

Active cholangiocarcinoma research projects at Imperial College London

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Introduction

Cholangiocarcinoma (CC) is a liver cancer arising in the bile ducts, a system of tube-like structures in the liver that produce and carry bile from the liver to the gut to help digest food and excrete waste products. CC kills over 1000 people per year. Most patients present too late for surgery - the only known cure. Since 1968 there has been a 15-fold increase in death rates from CC in the UK. This is a worldwide trend across industrialised countries. Our research group at Imperial College London has a proven track record in CC research.

Firstly, a lay summary is provided of all our active CC related research projects. Detailed, scientific project summaries are then presented for our genetic & proteomic projects. Finally, our group's published work on CC, including scientific papers, is listed.

Summary of current and planned projects

Biliary transporter gene study, underway

The cause of CC and its recent increase are unknown. Our group, and others around the world, have proposed that the risk of CC may be increased if a patient's biliary transporter proteins are abnormal. These transporter proteins line the bile duct wall and push bile and toxins into the bile duct. It is postulated that abnormalities in these transporter proteins may lead to accumulation of toxins within the wall of the bile duct and predispose to cancerous changes. Specific genes produce biliary transporter proteins and it is possible to study variations of these genes in a patient's DNA in the laboratory. We are undertaking the largest ever study of biliary transporter genes in patients with cholangiocarcinoma.

Epidemiological project, planning stage

Previous work by our group has shown higher rates of cholangiocarcinoma in some areas in the UK than others. The reasons for this clustering phenomenon are not clear. It may relate to environmental factors that predispose to CC development. We would like to undertake a detailed epidemiological study into the causes of this clustering as it may lead to a better understanding of the causes of CC.

Proteomic project, starting imminently

CC is difficult to accurately diagnose due to a lack of sensitive and specific blood tests. Even the most detailed radiological scans cannot accurately differentiate between benign and malignant disease of the bile ducts, or between CC and other types of liver tumours. This differentiation is important, as the treatment is very different depending on whether bile duct disease is benign or cancerous. Consequently, there is a need for accurate diagnostic biomarkers of CC. There has been recent interest in measuring variations in the levels of different protein to distinguish various diseases. Our proteomic study is based on successful pilot data from our department. We will analyse differences in the protein profiles in blood from patients with non-cancerous bile duct disease, CC and other liver tumours. The technique used, surface-enhanced laser desorption/ionization time-of-flight mass

spectroscopy (SELDI-TOF MS), is a well-recognised way to assess protein profiles in samples. This study could lead to the discovery of new diagnostic markers for CC and other liver tumours, enabling patients to be accurately diagnosed and receive appropriate therapy at an early stage. This would be a major advance and could translate into improved patient survival.

Biliary probe project, underway

Our group is collaborating with the engineering department of Imperial College London on a new device to enhance the quality of MRI scans of the bile duct. This exciting project may lead to more rapid and reliable diagnosis of CC and differentiation from benign bile duct disease.

Detailed scientific summaries for Proteomic & Genetic studies

Understanding cholangiocarcinogenesis

The aetiopathogenesis of CC is unknown, but gene/environment interactions are likely to be involved. Transporter proteins expressed on the apical surface of hepatocytes and cholangiocytes govern the rate of bile flow and dysfunction of the transporters is a leading cause of cholestasis (reduction in the bile flow). We hypothesise that genetic variations in transporter proteins affect susceptibility to CC by causing cholestasis, thus increasing the exposure of cholangiocytes lining the bile ducts to harmful toxins.

Key scientific objectives and challenges

The proteomic study is based on successful pilot data from our department. Differences in plasma protein profiles from patients with non-cancerous bile duct disease, CC and other liver tumours, including HCC and metastatic liver cancer, will be analysed. Our objective is to identify biomarkers that distinguish between these groups, using SELDI-TOF MS, a well-recognised way to assess protein profiles in biofluids. After identifying potential biomarkers, the challenge will be to purify and characterise these proteins, involving techniques in which we have proven. In parallel studies, gene sequencing will be used to ascertain the frequency of allelic variants in the major biliary transporter proteins in CC patients, compared with age, sex and ethnically-matched controls. Linking genetic alterations to protein expression profiles will be a major challenge.

Potential medical implications

This will be a translational study. The identification of accurate diagnostic biomarkers for liver cancer will allow patients to be correctly diagnosed and receive appropriate therapy. This could translate into improved patient survival. Unravelling mechanisms that predispose to CC is important for detecting at-risk individuals. These studies may also identify targets for future biological therapies, a much needed major breakthrough in liver cancer.

Plan of Investigation: Proteomic Study

Purpose: To investigate whether protein profiles can differentiate between benign (primary sclerosing cholangitis, PSC and gallstone-related) and malignant biliary disease (cholangiocarcinoma, CC), as well as between CC and other common liver tumours including hepatocellular carcinoma (HCC) and colorectal liver metastases.

Background: CC is the commonest cause of death from primary liver cancer in the UK, causing over 1000 deaths per year.¹⁻³ Since 1968 there has been a 15-fold increase in mortality from CC in the UK,³ with a similar global trend across industrialized countries.^{2,4} This was first brought to international attention by our group.^{2,3} Mortality from CC is high as most patients present too late for surgical resection, the only cure, and the cancer is resistant to chemo/radiotherapy. Furthermore, CC is difficult to diagnose, particularly in the background of chronic bile duct inflammation, the commonest known predisposing factor. PSC is the commonest known risk factor for CC, accounting for up to 20% of cases. Most CCs are sporadic.¹ Diagnosis of CC currently relies on imaging with computed tomography or

magnetic resonance.⁵ However, these modalities cannot distinguish benign from malignant biliary strictures, or differentiate CC from other liver tumours such as metastases or HCC. Histology is difficult to obtain and even if available, accurate differentiation between CC and other adenocarcinomas is problematic.⁶ Serum tumour markers, e.g. CA19.9 and CEA, have poor specificity and sensitivity.⁵ Liver transplantation is accepted therapy for HCC or PSC, but once CC has developed, as it does in up to one third of PSC cases, transplantation is contraindicated. Thus, therapies for these differential diagnoses are radically different, potentially involving surgical resection, liver transplantation, endoscopic intervention, chemotherapy or palliation. Consequently, there is a need for accurate biomarkers that distinguish CC from benign biliary disease, as well as from other liver tumours.

Proteomic profiling:^{7,8} We were the first group to analyse human bile for markers of hepatobiliary tumours, in metabonomic studies using nuclear magnetic resonance spectroscopy. The combination of this technique and the target biofluid did not yield the diagnostic information that was hoped for.⁹ However, the human proteome is recognised as a rich source of potential disease biomarkers. Proteomic profiling of plasma has been used to study potential biomarkers in various malignancies, including CC.¹⁰ In particular, affinity-based arrays, such as the protein chips analysed via surface-enhanced laser desorption/ionization time-of-flight mass spectroscopy (SELDI-TOF MS) have been used to successfully identify malignant patterns in plasma from ovarian, hepatocellular, pancreatic, prostate and breast carcinomas.

Pilot data: We carried out a study comparing proteomic profiles in plasma of 10 CC patients with 9 patients with benign biliary disease (gallstones) using a combination of hydrophobic (H50) and carboxymethyl (CM10) Protein Chips. Univariate analysis with the non-parametric Mann-Whitney-U test revealed several individual peaks that distinguished CC and benign disease (Figure 1). Using multiple regression analysis a two-biomarker panel (m/z peaks 2701 and 3311) was found to distinguish CC from non-malignant samples with 100% sensitivity and specificity ($p=0.0002$, ROC AUC=1.0;) [Khan et al., full manuscript in process; Work presented and published as abstracts at the following annual conferences in 2008: **National Cancer Research Institute**, Birmingham, UK; **British Association for the Study of Liver Disease**; Edinburgh, UK; **American Association for the Study of Liver Disease**, San Francisco, USA 2008].

Aims: To analyse protein biomarker panels, obtained with SELDI-TOF MS, from the following cohorts, totalling 175 samples: **cancer** patients, including those with PSC-associated cholangiocarcinoma (n=50), non PSC-associated sporadic CC (n=50), hepatocellular carcinoma (n=50) or colorectal liver metastases (n=50); **non-cancer** patients including gallstone biliary disease (n=50) and PSC without CC (n=50); age and sex-matched healthy controls with no hepatobiliary disease (n=50). The majority of patients have already been identified.

Samples: The cohort sizes are in line with current studies of proteomic profiling in cancer. The statistical power of our approach may be estimated from the expected distribution of the levels of the putative protein biomarkers and the number of patient and control samples obtained. On the basis of a normalised distribution with a mean of 1.0 and a SD of 0.8, which is typical of previous measurements, the statistical power to detect a change of 2-fold at a confidence level of at least 0.05 will be 95%. Plasma samples will be analyzed in duplicate by SELDI-TOF MS, using a Protein Biology System Ilc Reader, calibrated for mass accuracy using "all in 1" peptide and protein standards (BioRad). Samples will be analysed on a variety of Protein Chip arrays, including H50 and CM10.

Statistical analysis: The analysis of data will be performed using a combination of the software provided by the manufactures to detect the protein peaks (PEAKS), Excel spreadsheets to format the data and perform statistical tests of significance (Student's t-test)

and more dedicated software (e.g. SPSS, STATISTICA) to perform further analyses including Univariate, Multivariate, Principal Components and Discriminant Function analyses. The diagnostic performance of putative protein biomarkers will be assessed in terms of their sensitivity and specificity in the form of receiver operator characteristic curves. Comparison of serum biochemistry and measures of synthetic liver function will be made between the two groups. Whether the addition of standard tumour markers, such as CA19.9, CEA, CA-125 and AFP, adds discriminatory power will also be assessed.

Identification of plasma protein biomarkers: Significant peaks will be purified by sequential ion exchange, reverse-phase chromatography and SDS-PAGE. Protein identification will be achieved by nanoflow liquid chromatography tandem MS analysis of trypsin-digested peptides in combination with database searching (SEQUEST software). We have previously used these techniques to successfully identify protein biomarkers.¹⁰

Translational study: This work is intended to be a translational study, potentially leading to new diagnostic biomarkers for CC and other liver tumours, thus enabling patients to be accurately diagnosed and receive appropriate therapy. This would be a major advance, in the UK and internationally, and could translate into improved patient survival.

Plan of investigation: Genomic study

Background: Chemical toxins have been linked to CC. Our group reported pro-mutagenic DNA adducts in human CC tissue, demonstrating exposure to DNA-damaging agents.¹² Thorotrast, a radiological contrast agent banned in the 1960s for its carcinogenic properties, has been strongly associated with CC, increasing the risk to 300 times that of the general population.¹³ Associations have also been made with by-products from the chemical industry, including dioxins and nitrosamines.¹⁴ Exposure to environmental toxins has increased in the past few decades,¹⁵⁻¹⁷ but given that only a small percentage of Western populations develop CC, it follows that genetic predispositions in host mechanisms that govern the metabolic response to these to pathophysiological stimuli may play a role in cholangiocarcinogenesis. We were the first group to analyse complete p53 DNA sequences in biliary tract cancer, looking for a xenobiotic mutational signature.¹⁸

Hepato-biliary transport: The hepatobiliary system is the major route of metabolism and excretion for genotoxic, potentially carcinogenic environmental toxins. Bile flow and constituents vary between individuals. A reduction in the flow of bile (cholestasis) and abnormal biliary constituents may result in increased exposure of cholangiocytes lining the biliary epithelial cells to harmful xenobiotics. Biliary excretion of bile salts and toxins is performed by transporters expressed on the apical surface of hepatocytes and cholangiocytes.¹⁹ These biliary transporters also govern the rate of bile flow and dysfunction of the transporters is a leading cause of cholestasis.²⁰ The major biliary transporters include the bile salt excretory pump (BSEP), the MDR related proteins (MRP1 & MRP3) and products of the familial intrahepatic cholestasis gene (FIC1) and multidrug resistance genes (MDR1 & MDR3). BSEP (ABCB11 gene) is responsible for the active transport of bile acids across the hepatocyte canalicular membrane into bile, and secretion of bile acids is a major determinant of bile flow.^{20,21} Preliminary proteomic data from our group has shown up-regulation of BSEP in CC tissue (*manuscript in preparation*). MDR1 mediates the canalicular excretion of xenobiotics and cytotoxins. MDR 3 encodes a phospholipid transporter protein that translocates phosphatidylcholine from the inner to the outer leaflet of the canalicular membrane. Polymorphisms in the MDR3 gene may lead to a reduction in MDR3 protein function and biliary PtC levels increasing the risk of CC development.

Aims: Using gene-sequencing studies, the frequency of genetic variations of CYPs 1-3, UGTs 1-2; and biliary transporters BSEP, MRP1, MRP3, FIC1, MDR1 and MDR3 in CC patients will be compared with healthy age/sex and ethnically matched controls.

Methods: DNA will be obtained from whole blood samples or from stored histological material using commercially available extraction kits. Variant functional alleles will be genotyped and standard statistical analysis employed to detect over or under-representation, compared to a normal population, with checks for genotyping errors and population stratification). Genotyping will be performed with allelic discrimination assays on the Taqman system and sequencing on the 3100 genetic analyser. Patients and controls will be divided into two cohorts and associations sought by comparison of allele frequencies in the first cohorts using simple chi-squared tests. Positive associations will be confirmed in the second cohorts. Figure 2 shows the number of samples required to detect a susceptibility locus with an allele frequency of 5 to 40%, and which influences susceptibility to CC with varying odds ratios.

Figure 1. Spectra from two samples (one CC and one control; 1 in 50 dilution with 20% ACN on H50 Protein-Chips) over mass range of 2-10kDa showing four significantly different peaks.

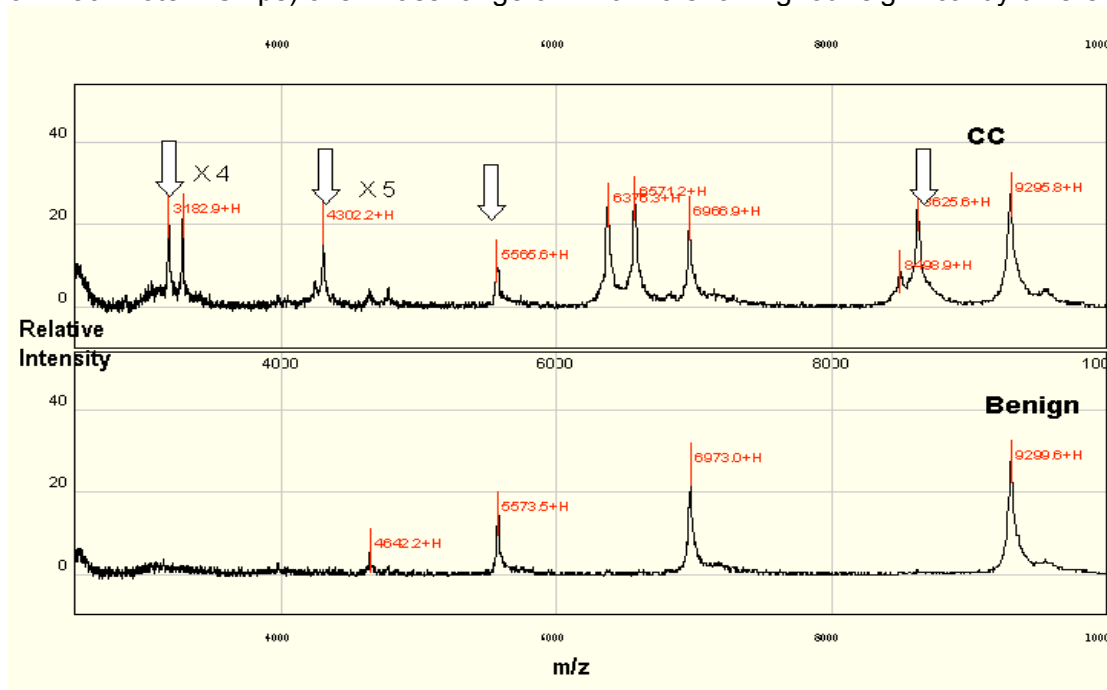
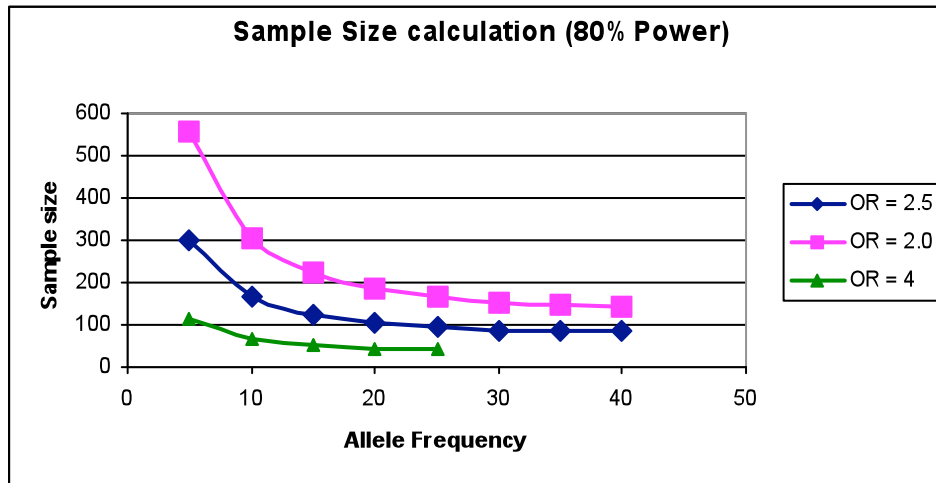


Figure 2. Number of samples required to detect a susceptibility locus with an allele frequency of 5 to 40% with odds ratios of 2.0, 2.5 and 4.0. This demonstrates that 116 samples will give >80% power to detect an allele with a population frequency of 5% that influences susceptibility with an odds ratio of 4.0. We currently have 150 cholangiocarcinoma DNA samples and anticipate that this cohort size will increase during the course of the research project.



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