Metabolic Profiling of Bile in Cholangiocarcinoma Using In Vitro Magnetic Resonance Spectroscopy


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Short title: Metabolic Profiling of CCA

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Abstract

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

**Methods:** Magnetic resonance spectra were acquired from the bile of five CCA patients and compared to disease 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 to the benign disease groups (p=0.002). 7β primary bile acids were significantly increased (p=0.03), and biliary phosphatidylcholine levels were reduced (p=0.01), in bile of patients with CCA compared to bile from gallstone patients.

These compounds were also of primary importance in the multivariate analysis: partial least squares discriminant analysis (PLS-DA) was able to differentiate the cohorts.

**Conclusion:** These preliminary data suggest that altered bile acid and phosphatidylcholine metabolism plays an important role in CCA aetiopathogenesis, and that specific metabolites may have potential as future biomarkers.
Key words: Cholangiocarcinoma, MRS, bile acids, phosphatidylcholine, metabonomic

Introduction

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

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 can be reliably obtained. Although in widespread use, serum CA 19-9 has a poor sensitivity (50-60%) for the diagnosis of CCA. Cytological examination of bile also has a poor detection rate for malignant biliary disease. Despite improvements in biliary cytological examination using digital image analysis (DIA) and fluorescent in situ hybridisation (FISH), distinguishing benign from malignant ductular epithelium remains sub-optimal. Tissue biopsies often yield negative results, owing to marked fibrosis and because no pathognomic 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
cholecysto-pancreatography (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 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-homogenous 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. In *vitro* MRS studies on bile have provided information on composition, structure and function, as well as the metabolism and biliary excretion of xenobiotics. A major advantage of the technique is that the sample can be studied intact, allowing for subsequent study as required. Recently, MRS studies on bile in patients with pancreatic carcinoma observed an alteration in bile composition, as well identifying a potential cancer biomarker.

However, bile collected at ERCP is frequently contaminated by the contrast agent used and two previously published studies investigating the metabolic composition of bile from CCA patients using MRS have been flawed owing to such contamination, resulting in dominating spectral resonances, which may confound the measurement of metabolites. 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 to patients with benign biliary disease, using in vitro proton (1H) MRS. The predominant lipid metabolites, bile acids and phosphatidylcholine, were selected for specific study due to their proposed role in cholangiocarcinogenesis. A further aim was to identify potential disease markers in bile, in order to improve diagnosis and prognosis of CCA.

METHODS

This study was approved by the Research Ethics Committee of Hammersmith Hospital, London (HHREC number AM1073/0086). The study conformed to the 1975 Declaration of Helsinki ethical guidelines. Written, informed consent was obtained from all patients. 4mL 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 transferred to a 5 mm glass NMR tube. A sealed 4mm stem coaxial NMR insert, containing 50 μL of an internal reference standard solution (35μL of sodium trimethylsilyl-[2H4] propionate (TSP) 1 mg/mL dissolved in deuterium oxide) was placed inside the NMR tube.

MR data acquisition
In vitro ¹H MRS was performed using a JEOL ECP+ 500 MHz MR spectroscopy system and an 11.7 Tesla superconducting magnet. The ¹H MR spectra were obtained using a pulse-collect sequence (90° 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. MR 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 due to phase inversion of some of the coupled signals.

Spectral interpretation and analysis

The data were processed using the KnowItAll Informatics System v7.9 (Bio-Rad, 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 (δ 0.00 ppm) ¹⁶. The cluster of peaks δ 0.7 - 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 phosphatidylcholine, and assigned to the choline head group and (CH₂)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, v14.0. 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 v7.9, each spectrum was divided into smaller regions (bins), of $0.04 \pm 0.02$ ppm, ensuring no overlap across binned regions. These regions were integrated and normalised to the total spectral signal, and the data mean-centred before multivariate analysis.

Principal Component Multivariate Analysis (PCA)
PCA is an ‘unsupervised’ technique in that it does not rely on a priori knowledge of the cohort to which samples belong. It allows visualisation of clustering within a multivariate dataset through data reduction, to identify and visualise 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 visualisation of the metabolites responsible for the variation seen in the scores plot.

Partial least squares discriminant analysis (PLS-DA)
Data were then analysed by partial least squares discriminant analysis (PLS-DA) using Pirouette v4.0 (Infometrix, USA). This linear regression technique relates the
NMR spectroscopic variables, corresponding to metabolites, to the class membership of the sample, allowing the identification and visualisation 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 minimise the possible influence of inter-individual variation. Validation of the discriminatory power of each model was carried out through 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).

RESULTS

Patient demographics

Five inoperable CCA patients (four male, one female, mean (range) age 73 (58-82) years) were recruited. Three were hilar bismuth grade 3 tumours and bile was collected downstream of the lesion. Two were distal tumours and bile was collected upstream. Of the five CCA patients only two had an elevated CA19-9 (240 and 26954 U/mL respectively). Histological diagnosis was confirmed in two patients. In the three other patients diagnosis was based on clinical presentation and imaging findings. Twenty patients with non-malignant biliary disease (seven choledocholithiasis [gallstones], eight sphincter of Oddi dysfunction (SOD) and five 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 clinico-
pathological details of patients are summarised in Table 1. The age and gender of the cohort were not normally distributed. The four groups were therefore analysed separately, in addition to being combined.

**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 Figure 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 (Figure 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 the benign biliary disease groups ($p=0.002$, Kruskal Wallis test). The level of these glycine-conjugated bile acids was higher in the CCA group, when compared to patients with SOD and PSC: median (interquartile range) 1.00 units (u) (0.78-1.59 u) vs 0.70 u (0.47-0.85 u) and 0.56 u (0.44-0.74 u) ($p=0.030$ and $0.056$ respectively). In contrast, there was no difference when comparing 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 in the CCA group was greater than in other patient groups at 1.11, while in the non-cancer groups, this glycine-to-taurine-conjugated bile acid ratio was reduced: SOD (0.82), PSC (0.64) and gallstones (0.97). 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 gallstones: median (interquartile range) 0.76 u (0.56-1.32 u) vs 0.44 u (0.09-0.71 u) \( (p=0.030) \). Levels of the peaks assigned to the choline moiety of phosphatidylcholine (PtC) head group \( \delta \) 3.24 ppm and the \((\text{CH}_2)_n\) tail group \( \delta \) 1.29 ppm were significantly lower in the bile from patients with CCA, when compared to bile from the gallstone control group: median (interquartile range) 4.03 u (3.94-4.66 u) vs 4.61 u (4.32-6.09 u) \( (p=0.018) \) and 24.99 u (22.68-27.06 u) vs 29.08 u (25.10-34.61 u) \( (p=0.010) \) respectively.

**Multivariate pattern-recognition analysis**

The PCA scores plot in Figure 3 depicts a degree of separation between the four patient groups. The loadings plots for principal components 1 and 2 were dominated by the resonance corresponding to the tail group of PtC, \( \delta \) 3.20 ppm. When comparing CCA patients to non-malignant controls, the greatest degree of separation was seen with SOD (Figure 3). Using OSC-PLS-DA modelling with “leave-one-out” cross validation, the CCA samples were discriminated from all 20 non-malignant controls with a sensitivity of 80% and specificity of 95%, positive predictive value 80%, negative predictive value 95% (Table 2): one CCA sample and one non-cancer patient were incorrectly predicted. The major metabolites contributing to this separation were PtC, H-18 bile acids (resonance \( \delta \) 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 samples being contaminated with contrast agent, and by the marked cholestastic nature of bile samples studied $^{11,13}$. Despite a small and heterogeneous sample population, the data presented have shown important differences in the biliary metabolic profile when comparing malignant versus benign biliary disease. The patient study groups could be distinguished using both the unsupervised PCA and the supervised OSC-PLS-DA.

The major findings of this study were significant differences in the levels of primary bile acids, their glycine conjugates and phosphatidylcholine in bile from patients with CCA when compared to bile from non-malignant control samples. When analysed using univariate statistical methods, normalised signal levels of glycine-conjugated bile acids were significantly higher in CCA bile, compared to 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 to SOD. Also of note, the ratio of glycine- to taurine-conjugated bile acids was greater in the CCA bile, when compared to controls. The ratio of conjugated to unconjugated bile acids, and the ratio of glycine to taurine bile acid conjugates, varies 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. 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.

Both PCA and PLS-DA highlighted PtC as the major metabolite in bile distinguishing the CCA group from the disease control groups. PtC levels were significantly lower in the CCA bile, when compared to bile from patients with gallstones. We have previously shown differences in phospholipid metabolites that may help distinguish between malignant and non-malignant causes of pancreaticobiliary obstruction; a reduced PtC resonance was seen in the bile from the majority of patients with hepatobiliary cancer, compared to bile from patients with non-malignant indications for ERCP. This finding has also more recently been confirmed by Albiin and co-workers when comparing bile from patients with CCA to PSC. An *in vivo* NMR study of bile after biliary decompression suggested that altered metabolism of phosphorus-containing metabolites may be useful indicators of malignancy in jaundiced patients with hepatobiliary disease. PtC, a cytoprotective and dominant biliary phospholipid, is synthesised in the hepatocyte and transported into the biliary canaliculus by the flippase multidrug resistant protein 3 (MDR3). Animal studies have shown that MDR2 knockout mice (*mdr 2-/-*) develop CCA after prolonged bile acid exposure. 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 maybe predisposed to developing CCA.
In conclusion, these preliminary data have highlighted the potential importance of bile acid and phosphatidylcholine metabolism in patients with cholangiocarcinoma. 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.

**List of abbreviations**

CCA cholangiocarcinoma  
ERCP endoscopic retrograde cholangiopancreatography  
MDR multidrug resistance protein  
MRS magnetic resonance spectroscopy  
NMR nuclear magnetic resonance  
OSC orthogonal signal correction  
PCA principal component analysis  
PLS-DA partial least square- discriminant analysis  
PSC primary sclerosing cholangitis  
PtC phosphatidylcholine

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