partial least squares projection to latent structures with discriminant analysis (PLS-DA) and orthogonal projection to latent structures with discriminant analysis (OPLS-DA) using SIMCA-P 12.0 software (Umetrics, Umeå, Sweden).

Results

Metabonomic profiling of bile samples and multivariate analysis. The bile samples were characterized by LC/MS in the positive ion mode and the mass spectra of the bile samples were obtained from all three groups. The mass spectral data were then processed by multivariate analysis. The PLS-DA model was subsequently used for data analyses. The results revealed high-grade quality control and clear separation between the disease groups (biliary tract cancer and benign biliary tract diseases) and the normal control group and the results also demonstrated clear separation between the biliary tract cancer group and the benign biliary tract disease group in the metabolomic 2D scores plot. The majority of the cancer and benign samples appeared clustered in their respective regions with only a few overlaps between them (Fig. 1).

The 3D scores plot demonstrated similar results. A validated PLS-DA model was used to evaluate the goodness of fit of the model. Generally, a model is considered to have a good fit if $R2$ intercepts are <0.4 and $Q2$ is <0.05 in the permutation assessment, with 200 iterations (21,22). In the present study, the $R2$ intercepts were <0.4 and the $Q2$ was <0 using PLS-DA, demonstrating that the model was well-fit and statistically valid (Fig. 2).

To further address the substantial clinical difficulties in differentiating between biliary tract cancer and benign biliary tract diseases, an OPLS-DA model was used for discrimination of the two disease groups and to select potential biomarkers. The analytical results revealed that the cancer and benign groups were well differentiated (Fig. 3).

The S-plot, which visualizes the covariance and correlation among metabolites, is usually used to identify important metabolites (23). As shown in Fig. 4, the S-plot revealed the metabolites which most reliably assisted in the differentiation of the two groups.

The variable importance in the projection (VIP) in the OPLS-DA model is a predominant parameter for the detection of potential biomarkers (24) and, in the present study, the different metabolite VIP values reflect the correlation between the metabolites and the discrimination of the biliary tract cancer and the benign biliary tract disease groups. The potential biomarkers, which discrimination was mainly attributed to, were selected from the S-plot according to the VIP values and, using the Metlin Metabolite Database (http://metlin.scripps.edu), Human Metabolome Database (http://www.hmdb.ca/), relevant literature (25,26) and comparison with a standard substance, the potential biomarkers were identified (Table I).

The representative variable averages of the potential biomarkers in the biliary tract cancer group and in the benign biliary tract disease group are shown in Figs. 5 and 6. As shown in Fig. 5, the variable averages of lysophosphatidylcholine

Table I. Summary of the metabolites indicating significant changes in bile between the benign and cancer groups.

RT (min)_m/z	VIP	Identification results	Cancer (vs. benign)
11.58_496.3385	21.4074	Lyso-PC 16:0, [M+H]$^+$	↓
11.53_991.6724	13.1144	Lyso-PC 16:0, [M+H]$^+$	↓
11.32_496.3387	7.8017	Lyso-PC 16:0 isomer, [M+H]$^+$	↓
7.57_899.6366	7.71738	GCDCA, [2M+H]$^+$	↑
12.29_524.3703	7.69633	Lyso-PC 18:0, [M+H]$^+$	↓
4.51_462.2664	7.39132	TUCA, [M-3H$_2$O+H]$^+$	↑
2.02_120.0801	6.87389	Phenylalanine, [M+H]$^+$	↓
5.71_464.2823	5.85991	TUDCA, [M-2H$_2$O]$^+$	↑
7.56_450.3208	5.34161	GCDCA, [M+H]$^+$	↑
5.54_412.2835	4.93109	GCA, [M-3H$_2$O+H]	↑
5.53_430.2947	4.82189	GCA, [M-2H$_2$O+H]$^+$	↑
3.97_480.2770	4.38365	TUCA isomer, [M-2H$_2$O]$^+$	↑
4.51_480.2769	4.15222	TUCA, [M-2H$_2$O]$^+$	↑
7.57_414.2993	4.08688	GCDCA, [M-2H$_2$O+H]	↑
3.49_286.2004	4.0158	2-Octenoylcarnitine, [M+H]$^+$	↓
6.06_464.2821	3.74324	TCDCA, [M-2H$_2$O+H]$^+$	↑
11.09_520.3395	3.59733	Lyso-PC 18:2, [M+H]$^+$	↓
2.28_188.0698	3.42225	Tryptophan, [M+H]$^+$	↓
4.52_498.2880	3.3963	TUCA, [M-H$_2$O]$^+$	↑
5.54_948.6544	3.36625	GCA, [2M+NH$_4$]$^+$	↑

RT, retention time; VIP, variable importance in the projection; ↓, decreased; ↑, increased; Lyso-PC, lysophosphatidylcholine; GCDCA, glyco-chenodeoxycholic acid; TUCA, tauroursocholic acid; TUDCA, tauroursodeoxycholic acid; GCA, glycocholic acid.
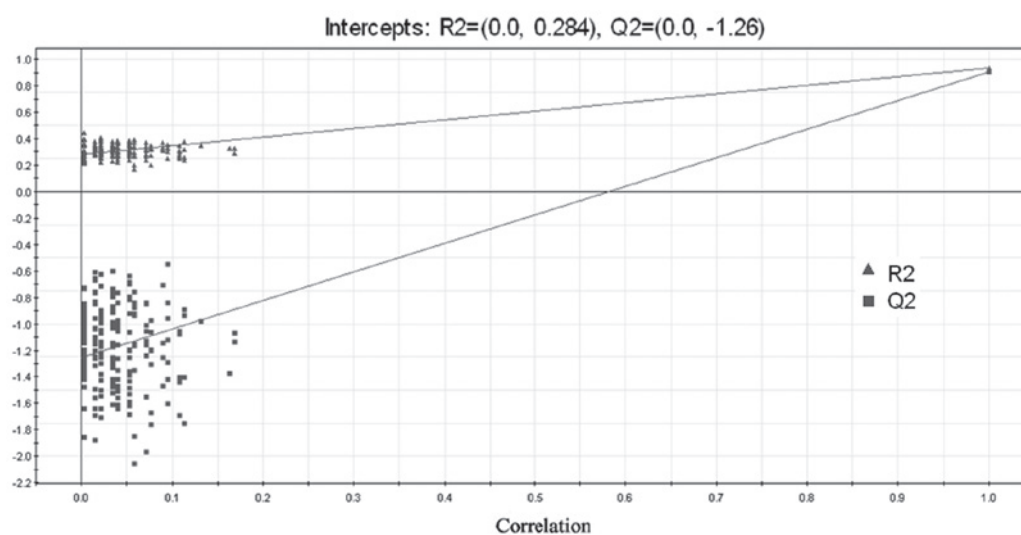

Figure 2. Validation plot of partial least squares projection to latent structures with discriminant analysis for discrimination of the normal control group, biliary tract cancer group and benign biliary tract disease group. The R2 intercepts were <0.4 and the Q2 was <0 in the permutation test with 200 iterations, demonstrating a good fit and statistically valid model.

(lyso-PC) 16:0 were markedly reduced in the biliary tract cancer group compared with those in the benign biliary tract disease group. Conversely, the variable averages of glycochenodeoxycholic acid (GCDCA) were significantly augmented in the biliary tract cancer group compared withthose in the benign biliary tract disease group (Fig. 6).

Discussion

Biliary tract cancer comprises a group of malignant biliary diseases, which have a high mortality rate (2-4). Surgical excision of the tumor is the only option to improve the survival rate of patients with these diseases, due to the
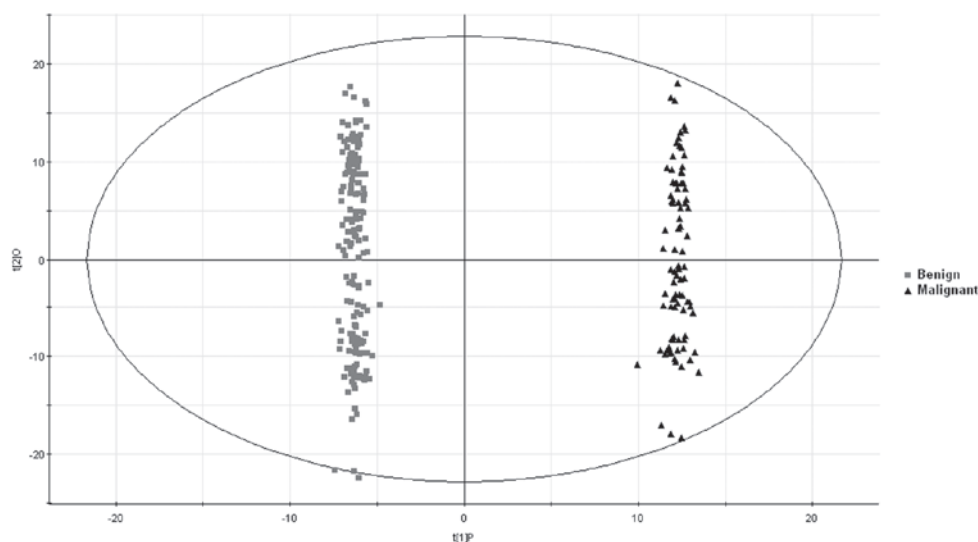
Figure 3. OPLS-DA score plot of benign and cancer samples. An OPLS-DA model was used for the discrimination of the two disease groups. Results revealed that the cancer and benign groups were well differentiated. Benign, bile samples from patients with benign biliary tract diseases; Malignant, bile samples from patients with biliary tract cancer. OPLS-DA, orthogonal projection to latent structures with discriminant analysis. t, component in metabolic study.
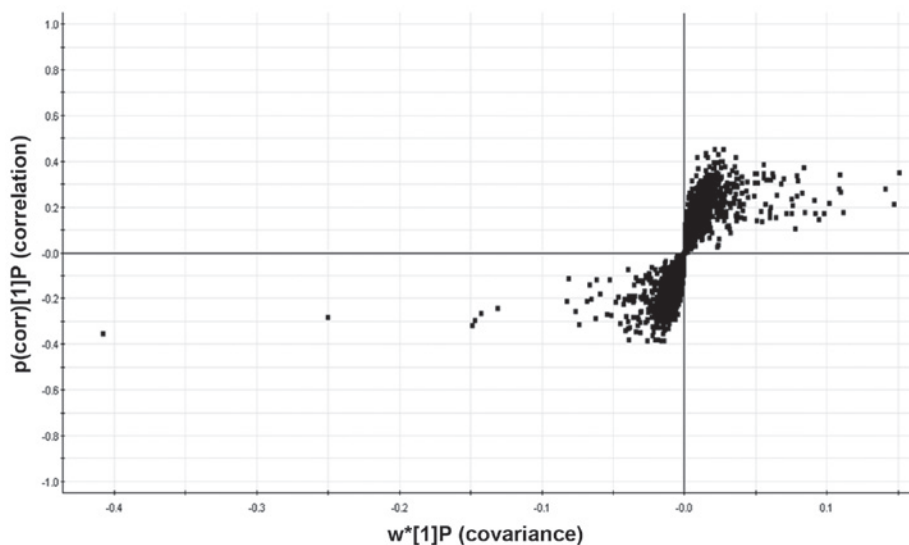

Figure 4. Orthogonal projection to latent structures with discriminant analysis S-plot of the mass spectra of bile samples from patients with benign biliary tract diseases and patients with biliary tract cancer. The metabolites on the outermost verge of the S-plot contributed most in differentiating between the two groups, assisting in the selection of potential biomarkers together with variable importance in the projection values.

insensitivity or lack of response to chemotherapy or radiotherapy. However, the prognosis of patients who suffer from biliary tract cancer remains poor as diagnosis of the disease is generally late. This late diagnosis is partly the result of the disease characteristics, which include asymptomatic traits or nonspecific clinical features; however, it is mainly attributed to the lack of powerful and sensitive diagnostic tools (6). Previously, the diagnosis was primarily dependent on the serum tumor markers CA19-9 and CEA as well as radiological imaging, which have low levels of sensitivity and specificity. Tissue diagnosis was also rare due to the location and size of the tumor and the risk of possible hemorrhage or bile leakage (7,8). In recent years, brush and scrape biopsy and cytological examinations of bile have been used to establish a tissue diagnosis with widespread application of ERCP;

however, their use for differentiating between cancer and benign diseases is limited due to insufficient sensitivity and benign results are often considered to be unreliable (27-30). Therefore, novel diagnostic methods are required and of high importance.

Metabolomics is a novel and promising tool, which has emerged in recent years. It has been identified as an effective tool for the screening of biomarkers and disease diagnosis (14,16). Techniques used in metabolomic studies generally include nuclear magnetic resonance spectroscopy, Fourier-transform infrared spectroscopy and gas chromatography/mass spectrometry or LC/MS (31). LC/MS is regarded as an ideal tool for organic metabolites and the screening of biomarkers among them due to its prominent advantages, including reproducible quantitative analysis and
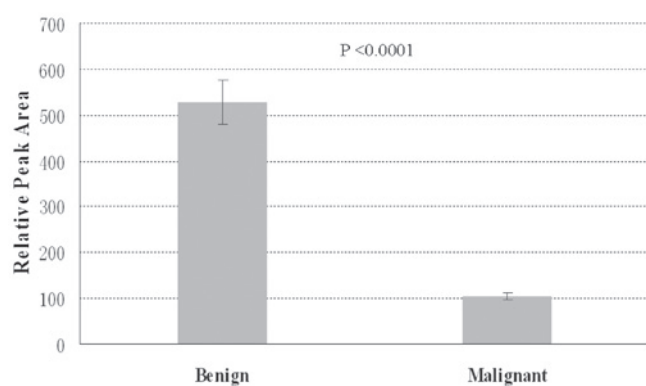
Figure 5. Variable averages of lysophosphatidylcholine 16:0 in the benign biliary tract disease group (benign; 529.49±48.92, n=61) and in the biliary tract cancer group (malignant: 103.96±8.17, n=32). Data are presented as the mean ± standard deviation.
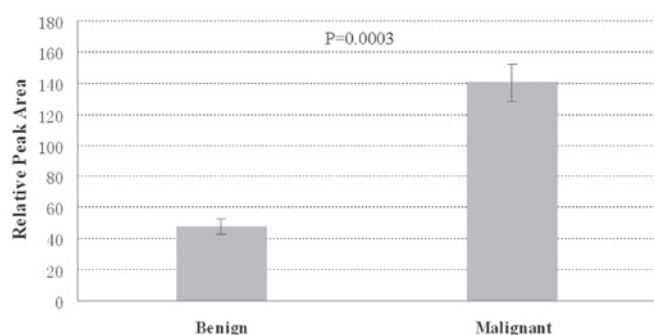

Figure 6. Variable averages of glycochenodeoxycholic acid in the benign biliary tract disease group (benign; 47.94±4.84, n=61) and in the biliary tract cancer group (malignant; 140.27±12.24, n=32). Data are presented as the mean ± standard deviation.

the ability to analyze biofluids with high levels of molecular complexity (32). Previous studies have demonstrated the potential for metabolomics as a novel reliable diagnostic approach for hepatocellular carcinoma as well as pancreatic, breast and prostate cancer (18-20). The few previous investigations into the application of the metabolomic approach on biliary tract cancer consisted of small sample sizes, discrepant results and different methods (33-35). In the present study, an LC/MS-based metabolomic method was used to examine the metabolite patterns of bile samples from a large study sample of 115 individuals. In addition, normal bile samples were included as a control, which, to the best of our knowledge, had not been included in previous similar metabolomic investigations of bile. The metabolomic analytic results demonstrated a clear separation between the disease groups (biliary tract cancer and benign biliary tract diseases) and the normal control group in the PLS-DA model. The results also revealed clear separation between the biliary tract cancer group and benign biliary tract disease group in the metabolomic 2D and 3D score plots. These results reflected the evident efficacy of the present study and the applied model, which was further confirmed using a validated model in the permutation assessment with 200 iterations, R2 intercepts <0.4 and the Q2<0, demonstrating that the model was good-fit and statistically valid.

In diagnosing biliary tract diseases, the differential diagnosis between biliary tract cancer and benign biliary tract diseases is particularly challenging. It is often difficult to provide an exact diagnosis for a patient with biliary tract stricture as biliary tract cancer or long term biliary inflammation can lead to biliary tract stricture (36). The present study aimed to assist in overcoming this clinical difficulty. The OPLS-DA model was further applied to discriminate between the disease groups in the present study and the results revealed that the cancer and benign groups were well differentiated. Furthermore, potential biomarkers with the greatest contribution to this discrimination were then selected from the S-plot according to the VIP values and were identified. The results revealed significantly lower levels of lyso-PC 16:0, lyso-PC 16:0 isomer, lyso-PC 18:0, phenylalanine, 2-octenoylcarnitine, lyso-PC 18:2 and tryptophan and significantly higher levels of GCDCA, touroursocholic acid (TUCA), touroursodeoxycholic acid (TUDCA), glycocholic acid (GCA), TUCA isomer and taurochenodeoxycholic acid (TCDCA) in the bile from biliary tract cancer patients compared with that of patients with benign biliary tract diseases.

Lyso-PC is one of the major lysophospholipids and is mainly generated by hydrolysis of phosphatidylcholine. Phosphatidylcholine reduction has been observed in the bile from patients with biliary tract cancer or cholangiocarcinoma (34,35,37). Phosphatidylcholine is considered to be the major and dominant biliary phospholipid and is essential for membrane structure, signal transduction and lipoprotein metabolism (38). It is synthesized in the hepatocytes and is subsequently transported into the biliary canaliculus by flippase multidrug-resistant protein 3 (39). Phosphatidylcholine is cytoprotective towards the biliary epithelium and reduces the cellular toxicity of bile acids (40,41). The reduction of phosphatidylcholine in the bile exposes the biliary epithelium to 'toxic' bile and predisposes it to biliary malignancies (42). The correlation between lyso-PC and biliary malignancy remains to be elucidated; however, a significant reduction in lyso-PC was observed in the bile of patients with biliary tract cancer patients compared with that of patients with benign biliary tract diseases in the present study. The mechanism underlying this finding requires further elucidation, which following investigations aim to focus on.

Phenylalanine and tryptophan are two of the essential amino acids involved in protein synthesis in the human body, which are aromatic amino acids. No previous studies have investigated the role of phenylalanine in biliary tract cancer, whereas one revealed that the expression of tryptophan hydroxylase 1 increases and monoamine oxidase A decreases in cholangiocarcinoma, resulting in increased secretion of serotonin from the cholangiocarcinoma and increased serotonin in the bile from cholangiocarcinoma patients (43). No other observations have been made regarding tryptophan and biliary tract cancer and the previous tryptophan study did explain the significantly lower level of tryptophan in the bile from patients with biliary tract cancer, which was observed in the present study. Thus, to the best of our knowledge, the present study was the first to demonstrate a correlation between phenylalanine and tryptophan and biliary tract cancer.

Of note, only a few previous studies have investigated 2-octenoylcarnitine and no studies have examined its role in

biliary tract cancer (44,45). In the present study, 2-octenoyl-carnitine was markedly decreased in the bile from patients with biliary tract cancer and was examined as a potential biomarker for biliary tract cancer for the first time, although similar studies are required to repeat and verify the findings and to examine the detailed mechanism.

GCDCA, TUCA, TUDCA, GCA, TUCA isomer and TCDCA are all taurine- or glycine-conjugated bile acids. Compared with the reduced metabolites, a significant elevation of these bile acids in the bile from patients with biliary tract cancer compared with that from patients with benign biliary tract diseases has been observed in previous studies. Sharif *et al* (34) reported that primary bile acids and their glycine-conjugates are significantly increased in patients with cholangiocarcinoma compared with those in benign disease groups. AbdAlla *et al* (35) observed a similar result to that of the present study, with taurine- and glycine-conjugated bile acids significantly elevated in bile from patients with cholangiocarcinoma. It is understood that an imbalance of lipids and bile acids in bile may have a pathogenic role in cholangiocarcinogenesis. Markedly increasing bile acids disrupt the balance and cellular toxicity of bile acids, which may lead to carcinogenesis through oxidative DNA damage and DNA mutation (46-48).

In conclusion, the present study aimed to assist in overcoming the substantial difficulties faced by clinicians in the differential diagnosis between biliary tract cancer and benign biliary tract diseases. The results of the present study demonstrated that the LC/MS-based metabolomic method is a potent and promising approach for discriminating between biliary tract cancer and benign biliary tract diseases and in identifying specific metabolites, which may have potential as a novel biomarkers for the early detection of biliary tract cancer.

Acknowledgements

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