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ORIGINAL ARTICLE

Metabolic profiling of bile in cholangiocarcinoma using *in vitro* magnetic resonance spectroscopy

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Abstract

Objectives: Cholangiocarcinoma (CCA) has a poor prognosis and its aetiology is inadequately understood. Magnetic resonance spectroscopy (MRS) of bile may provide insights into the pathogenesis of CCA and help identify novel diagnostic biomarkers. The aim of this study was to compare the chemical composition of bile from patients with CCA with that of bile from patients with benign biliary disease.

Methods: Magnetic resonance spectra were acquired from the bile of five CCA patients and compared with MRS of control bile from patients with benign biliary disease (seven with gallstones, eight with sphincter of Oddi dysfunction [SOD], five with primary sclerosing cholangitis [PSC]). Metabolic profiles were compared using both univariate and multivariate pattern-recognition analysis.

Results: Univariate analysis showed that levels of glycine-conjugated bile acids were significantly increased in patients with CCA, compared with the benign disease groups ($P = 0.002$). 7β primary bile acids were significantly increased ($P = 0.030$) and biliary phosphatidylcholine (PtC) levels were reduced ($P = 0.010$) in bile from patients with CCA compared with bile from gallstone patients. These compounds were also of primary importance in the multivariate analysis: the cohorts were differentiated by partial least squares discriminant analysis (PLS-DA).

Conclusions: These preliminary data suggest that altered bile acid and PtC metabolism play an important role in CCA aetiopathogenesis and that specific metabolites may have potential as future biomarkers.

Keywords

bile acids, cholangiocarcinoma, metabonomic, magnetic resonance spectroscopy, phosphatidylcholine

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Introduction

Cholangiocarcinoma (CCA) is the leading cause of death from a primary liver tumour in many developed countries.[1,2] Although a relatively rare cancer, the incidence and mortality of CCA have been increasing on a global scale for reasons which remain to be determined.[1–3] Cholangiocarcinoma has a high mortality and poor prognosis, with a 5-year survival of <5%, as a result of its late clinical presentation. Consequently, most tumours are at an advanced stage at the time of diagnosis.[3]

Confirmation of CCA diagnosis is often difficult and is currently based on a high index of clinical suspicion, depending on serum levels of the cancer antigen tumour marker, carbohydrate antigen (CA) 19-9, and hepatobiliary imaging, with additional histological or cytological verification if tumour tissue or bile can be reliably obtained. Although in widespread use, serum CA19-9 has a poor sensitivity (50–60%) for the diagnosis of CCA.[4,5] Cytological examination of bile also has a poor detection rate for malignant biliary disease.[6] Despite improvements in biliary cytological examination using digital image analysis (DIA) and fluorescent *in situ* hybridization (FISH), the ability to distinguish

benign from malignant ductular epithelium remains suboptimal.[7] Tissue biopsies often yield negative findings as a result of marked fibrosis and because no pathognomonic immunohistochemistry exists.

In order to improve diagnosis and prognosis, there is a pressing need to identify biological markers for early disease detection, as well as to enhance the understanding of disease aetiopathogenesis. Sampling of bile for diagnostic purposes has become common clinical practice since the introduction of endoscopic retrograde cholangiopancreatography (ERCP). Exposure of the biliary epithelium to bile and its constituents make bile an ideal biofluid for analytical profiling studies in malignancy of the biliary tract. The analysis of the metabolic profile of bile may therefore provide insights into the pathogenesis of CCA, as well as identifying biochemical disease markers.

Magnetic resonance spectroscopy (MRS) is a non-invasive and sensitive analytical technique which can determine both chemical composition and molecular structural information from non-homogeneous biological samples without *a priori* knowledge. Specific metabolites of interest may be quantified and analysed using conventional univariate statistical methods; the data may also be analysed using a multivariate, pattern-recognition approach with techniques such as principal components analysis (PCA) and partial least squares discriminant analysis (PLS-DA). This has been termed a 'metabonomic' or 'metabolomic' approach.[8] *In vitro* MRS studies on bile have provided information on composition, structure and function, as well as on the metabolism and biliary excretion of xenobiotics.[9–11] A major advantage of the technique is that the sample can be studied intact, which allows for subsequent study as required. Recently, MRS studies on bile in patients with pancreatic carcinoma observed an alteration in bile composition and also identified a potential cancer biomarker.[12]

However, bile collected at ERCP is frequently contaminated by the contrast agent used; two previously published studies investigating the metabolic composition of bile from CCA patients using MRS were flawed by such contamination, which resulted in dominating spectral resonances that may confound the measurement of metabolites.[11,13] A secondary major drawback of these studies is that bile samples were analysed from patients with marked cholestasis, which could partially account for the spectral differences observed.

In this study, uncontaminated bile was analysed from non-cholestatic patients. Our aims were to assess and quantify differences in the chemical composition of bile from patients with cancer of the biliary tree, compared with bile from patients with benign biliary disease, using *in vitro* proton (^{1}H) MRS. The predominant lipid metabolites, bile acids and phosphatidylcholine (PtC), were selected for specific study, owing to their proposed role in cholangiocarcinogenesis.[11,14,15] A further aim was to identify potential disease markers in bile in order to improve the diagnosis and prognosis of CCA.

Materials and methods

This study was approved by the Research Ethics Committee of Hammersmith Hospital, London (HHREC no. AM1073/0086). The study conformed to the ethical guidelines outlined in the 1975 Declaration of Helsinki. Written, informed consent was obtained from all patients. Four millilitres of contrast-free bile were obtained at ERCP, after an overnight fast, from 25 patients with malignant and benign conditions of the biliary tree.

MR sample preparation

Bile samples, stored at –80 °C and protected from light, were thawed to room temperature and pH was measured; 600 µl of bile were then transferred to a 5-mm glass nuclear magnetic resonance (NMR) tube. A sealed 4-mm stem coaxial NMR insert, containing 50 µl of an internal reference standard solution (35 µl of sodium trimethylsilyl-[$^2H_4$] propionate [TSP] 1 mg/ml dissolved in deuterium oxide), was placed inside the NMR tube.

MR data acquisition

In vitro ^{1}H MRS was performed using an ECP+ 500-Mhz NMR spectroscopy system (JEOL Instruments, Tokyo, Japan) and an 11.7-Tesla superconducting magnet. The ^{1}H MR spectra were obtained using a pulse-collect sequence (90-degree pulse angle, acquisition duration 4.4 s, relaxation delay 20 s, 32 data collects), in combination with a water presaturation technique to reduce the dominant water signal. Magnetic resonance spectra were also acquired using the Hahn spin-echo (relaxation delay 2 s, time to echo 135 ms, 64 data collects) to aid metabolite peak assignments caused by the phase inversion of some of the coupled signals.

Spectral interpretation and analysis

The data were processed using the KnowItAll Informatics System Version 7.9 (Bio-Rad Laboratories, Philadelphia, PA, USA). Free induction decays were zero-filled, and multiplied by an exponential line-broadening function of 0.8 Hz and then subjected to Fourier transformation. The MR spectra were manually phased and a baseline correction was applied. Peaks were assigned on the basis of published literature relative to TSP ($\delta = 0.00$ ppm).[16] The cluster of peaks δ 0.70–1.10 ppm was assigned as one region with contributions from H-19 bile acid proton, H-21 bile acid proton and cholesterol. The peaks δ 3.09 ppm and δ 3.57 ppm were assigned to the taurine moiety in taurine-conjugated bile acids. The peak δ 3.71 ppm was assigned to the glycine moiety of glycine-conjugated bile acids. The peaks δ 3.24 ppm and δ 1.29 ppm were the most prominent resonances attributable to PtC, and assigned to the choline head group and $(CH_2)_n$ chain, respectively.

For relative quantification, peak areas were manually integrated and expressed in arbitrary units (u), as a percentage ratio to the total spectral signal (range δ 10.00–0.20 ppm). The residual water signal (range δ 5.20–4.50 ppm) was excluded from the analysis.

Univariate statistical analysis was carried out using SPSS for Windows, Version 14.0 (SPSS, Inc., Chicago, IL, USA). The non-parametric Kruskal–Wallis test was used for comparisons of metabolite concentrations between the different disease groups. The Mann-Whitney *U*-test was used for comparisons between two independent disease groups.

Multivariate pattern-recognition analysis

Using an 'intelligent bucketing' algorithm in KnowitAll Version 7.9, each spectrum was divided into smaller regions (bins), of 0.04 ± 0.02 ppm, ensuring no overlap across binned regions. These regions were integrated and normalized to the total spectral signal and the data were mean-centred before multivariate analysis.

Principal component multivariate analysis

Principal component analysis is an 'unsupervised' technique in that it does not rely on *a priori* knowledge of the cohort to which samples belong. It facilitates the visualization of a multivariate dataset through data reduction, to identify and visualize inherent patterns of variance within the dataset. The first principal component is essentially a linear combination of the original variables, explaining the maximum amount of variance in the dataset; the second principal component describes the second greatest, and so on. Each sample is represented in the PCA scores plot generated; inspection of the corresponding loadings plot allows visualization of the metabolites responsible for the variation seen in the scores plot.

Partial least squares discriminant analysis

Data were then analysed by PLS-DA using Pirouette Version 4.0 (Infometrix, Inc., Woodinville, WA, USA). This linear regression technique relates the NMR spectroscopic variables, corresponding to metabolites, to the class membership of the sample, allowing the identification and visualization of metabolites responsible for differences between classes. It is thus 'supervised'. The data filtering technique of orthogonal signal correction (OSC) was used to remove variation in the spectra not directly related to the physiological condition being studied, and to minimize the possible influence of inter-individual variation.[17,18] The discriminatory power of each model was validated using a cross-validation technique whereby each sample in turn was excluded from the analysis, a model was created from the other samples and the class membership of the excluded sample was predicted ('leave-one-out' cross-validation).[19,20]

Results

Patient demographics

Five inoperable CCA patients (four male, one female; mean age 73 years, range 58–82 years) were recruited. Three had hilar bismuth grade 3 tumours and bile was collected downstream of the lesion. Two had distal tumours and bile was collected upstream. Of the five CCA patients, only two had an elevated CA19-9 (240 U/ml and 26 954 U/ml, respectively). Histological diagnosis was confirmed in two patients. Diagnosis in the other three patients was based on clinical presentation and imaging findings. Twenty patients with non-malignant biliary disease (seven with choledocholithiasis [gallstones], eight with sphincter of Oddi dysfunction [SOD] and five with primary sclerosing cholangitis [PSC] with biliary strictures) were included in the study. Importantly, bile samples were stratified according to ambient serum bilirubin levels. The mean serum bilirubin level for all the bile samples analysed was 16.2 mmol/l (standard deviation 13.2 mmol/l). The normal reference range was 3–17 mmol/l. The clinicopathological details of patients are summarized in Table 1. The age and gender of the cohort were not normally distributed. The four groups were therefore analysed separately and in combination.

Proton NMR spectroscopy of bile samples

The human bile ^{1}H MR spectrum is dominated by broad resonances arising from bile acids, phospholipids and cholesterol. A representative ^{1}H MR spectrum of bile from a patient with CCA is shown in Fig. 1. The region of interest δ 3.00–4.00 ppm has been expanded to illustrate the assignment to resonances arising from taurine- and glycine-conjugated bile acids (Fig. 2).

Univariate analysis

Biliary levels of glycine-conjugated bile acids (δ 3.71 ppm, relative to the total spectral integral) were significantly higher in patients with CCA than in the benign biliary disease groups (P = 0.002, Kruskal–Wallis test). The level of these glycine-conjugated

Table 1 Clinico-pathological details of patients and bile samples (mean and range). Kruskal–Wallis tests showed significant differences between the four disease groups according to age (P = 0.011) and gender (P < 0.001)

	CCA	Gallstones	SOD	PSC
Age, years	73 (58–82)	64 (45–79)	47 (27–62)	45 (28–66)
Sex, M : F	4 : 1	0 : 7	0 : 8	5 : 0
Bilirubin, μmol/l	16 (8–31)	22 (5–57)	10 (2–33)	19 (8–42)
ALP, IU/l	637 (69–2423)	222 (76–426)	96 (50–130)	242 (63–325)
pH of bile	7.80 (7.26–8.40)	7.80 (7.50–8.10)	7.78 (6.8–8.45)	8.02 (7.50–8.70)

CCA, cholangiocarcinoma; SOD, sphincter of Oddi dysfunction; PSC, primary sclerosing cholangitis; M, male; F, female; ALP, alkaline phosphatase

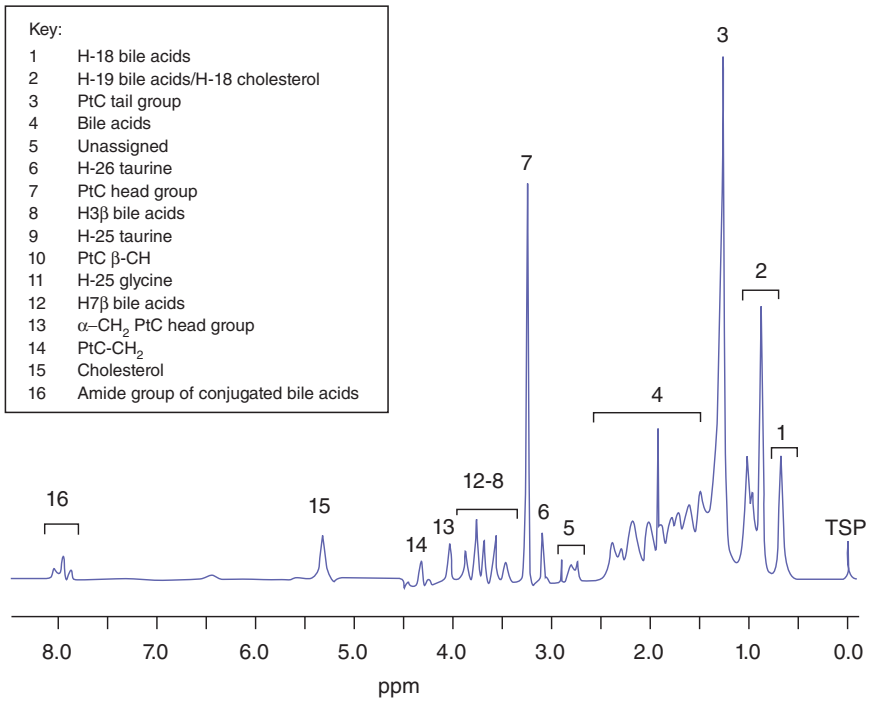

Figure 1 Typical 500 MHz proton MR spectrum of bile from a patient with cholangiocarcinoma illustrating the predominant metabolites

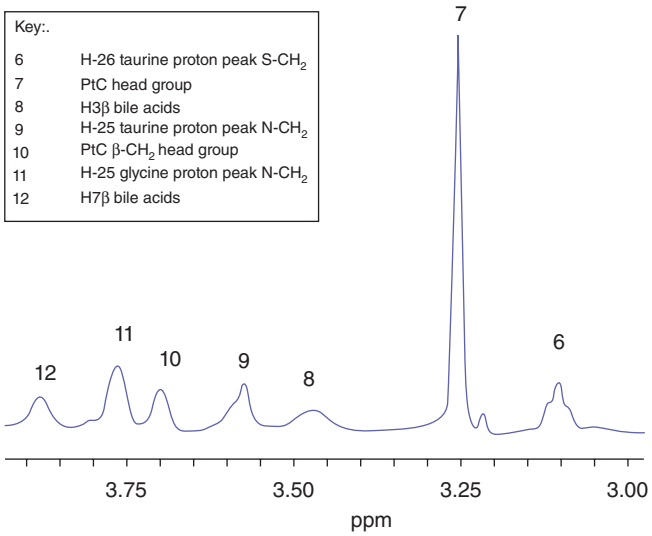

Figure 2 Relevant peak assignments in the proton MR spectrum of CCA bile in the expanded region 3.00–4.00 ppm

bile acids was higher in the CCA group than in patients with SOD and PSC, at a median of 1.00 u (interquartile range [IQR] 0.78–1.59 u) vs. 0.70 u (IQR 0.47–0.85 u) ($P = 0.030$) and 0.56 u (IQR 0.44–0.74 u) ($P = 0.056$), respectively. By contrast, there was no difference in the levels of taurine-conjugated bile acids (δ 3.57 ppm) across the patient groups ($P = 0.12$, Kruskal–Wallis test). However, the relative ratio of glycine- to taurine-conjugated bile acids was greater in the CCA group, at 1.11, than in the other patient groups, in which it was reduced to 0.82 in the SOD group, 0.64 in the PSC group and 0.97 in the gallstones group. Median levels of the 7β primary bile acids (cholic acid and chenodeoxycholic acid, δ 3.45 ppm) were significantly elevated in the bile of patients with CCA vs. that of patients with gallstones: median 0.76 u (IQR 0.56–1.32 u) vs. 0.44 u (IQR 0.09–0.71 u) ($P = 0.030$). Levels of the peaks assigned to the choline moiety of the PtC head group δ 3.24 ppm and the $(CH_2)_n$ tail group δ 1.29 ppm were significantly lower in bile from patients with CCA compared with bile from the gallstone control group, at a median of 4.03 u (IQR 3.94–4.66 u) vs. 4.61 u (IQR 4.32–6.09 u) ($P = 0.018$) and a median of 24.99 u (IQR 22.68–27.06 u) vs. 29.08 u (IQR 25.10–34.61 u) ($P = 0.010$), respectively.

Multivariate pattern-recognition analysis

Principal component analysis of all samples revealed no significant outliers. The analyses demonstrated the most notable clustering when CCA samples were compared with SOD samples (Fig. 3). Models were then constructed using OSC-PLS-DA to investigate the ability of this technique to distinguish between the cohorts studied. Using this method, with 'leave-one-out' cross-validation, the CCA samples were discriminated from all 20 non-malignant samples with a sensitivity of 80%, specificity of 95%, positive predictive value of 80% and negative predictive value of 95% (Table 2) (one CCA sample and one non-cancer patient were incorrectly predicted). The major metabolites contributing to this

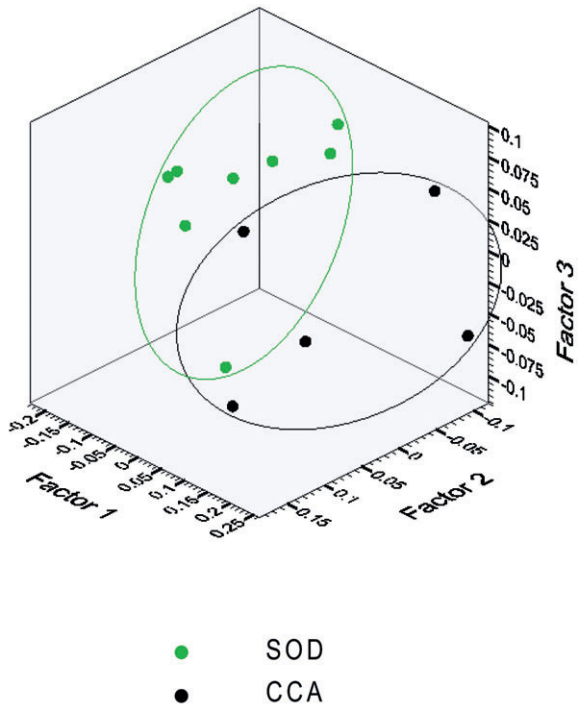

Figure 3 Principal component analysis demonstrated the most notable clustering when cholangiocarcinoma (CCA) samples were compared with sphincter of Oddi dysfunction (SOD) samples

separation were PtC, H-18 bile acids (resonance δ 0.70 ppm) and taurine-conjugated bile acids.

Other OSC-PLS-DA models were also constructed, comparing CCA with each of the control groups: the excellent discrimination achieved is demonstrated in Table 2, which shows the predictive abilities of the models constructed. The major discriminatory metabolites for these models were: glycine- and taurine-conjugated bile acids (CCA vs. PSC); PtC and taurine-conjugated bile acids (CCA vs. gallstones), and PtC (CCA vs. SOD).

Discussion

In this MR study, 25 contrast-free, non-cholestatic bile samples were analysed and compared using ^{1}H MRS. Previous studies have been hampered by the contamination of samples with contrast agent, and by the marked cholestastic nature of the bile samples studied.[11,13] Despite a small and heterogeneous sample population, our data demonstrated important differences in the biliary metabolic profile in comparisons between malignant and benign biliary disease. The patient study groups could be clearly distinguished using OSC-PLS-DA. The major findings of this study were significant differences in the levels of primary bile acids, their glycine conjugates and PtC in bile from patients with CCA compared with bile from non-malignant control groups. When analysed using univariate statistical methods, normalized signal levels of glycine-conjugated bile acids were significantly higher in CCA bile, compared with controls; these metabolic differences contributed strongly to the PLS-DA models and thus corroborated the findings. The greatest difference was seen when comparing CCA bile with SOD bile. It is also worth noting that the ratio of glycine- to taurine-conjugated bile acids was greater in the CCA bile compared with control bile. The ratio of conjugated to unconjugated bile acids, and the ratio of glycine to taurine bile acid conjugates, vary in specific hepatobiliary diseases and cholestasis,[21,22] although until now there have been no published reports quantifying bile acid conjugates in bile from patients with CCA.

In vitro studies have shown that bile acids play a role in CCA aetiopathogenesis and, furthermore, bile acid abnormalities and toxicity have been implicated in other biliary diseases, such as primary biliary cirrhosis and PSC, in addition to colorectal carcinoma.[23–25] The *in vitro* MR data presented in this study suggest that primary bile acids and their glycine conjugates may be elevated in CCA bile in the absence of clinical cholestasis and therefore these agents are potential disease biomarkers, requiring further evaluation in larger-scale studies.

Multivariate analysis highlighted PtC as the major metabolite in bile distinguishing the CCA group from the disease control groups. Levels of PtC were significantly lower in CCA bile compared with bile from patients with gallstones. We have previously shown differences in phospholipid metabolites that may help to distinguish between malignant and non-malignant causes of pancreaticobiliary obstruction; a reduced PtC resonance was seen in bile from the majority of patients with hepatobiliary cancer compared with bile from patients with non-malignant indications for ERCP.[11] This finding has also more recently been confirmed by Albiin and co-workers in a comparison of bile from patients with CCA with bile from patients with PSC.[13] An *in vivo* NMR study of bile after biliary decompression suggested that the altered metabolism of phosphorus-containing metabolites may be a useful

Table 2 Predictive abilities of the orthogonal signal correction partial least squares discriminant analysis (OSC-PLS-DA) models constructed

	CCA vs. all non-malignant	CCA vs. SOD	CCA vs. PSC	CCA vs. GS
Sensitivity	80%	80%	100%	100%
Specificity	95%	100%	100%	86%
PPV	80%	100%	100%	83%
NPV	95%	89%	100%	100%

CCA, cholangiocarcinoma; SOD, sphincter of Oddi dysfunction; PSC, primary sclerosing cholangitis; GS, gallstones; PPV, positive predictive value; NPV, negative predictive value

indicator of malignancy in jaundiced patients with hepatobiliary disease.[26] Phosphatidylcholine, a cytoprotective and dominant biliary phospholipid, is synthesized in the hepatocyte and transported into the biliary canaliculus by the flippase multidrug resistant protein 3 (MDR3).[27] Animal studies have shown that MDR2 knockout mice (*mdr 2–/–*) develop CCA after prolonged bile acid exposure.[28] In humans, genetic mutations in the *ABCB4* gene (which encodes the protein MDR3) lead to reduced or absent phospholipid export into the bile, thus exposing the biliary epithelium to 'toxic' bile, and it has been postulated that these individuals may be predisposed to developing CCA.[23,29]

In conclusion, these preliminary data have highlighted the potential importance of bile acid and PtC metabolism in patients with CCA. These findings suggest important alterations in biomolecular mechanistic pathways in CCA and require further evaluation. The putative role of bile acids as potential disease biomarkers is novel and requires further validation in larger studies in order to improve current diagnostic techniques aimed at distinguishing malignant and benign biliary pathology. This remains a major challenge for the clinician.

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Conflicts of interest

None declared.

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