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A new NMR-based metabolomics approach for the diagnosis of biliary tract cancer

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Background & Aims: Biliary tract cancer is highly lethal at presentation, with increasing mortality worldwide. Current diagnostic measures employing multiple criteria such as imaging, cytology, and serum tumor markers are not satisfactory, and a new diagnostic tool is needed. Because bile is a cognate metabolite-rich bio-fluid in the biliary ductal system, we tested a new metabolomic approach to develop an effective diagnostic tool.

Methods: Biles were collected prospectively from patients with cancer ($n = 17$) or benign biliary tract diseases ($n = 21$) with percutaneous or endoscopic methods. Nuclear magnetic resonance spectra (NMR) of these biles were analyzed using orthogonal partial least square discriminant analysis (OPLS-DA).

Results: The metabolomic 2-D score plot showed good separation between cancer and benign groups. The contributing NMR signals were analyzed using a statistical TOCSY approach with verification. The diagnostic performance assessed by leave-one-out analysis exhibited 88% sensitivity and 81% specificity, better than the conventional markers (CEA, CA19-9, and bile cytology).

Conclusion: The NMR-based metabolomics approach provides good performance in discriminating cancer and benign biliary duct diseases. The excellent predictability of the method suggests that it can, at least, increase the currently available diagnostic approaches.

Keywords: Bile; Biliary tract cancer; Metabolomics; Diagnosis.
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Abbreviations: NMR, nuclear magnetic resonance spectra; OPLS-DA, orthogonal partial least square discriminant analysis; STOCSY, statistical total correlation spectroscopy; CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; PTC, percutaneous transhepatic cholangiography; ERCP, endoscopic retrograde cholangiopancreatography; PTBD, percutaneous transhepatic biliary drainage; ENBD, endoscopic nasobiliary drainage.

Introduction

Biliary tract cancer arises from epithelial cells of the intrahepatic and extrahepatic bile ducts. Although this type of cancer is not very common, it is highly lethal, since most are locally advanced at presentation. Its incidence increases with age, and the mortality is increasing worldwide [1–4]. Patients with biliary tract cancer often present painless jaundice, pruritus, and/or anorexia. Hepatic resection and liver transplantation are the only curative options for this cancer, but the recurrence rate is high.

The diagnosis of biliary tract cancer is usually done based on a combination of radiologic, histological, and tumor marker evidence, because each of these approaches alone has drawbacks. Tissue diagnosis, which could confirm the presence of cancer cells, cannot be generally performed due to tumor location, size, and desmoplastic characteristics [5–7]. For example, obtaining tissues through percutaneous fine needle aspiration is frequently not possible, since many of these tumors are located in the liver hilum amid large vascular structures [8,9].

Serum tumor markers, including carbohydrate antigen 19-9 (CA19-9) and carcinoembryonic antigen (CEA), have been used to diagnose biliary tract cancer [10–12]. These proteins are oncofetal antigens found at high levels in the fetal small intestine and gastrointestinal tumors. CA19-9 is mainly used in pancreatic and biliary tract cancer diagnosis, with sensitivities of about 80% and 60%, respectively [10,11]. However, it can also be elevated in other malignancies such as pancreatic, colon, lung, and breast cancers, and other benign conditions such as pancreatitis, bile stasis, cholangitis, and inflammatory bowel disease. CEA is normally found in embryonic entodermal tissues and fetal gastrointestinal tissues, but also elevated in adult cancers, such as pancreatic, stomach, lung, and hepatobiliary cancers [13]. Therefore, these serum markers alone are not sufficient to diagnose biliary tract cancers, and other benign biliary duct complications can compromise their utility [14].

Bile cytology has been used widely for the diagnosis, because bile can be obtained relatively easily with Percutaneous Transhepatic Cholangiography (PTC) and Endoscopic Retrograde CholangioPancreatography (ERCP). However, ERCP cytology alone gives a low sensitivity of 35% [15], and additional brushing step was reported to improve the sensitivity [16]. This brush cytology is now the most common tissue sampling technique and it can be

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performed for most biliary strictures detected by endoscopic cholangiography. Even with the brushing step, the reported sensitivity is still low and variable, with its mean value around 60% [17–20]. Moreover, the additional procedure could increase the risk of infection [21]. Overall, diagnosis of biliary tract cancer, especially, differentiating it from benign clinical conditions, is quite difficult, and new diagnostic approaches are highly needed [22].

Recently, a new "-omics" approach, called metabolomics, has emerged as a promising tool to differentiate individuals in disease or toxic conditions [23]. Compared with other omics approaches, metabolomics deals with smaller molecular metabolites in the body these change depending on the subject's environmental states. It can be applied to any bio-fluid, such as urine, serum, saliva, or bile, and is particularly useful for organs that store or produce small molecular metabolites. Metabolomics can be readily employed for new diagnostic approaches, as first shown in a study with 36 coronary heart disease patients, where it showed its utility as a rapid and non-invasive diagnostic tool with high sensitivity and specificity [23,24]. Metabolomics has subsequently shown promising results in diagnosing several cancers, such as those in breast, ovary, and prostate [25].

Here, we have applied pattern recognition techniques and expert data analysis to NMR spectra of biles taken from individuals with biliary tract cancer or benign biliary tract diseases. The objective of this study was to evaluate the performance of metabolomic diagnosis of biliary tract cancer in comparison with the conventional diagnostic tools including serum tumor markers (CA19-9, CEA) and bile cytology. Our approach gave good distinction between the cancer and benign diseases and better sensitivity and specificity than the other approaches. This metabolomic approach may become a reliable and convenient diagnostic tool for biliary tract cancer.

Patients and methods

Patients

Informed consent was obtained from every patient enrolled in this study and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki. The study was approved before initiation by the Institutional Review Board at the Inha University Medical School and Hospital.

We prospectively obtained bile samples from patients with biliary tract cancer and benign biliary tract diseases at the Inha University Hospital (Incheon, Korea) between January, 2006, and August, 2007. Patients with severe biliary sepsis were excluded from this study. This study included 17 patients with biliary tract cancer and 21 patients with benign biliary tract disease (Table 1). The patient groups were not matched on gender, age or disease stages to maximize patient diversity. There were no exclusion criteria except for biliary sepsis, which severely distorts metabolite profiles but can be easily diagnosed with other methods as reported previously [26].

Assays and bile cytology

Serum CA19-9 and CEA were assayed with an immunoradiometric method and a commercially available ELSA-CA19-9 and ELSA2-CEA (Cisbio International, Bedford, MA). For patients with biliary tract cancer, routine diagnostic procedures included abdominal CT scans or ultrasound. Upon identifying a duct stricture, we performed percutaneous transhepatic biliary drainage (PTBD) or endoscopic nasobiliary drainage (ENBD) as needed.

Sample collection

Bile samples were collected by PTBD, ENBD or during operation. The collected biles were frozen at -80 °C immediately and freeze-dried *in vacuo*. Ten milligrams of the dried samples were re-solubilized into 500 μl of a D_2O + CD_3OD mixture containing 10 mM sodium phosphate (pH 6.0). Insoluble material was removed by centrifugation, and 0.025% TSP was added for chemical shift referencing and normalization.

Table 1. Clinical patient characteristics.

	Biliary tract cancer	Benign biliary tract disease[f]
Clinical parameters	(*n* = 17)	(*n* = 21)
Gender (M:F)	4.7:1	1.3:1
Age, years[a]	70.4 ± 10.6	59.4 ± 15.5
Methods of bile sampling		
PTBD[†]	13	2
ENBD[†]	3	14
Operation	1	5
Cancer stages[b]		
I	Ia: 5, Ib: 2	
II	IIa: 3, IIb: 2	–
III	IIIa: 1, IIIb: 2, 11 Ic: 2	–
Diagnosis of cancer[c]		
Operation[d]	5	–
Operation and Bile cytologie[d]	4	–
Bile cytology[d]	3	–
Clinical and radiological[e]	5	–

Out of 17 cancer patients, 9 patients were diagnosed by operation (Operation (5) + Operation and Bile cytology(4)). In addition, three un-operated patients showed positive in cancer cells in the drained bile. Therefore, total of 12 patients (71%) were diagnosed either by operation or bile cytology. Overall, the sensitivity of the bile cytology was 41%. For the rest of the cancer patients (five, 29%), histological examination (bile cytology, brushing cytology, guided fine needle aspiration) could not detect cholangiocarcinoma. However, radiological (cholangiography and abdominal CT), and clinical (obstructive jaundice, weight loss, abdominal pain or incidental abdominal mass detection) evidence justified the diagnosis of cholangiocarcinoma. Moreover, all of the patients died of cancer progression within one year of diagnosis, which gave additional support to our diagnosis.

[†] PTBD, Percutaneous transhepatic biliary drainage; ENBD, Endoscopic nasobiliary drainage.

[a] Values expressed as the mean + SD (range).

[b] According to American joint committee on cancer staging manual (2002, 6th Edition, Springer).

[c] All of the patients died of cancer progression within one year of diagnosis.

[d] These represent the gold standard of the biliary duct cancer diagnosis.

[e] Radiological evidence includes cholangiography and abdominal CT. Clinical evidence includes obstructive jaundice, weight loss, abdominal pain or incidental abdominal mass detection.

[f] One cancer patient had been treated with intrahepatic duct stone before the cholangiocarcinoma.

NMR measurements

All spectra were obtained by an NMR spectrometer (Bruker Biospin Avance 500) operating at a proton NMR frequency of 500.13 MHz. The acquisition parameters were essentially the same as previously reported [27,28]. The time domain data were Fourier transformed, phase corrected, and baseline corrected manually. This study made use of the NMR facility at Korea Basic Science Institute, which is supported by Bio-MR Research Program of the Korean Ministry of Science and Technology (E28080).

Metabolomics data analysis

To reduce the complexity of the NMR data for pattern recognition, the spectra were binned with 0.04 ppm width using an in-house Perl program. The signals were normalized against total integration values, and then, 0.025% TSP signal. The water and methanol regions were excluded. The numeric data were imported into statistical software. Matlab (MathWorks, Natick, MA), SIMCA-P version 11.0 (Umetrics, Sweden), Chenomx (Spectral database; Edmonton, Alberta, Canada) and Excel (Microsoft, Seattle, WA) programs were used for data analysis. Orthogonal projections to latent structure-discriminant analysis (OPLS-DA) were performed to distinguish cancer and benign patient groups. The statistical validation was performed using "*Y*-scrambling" validation, where the class mem-

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bership was shuffled 200 times randomly, and the resulting Q^2 and R^2 values were calculated. Prediction of the unknown samples was carried out by leave-one-out analysis, as reported previously [29]. The conceptual explanation of these methods is given in Supplementary material S4.

Results

Patient characteristics

The biliary tract cancer group included 13 Klatskin tumors, two CBD cancers, one gallbladder cancer, and one intrahepatic cholangiocarcinoma. There were 17 bile duct stones, two benign biliary strictures, one choledochal cyst, and one other disease in the benign biliary tract group. Patient characteristics of the two groups were different because of the epidemiology of biliary tract cancer (Table 1). Bile sampling was also different between the two groups because treatment options for the two groups differed.

NMR spectra and multivariate analysis

We obtained NMR spectra of bile samples from both patient groups. The general spectral features were similar, with large peaks in the aliphatic region (2.3–0.8 ppm) corresponding to the bile acids, cholesterol, fatty acids, and other lipid components, present abundantly in bile (Fig. 1). To analyze the NMR data holistically and to establish the prediction model for biliary tract cancer, we applied OPLS-DA multivariate analysis to the NMR data. The results revealed that analysis with signals upfield of 6.0 ppm gave better separation (data not shown), probably due to the aliphatic nature of the bile components. Therefore, we performed the subsequent analysis with the 0–6.0 ppm region signal. The OPLS-DA distinction model was obtained using one predictive (P_p) and four orthogonal components (P_o) (Fig. 2). The majority of the normal and cancer samples appear clustered in their respective regions with only a few overlaps between them. The model featured an overall goodness of fit, $R^2(Y)$, of 95% and an overall cross-validation coefficient, $Q^2(Y)$, of 91%. Out of the overall $R^2(X)$ value of 0.87, 60% was structured but uncorrelated to the response, and 27% was predictive. These results show that there is considerable variation within each group, but that our model can reliably differentiate between them, even with large structured noise.

Statistical TOCSY analysis

With the efficient separation of the cancer and benign groups, we further identified the variables responsible for the classification rules. We utilized statistical total correlation spectroscopy (STOCSY), which can show the modeled correlation $(P(\mathrm{corr})_p)$ as NMR lines, enabling straightforward interpretation of the variable contributions [27,30,31]. The STOCSY plot (Fig. 3) shows that important contributions for the separation come from signals at 1.50 (1), 1.06 (2), and 3.70 (3) ppm, which correspond to $-CH_2-$, $-CH_3-$, and $-CH_n-OR$ moieties that are common in bile acids. Therefore, differences in the bile acid composition are importantly related to the class differentiation. However, the $P(\mathrm{coor})_p$ values indicate that variations in these signals are not entirely responsible for the class difference. This is not very surprising, considering a previous report on coronary heart disease [23]. There, only 20–30% of the variance of the most important variables was related to the heart disease risk, but very high sensitivity and specificity were still obtained. Therefore, the remaining variations in our case should result from subtle individual chemical differences in bile acids, such as the position of the double bonds and bile-metabolite conjugation.

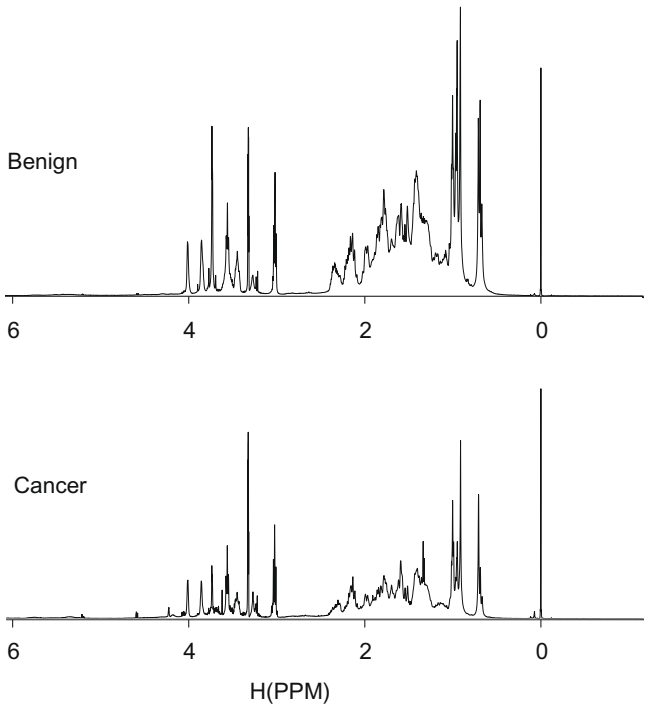

Fig. 1. Representative 500 MHz ^{1}H-NMR spectra of bile samples from a benign biliary tract disease patient (top) and a biliary tract cancer patient (bottom). The spectra were taken for samples in 500 μl of D_2O + CD_3OD mixture containing 10 mM sodium phosphate (pH 6.0) and 0.025% TSP as a chemical shift reference.

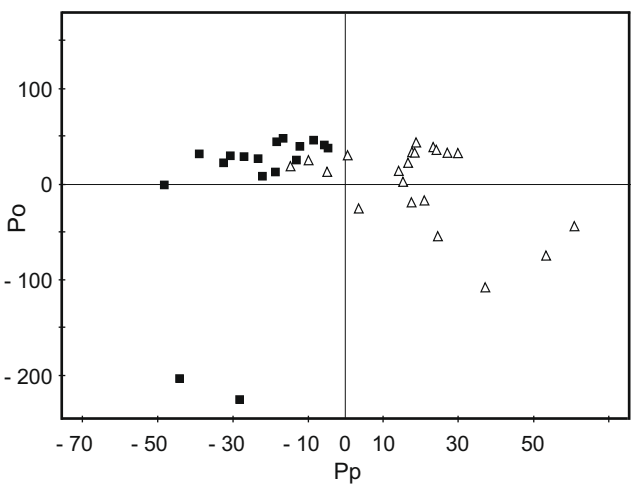

Fig. 2. Orthogonal projections to latent structure-discriminant analysis (OPLS-DA) score plot of benign and cancer samples. Open triangle: Benign samples; Filled box: Cancer samples. The model was obtained using one predictive and four orthogonal component, with $R^2(Y)$ of 95% and $Q^2(Y)$ of 91%. P_p represents the predictive component and P_o represents the orthogonal component.

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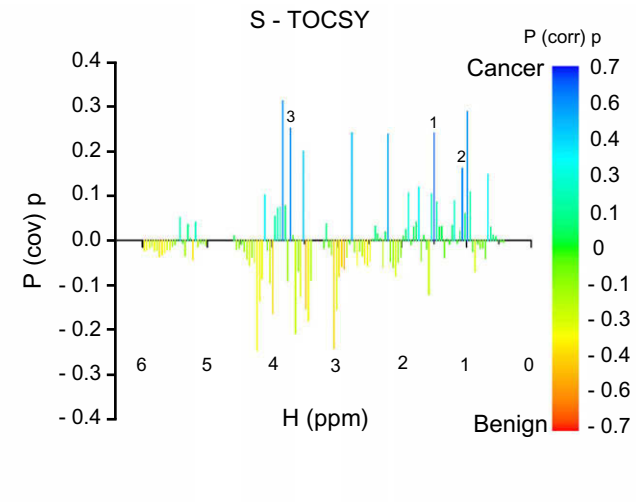

Fig. 3. Variable contributions from statistical total correlation spectroscopy (STOCSY). The color scale on the right indicates $P(\text{coor})_p$. The $P(\text{cov})_p$ represents the modeled covariance and $P(\text{coor})_p$ represents the modeled correlation. Peaks with labels are mentioned in the text (1: 1.50 ppm, 2: 1.06 ppm, 3: 3.70 ppm).

Statistical validation

The separation result of the cancer and normal patients was subjected to "*Y*-scrambling" statistical validation to test the possibility of chance correlation. We randomly permutated the *Y*-variable (cancer or benign group designation), re-built the statistical model, and observed the trends of the predictive power and goodness of fit at each step. Two hundred rounds of such reshuffling gave coherent decreases in both parameters and the extrapolated value of the Q^2 of −0.3 (Fig. 4), which shows that the separation model is statistically sound, and that its high predictability is not due to over-fitting of the data. Although the current study may not cover all the possible variations in the patients, such as the bile duct obstruction time, we believe our validation through randomization of the *Y*-variable suggests that those variations should be orthogonal to, and thus not be a major factor for our differentiation between the cancer and normal groups. Unrelated variations were most likely partitioned into the orthogonal components of the prediction model and, thus, should not affect the predictability.

Among the unrelated variations, gender and age could provide a large source of variation that may affect the differentiation. Therefore, we analyzed the patient data in subgroups that are

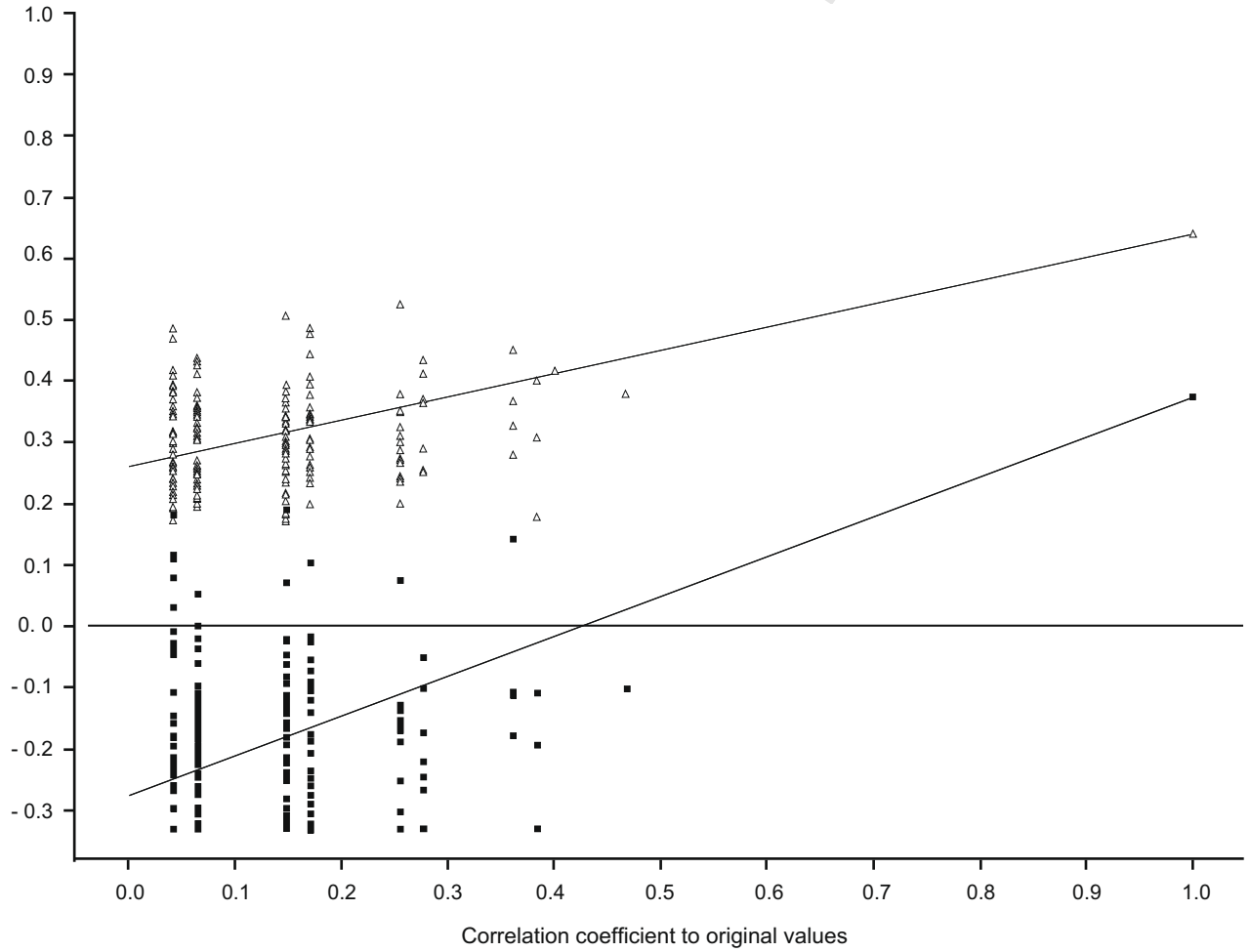

Fig. 4. Statistical validation of the OPLS-DA analysis result by "*Y*-scrambling". Two hundred permutations were performed, and the resulting R^2 and Q^2 values were plotted. Open triangle: R^2; Filled square: Q^2. The solid line represents the regression line for R^2 and the dashed line for Q^2.

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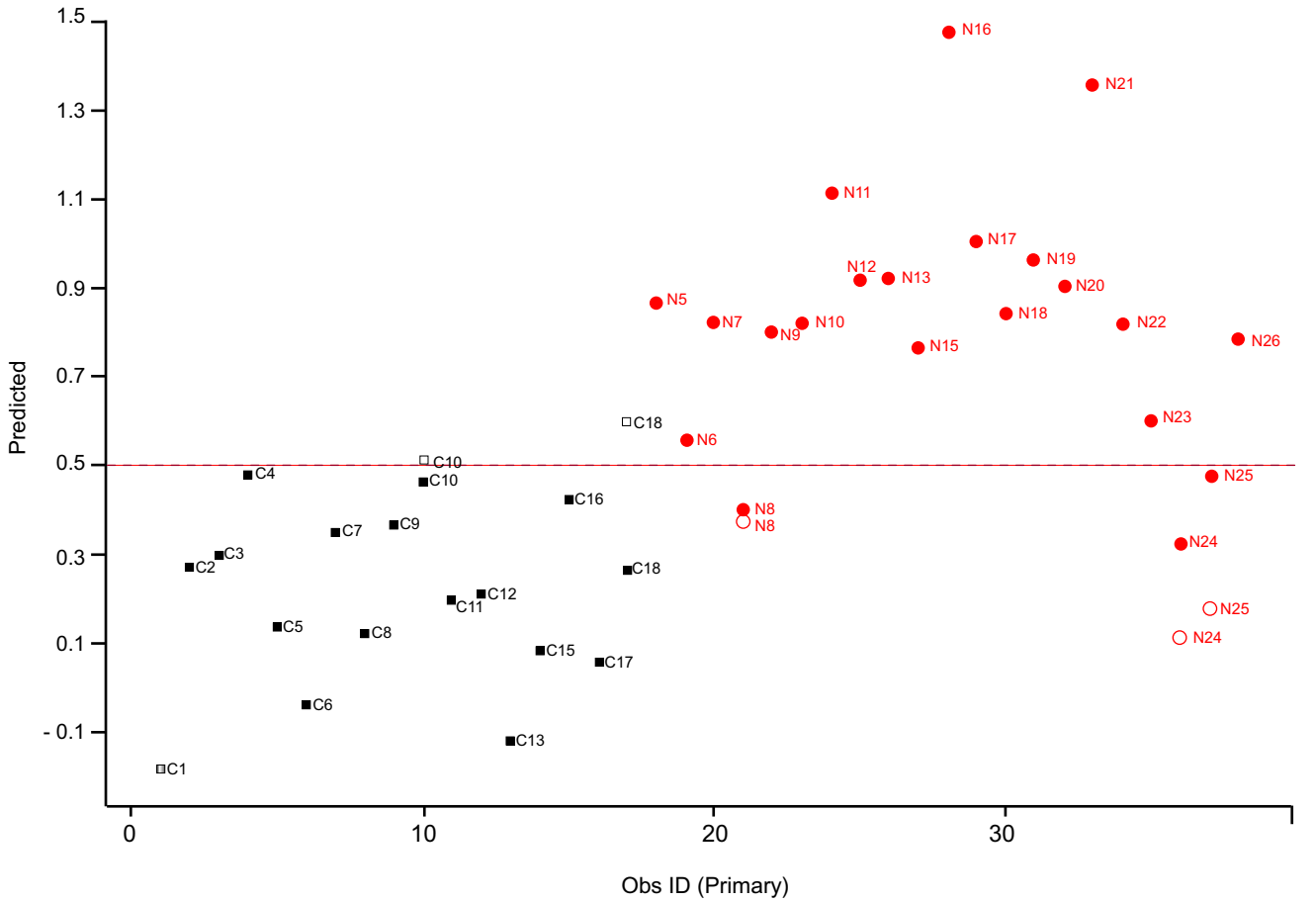

Fig. 5. Prediction of cancer and benign patients using leave-one-out analysis. One patient sample (unknown) was left-out at a time and an OPLS-DA prediction model was constructed with the rest of the data. The class membership of the left-out samples was predicted using an *a priori* cut-off value of 0.5 (dashed line) [23]. Cancer samples: black box; Benign samples: Red circle. The *Y* values of the filled symbols are from the analysis using the entire dataset. In the case of mis-classified samples, the predicted *Y* values from the leave-one-out analysis are also shown as open boxes (cancer patients) and open circles (normal patients).

not affected by these biases. First, we performed the differentiation with only male patients, as the cancer group is primarily male and the benign group has relatively even distribution. The actual result (see Supplementary Fig. S1A) exhibited very similar differentiation as our original model with all the patients (see Fig. 2), which confirms that our original differentiation is not based on the gender. If the original model had been influenced by the gender, the male-only analysis should have given much poorer, or even no, discrimination between the cancer and benign groups. We also tested the influence of age in our model. In separate analyses with younger (see Supplementary Fig. S1B) and older groups (see Supplementary Fig. S1C), the differentiation of cancer and benign groups were even better than the one with all the patients (see Fig. 2). As stated above, if our original model had been influenced mainly by age, the differentiation should have been much worse in each subgroup. These results again confirm the validity of our OPLS-DA approach which can effectively exclude these possible confounding factors in differentiating the groups based on the feature of interest (cancer status, in our case).

Prediction and diagnostic performance test

To estimate the actual performance of our OPLS-DA model in diagnosing biliary tract cancer, we performed a leave-one-out predictive test. For this, we left-out one patient sample at a time and constructed the OPLS-DA prediction model with the rest of the data (a training set). The prediction model was constructed with the same number of predictive and orthogonal components as the original OPLS-DA classification model. The class membership of the left-out sample was predicted using an *a priori* cut-off value of 0.5. This procedure was repeated until each and every sample had been tested once. Of the 21 benign disease samples, 18 were predicted correctly as benign, and of the 17 cancer samples, 15 were predicted correctly as cancer (Fig. 5). Therefore, our OPLS-DA metabolomics prediction model exhibited a sensitivity of 88% and a specificity of 81% for biliary tract cancer diagnosis, which is significantly better than conventional serum markers or cytology (Table 2).

Discussion

Biliary tract cancer is highly lethal and only surgical excision of the tumor can improve survival [32–35]. However, biliary tract cancer is often presented locally advanced, and the majority of the patients are elderly, with critical co-morbidity which increases the risk of operation. In this respect, it has been suggested that neither more advanced surgical techniques nor radi-

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Table 2. Comparison of the diagnostic performance between conventional and metabolomic.

Criteria [Reference]	CA19-9** [22]	CEA† [9]	Bile cytology [15,20]	Metabolomics [current study]
Sensitivity	81% (73%)	20% (68%)	41% (35–61††%)	88%
Specificity	53% (63%)	100% (82%)	N/A	81%

* The numbers indicate the values obtained from the patients enrolled in the current study. The numbers in the parenthesis are from the literature.
** Cut-off value of >37 U/mL (both reference and our study).
† Cut-off value of >5.2 ng/mL in primary sclerosing cholangitis patients and cut-off value of 6.0 ng/mL in our study.
†† 61% was obtained using brush cytology.

ation therapy is likely to improve survival [36]. Therefore, currently, efforts are being directed to prevention and reliable detection. However, current diagnostic tools, such as serum tumor markers and bile cytology, have limited utility for differentiating cancer and benign diseases, and new diagnostic methods are highly needed.

Here, we applied a metabolomics approach to biles obtained directly from patients in order to assess its accuracy and reliability in diagnosing biliary tract cancer. Our approach showed better performance in terms of both specificity and sensitivity than conventional data obtained from literature and our own patients (Table 2). Although CEA showed perfect specificity for our patients, its utility was significantly compromised due to its poor sensitivity. In general, sensitivity is more important than specificity in serious diseases such as cancer. Bile cytology, in theory, can deliver perfect specificity, as it directly observes cancer cells in the samples, but its reported sensitivity is rather poor to range between 35% and 40% [15,37]. We also obtained 41% sensitivity using bile cytology for our patients. Although brushing has been shown to increase cytology sensitivity by about 20% [17–20], it requires additional invasive steps such as ERCP or EST, which could increase the risk of pancreatitis [21]. The final sensitivity of brush cytology is still about 60%, significantly lower than our metabolomics results. Our metabolomics approach gave high values for both sensitivity and specificity. Therefore, we believe that the metabolomic diagnosis may be more clinically useful than conventional techniques in biliary tract cancer diagnosis. Obviously, as with any other new diagnostic approaches, there are limitations to our study. One such possibility is the effects of biliary infection without clinical evidence of sepsis on the metabolic profiles. This potential confounding factor, though, can be diagnosed by culture or PCR, and therefore, may be an interesting subject for later studies.

To get deeper insights into the metabolic difference, we analyzed our data with targeted metabolic profiling for four metabolites involved importantly in energy metabolic pathways: choline, lactate, citrate and glucose. We used student's independent *t*-test to see if the contents of these metabolites are statistically different between cancer and benign groups (see Supplementary Fig. S2). While choline ($p > 0.85$), lactate ($p > 0.79$), and glucose ($p > 0.24$) did not show any relevant differences, citrate level was statistically higher in cancer groups ($p < 0.05$). The higher content of citrate is interesting, as it is the starting molecule of the TCA cycle, the hallmark of the aerobic energy metabolism. Citrate is formed through a condensation reaction between oxaloacetate and acetyl CoA. The latter is also the precursor of the cholesterol which is metabolized into bile acids. The higher level of citrate in the cancer group might result from the low dependence of the cancer cells on the aerobic energy metabolism consuming citrate in the TCA cycle, consistent with the Warburg effect in cancer cells. High level of citrate is expected to affect the concentration of acetyl CoA, its immediate precursor, which in turn can affect the bile acid formation. As citrate also has $-CH_2-$ group, this is consistent with our initial suggestion on contributing signals .Although confirmation of the above will require detailed flux analysis of all the involved pathways, our metabolomic data provide an interesting initial evidence for the link between the energy metabolism and bile acid compositions in the cancer group.

In addition to our main goal of differentiating cancer and benign patients, we also tested if our approach can differentiate the various stages of the biliary duct cancer. Although individual differentiation of stages I, II, and III were not satisfactory (data not shown), we obtained a good separation between stages I and II combined against stage III (see Supplementary Fig. S3). Although the number of patients is not large for each group, these data suggest that it may be possible to differentiate between relatively early (I and II) and later stage biliary duct cancers with our metabolomic approach.

It should be noted that our metabolomics approach is "non-invasive", as it uses bile that had been drained for therapeutic purposes and required no separate collection steps. In contrast, brush bile cytology requires additional steps, and serum markers require blood drawing. This fact also alleviates ethical problems, the inconvenience of additional visits, or pain for sample collection for patients, providing a more convenient option.

An efficient diagnostic method is best developed with tissues or bio-fluids that are cognate to the organs of interest. For example, urine metabolites have been used to predict kidney cancer or allograft rejection [29,38]. Here, we used bile for biliary duct cancer diagnosis. Bile passes through the biliary duct before being secreted into the intestine, during which time it has direct contact with any surrounding cancer tissue. Especially in obstructive bile duct diseases, such as those targeted in this study, bile stays in the ducts for a long time, thus likely reflecting differences in the ductal epithelial cells. Conventional serum markers, such as CA-19-9 and CEA are detected from serum and, therefore, could reflect changes in other tissues, including colon or pancreatic cancers. Bile cytology, although using bile, may not always be able to retrieve cancer cells from the tissue, resulting in low sensitivity. Therefore, bile metabolomics seems more theoretically relevant for the biliary tract cancer diagnosis than those approaches.

Currently, biliary tract cancer is diagnosed by multiple criteria based on computerized tomography, magnetic resonance imaging, bile cytology, endoscopic ultrasonography, serum markers, and positron emission tomography. Our study shows that a metabolomics approach, by itself, can differentiate biliary tract cancer from benign diseases with high reliability. To the best of our knowledge, this study is the first report of a metabolomics diagnostic approach in the human hepatobiliary system outperforming other conventional clinical criteria. A study with larger patient groups and standardized protocols could eventually lead to a dependable diagnostic tool for biliary tract cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2009.11.002.

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