

Genetic Factors in the Pathogenesis of Cholangiocarcinoma

Christopher A. Wadsworth^a Peter H. Dixon^b Jason H. Wong^a
Michael H. Chapman^d Siobhan C. McKay^c Amar Sharif^a Duncan R. Spalding^c
Stephen P. Pereira^d Howard C. Thomas^a Simon D. Taylor-Robinson^a
John Whittaker^e Catherine Williamson^b Shahid A. Khan^a

^aDepartment of Hepatology and Gastroenterology, ^bInstitute of Reproductive and Developmental Biology, and
^cDepartment of Hepatobiliary Surgery, Imperial College London, ^dInstitute of Hepatology, University College
London Medical School, ^eLondon School of Hygiene and Tropical Medicine, University of London, London, UK

Key Words

Cholangiocarcinoma · Genetics · ABCB11 · ABCB4 · ABCC2 · ATP8B1 · NR1H4

Abstract

Background: Cholangiocarcinoma (CC) is increasing in incidence, but its pathogenesis remains poorly understood. Chronic inflammation of the bile duct and cholestasis are major risk factors, but most cases in the West are sporadic. Genetic polymorphisms in biliary transporter proteins have been implicated in benign biliary disease and, in the case of progressive familial cholestasis, have been associated with childhood onset of CC. In the current study, five biologically plausible candidate genes were investigated: *ABCB11* (BSEP), *ABCB4* (MDR3), *ABCC2* (MRP2), *ATP8B1* (FIC1) and *NR1H4* (FXR). **Methods:** DNA was collected from 172 Caucasian individuals with confirmed CC. A control cohort of healthy Caucasians was formed. Seventy-three SNPs were selected using the HapMap database to capture genetic variation around the five candidate loci. Genotyping was undertaken with a competitive PCR-based system. Confirmation of Hardy-Weinberg equilibrium and Cochran-Armitage trend testing were performed using PLINK. Haplotype frequencies were com-

pared using haplo.stats. **Results:** All 73 SNPs were in Hardy-Weinberg equilibrium. Four SNPs in *ABCB11* were associated with altered susceptibility to CC, including the V444A polymorphism, but these associations did not retain statistical significance after Bonferroni correction for multiple testing. Haplotype analysis of the genotyped SNPs in *ATP8B1* identified significant differences in frequencies between cases and controls (global p value of 0.005). **Conclusion:** Haplotypes in *ATP8B1* demonstrated a significant difference between CC and control groups. There was a trend towards significant association of V444A with CC. Given the biological plausibility of polymorphisms in *ABCB11* and *ATP8B1* as risk modifiers for CC, further study in a validation cohort is required.

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Introduction

Cholangiocarcinoma (CC) is a cancer that arises in the epithelial cells of the bile ducts and has features of cholangiocyte differentiation [1]. Max Durand-Fardel, a French physician, was the first author to describe CC. In 1840, he reported a series of four post mortem exami-

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Dr. Shahid A. Khan
Department of Hepatology, Imperial College London
10th Floor QEOM Building, St Mary's Campus, Praed Street
London W2 1NY (UK)
Tel. +44 207 886 6454/6254, E-Mail shahid.khan@imperial.ac.uk

nations of patients with jaundice who had rapidly succumbed to their disease. In 3 cases, examination revealed tumours of the pancreas obstructing the bile duct. In the final case, he reported 'a soft, whitish, cancerous material, enveloping and completely filling the bile duct but that did not extend beyond the wall of the duct'. He concluded that this represented a primary cancer of the extrahepatic bile duct [2].

Since 1968, there has been a 15-fold increase in mortality from CC in the UK, with a similar global trend across industrialised countries [3–5]. CC is now the commonest cause of death from primary liver cancer in the UK, causing over 1,000 deaths per year, exceeding even the mortality due to hepatocellular carcinoma [3]. The mortality rate of CC is equal to its incidence and is so high because presentation is usually too late for complete surgical resection, which is the only cure, and because the cancer is resistant to chemotherapy and radiotherapy. The median age at diagnosis of CC is 65 years and it rarely presents before the 5th decade, except in patients with primary sclerosing cholangitis (PSC) or uncommon genetic or congenital diseases of the biliary tract [6, 7]. The prognosis of CC is short, with a median survival of 3–6 months in patients receiving palliative care [8]. Overall, median survival in developed countries is less than 2 years [8, 9].

Environmental Toxin Evidence

Epidemiological studies have implicated a variety of toxins and occupational risks with CC, with varying strength of evidence. Thorotrast, an α -particle-emitting radiological contrast containing thorium oxide, was used and subsequently proscribed in the middle of the last century. Thorium oxide has a long half-life and is concentrated in the reticuloendothelial system. It has been incontrovertibly linked with CC and a number of other malignancies, with a latency period of 20–35 years [10]. Thorotrast exposure increases the risk of CC to 300 times that of the general population [6]. Epidemiological studies have also implicated tobacco smoking and alcohol use, although conflicting evidence also exists [11–15]. Occupational exposure to the manufacture of rubber, petrochemicals and varnishes is an independent risk factor for CC. These associations have been ascribed to exposure to chemical by-products, including dioxins and nitrosamines [16]. Pro-mutagenic DNA adducts have been identified in CC-adjacent human tissue, indicating exposure to DNA-damaging exogenous toxins [17]. A proposed

common pathway of these environmental risks is exposure of the biliary epithelium to oncogenic substances that have been excreted and concentrated in the bile, causing a sequence of genetic mutations thought to initiate and promote neoplasia [17].

Variation with Ethnicity

Incidence of CC varies with geography and ethnicity, in part independently. The population of northern Thailand has a very high rate of CC that has been clearly associated with endemic liver fluke infestation in that region. Chile also has a very high incidence, while Australia has a very low incidence; however, there is no clear variation in environmental risk factors between the two countries [6]. Studies of the US population have shown wide variation between different ethnic groups, with rates of 1.22/100,000 in Hispanic Americans and 0.17–0.5/100,000 in African Americans [18]. The variation in risk between different ethnic groups living in the same environment suggests a potential genetic influence on susceptibility to CC.

Genetic Factors in CC and Benign Biliary Disease

A number of genetic diseases are risk factors for CC. Lynch syndrome (HNPCC) and biliary papillomatosis, which are both genetic conditions, increase the risk of CC [19]. Biliary papillomatosis carries a huge (85%) risk of conversion to CC [20]. These inborn genetic mutations appear to offer a biliary epithelial substrate that is highly vulnerable to subsequent oncogenic factors.

Genetic defects leading to congenital abnormalities in bile salt transporter proteins, such as the bile salt exporter pump, (BSEP/ABCB11), familial intrahepatic cholestasis protein 1 (FIC1/ATP8B1) and multidrug resistant protein 3 (MDR3/ABCB4), cause cholestatic disease in infants. These defects result in unstable bile content and deconjugation of xenobiotics, previously conjugated in the liver [5, 21, 22]. Very early onset hepatocellular carcinoma and CC have been reported in children with progressive familial intrahepatic cholestasis (PFIC) type 2, caused by BSEP/ABCB11 deficiency [7, 23, 24].

Recent studies have demonstrated a number of polymorphisms in biliary transporter genes, including *ABCB4* and *ABCB11*, which increase the risk of cholelithiasis – a major risk factor for CC [25–28]. A recent study of a cohort of women with intrahepatic cholestasis of pregnancy

demonstrated an association between a SNP in *ABCB11* and the disease [29].

Genetic factors in PSC, the major risk factor for CC in Western populations, have been investigated extensively. Genetic variation in the human leukocyte antigen complex on chromosome 6p21 has been repeatedly associated with PSC. Studies of specific genes have been small and negative to date, including a study of *ABCB11* and *ABCB4* ($n = 37$) [30]. A recent national genome-wide association study in Norway demonstrated associations between polymorphisms in human leukocyte antigen-B, macrophage-stimulating 1, G protein-coupled bile acid receptor 1 and PSC [31]. Large-scale international genome-wide association studies into PSC are underway.

A number of studies of candidate genes in CC, each examining between 30 and 216 cases, have reported a positive association with the disease. These studies have generally been performed on populations from the Far East and have focussed on polymorphisms affecting enzymes of xenobiotic metabolism (glutathione S-transferase, *GST01*, N-acetyltransferase 1, *NAT2* and cytochrome P450 1A2, *CYP1A2*) and folate metabolism (methylene-tetrahydrofolate reductase, *MTHFR*) [32–35]. Several of these studies included gallbladder cancer, and even ampullary tumours, with CC cases. All have yet to be replicated in validation cohorts. A recent study explored the possible association of polymorphisms in G-protein signal transduction and apoptosis. One of the three SNPs studied was associated only with an improved prognosis in extrahepatic CC [36]. Genes modifying natural killer cell activation (natural killer cell receptor G2D, *NKG2D*) have been implicated in a number of neoplasms and have been investigated in PSC patients with CC. Two of the seven SNPs studied were associated with altered susceptibility to CC in PSC [37]. Polymorphisms in *NKG2D* have yet to be investigated in sporadic CC.

A recent study in a small cohort ($n = 60$) of patients found a polymorphism in the multidrug resistance-associated protein 2 (MRP2) gene *ABCC2* was associated with CC [38]. MRP2 is an ATP-binding cassette (ABC) biliary transporter expressed on the apical membrane of hepatocytes and cholangiocytes. The c.3972 C>T SNP genotype was significantly more frequent in CC (39.2%) versus controls (26.0%, $p = 0.022$) with an OR of 1.83 (95% CI: 1.09–3.08). MRP2 is a reasonable candidate susceptibility protein as it plays a critical role in the excretion of xenobiotics and polymorphisms in *ABCC2* have been associated with hepatocellular carcinoma. This is the only published study of biliary transporter proteins as risk factors for CC.

Canalicular Transporter Proteins

The hepatobiliary system is the major route of metabolism and excretion for genotoxic, potentially carcinogenic, endogenous and environmental toxins. Exposure to environmental toxins has increased in the past few decades and some of these toxins have been implicated in the pathogenesis of CC [39–41]. However, given that only a small percentage of Western populations develop CC, it follows that genetic differences in the host response to these pathological stimuli may play a role in cholangiocarcinogenesis.

Biliary excretion of bile salts, conjugated toxins and other components is performed by transporters expressed on the apical surface of hepatocytes and cholangiocytes [42]. These biliary transporters also govern the rate of bile flow, and dysfunction of the transporters is a leading cause of cholestasis [43]. Bile flow and constituents vary between individuals. The biliary proteins involved in the physiological production of bile have only been relatively recently described, and their functions in health and disease remain only partially understood.

A reduction in the flow of bile (cholestasis) and abnormal biliary constituents may result in increased exposure of cholangiocytes lining the biliary epithelium to xenobiotics and endogenous mutagens, such as hydrophobic bile acids. These bile acids have a strong detergent action that disrupts cell membranes. The formation of bile acids, phosphatidylcholine (PtC) and cholesterol into stable micelles in the bile serves to protect the biliary system from such damage [42]. Disequilibrium of biliary components is known to lead to biliary deconjugation of toxic species previously conjugated in the liver.

BSEP (*ABCB11*) is a member of the superfamily of ABC transporters. ABC proteins transport various molecules across extra- and intracellular membranes. ABC genes are divided into seven subfamilies (ABC1, MDR/TAP, MRP, ALD, OABP, GCN20, White). BSEP 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 [22, 43]. Underexpression of BSEP is implicated in PFIC type 2.

MDR3 (*ABCB4*) is a phosphatidylcholine floppase that translocates PtC from the inner to the outer leaflet of the canalicular membrane. PtC is critical in the formation of stable micelles in bile. Polymorphisms in *ABCB4* may lead to a reduction in MDR3 protein function and biliary PtC levels, resulting in unstable micelle formation. MDR3 deficiency has been implicated in PFIC type 3.

Table 1. Summary of the names, encoding genes, known functions and associated diseases of the proteins

Protein abbr.	Trivial name	Full name	Gene abbr.	Known function	Distribution in liver	Associated diseases
BSEP	bile salt exporter pump	ATP-binding cassette, sub-family B (MDR/TAP), member 11	<i>ABCB11</i>	bile acid transport	hepatocyte apical membrane	PFIC-2, HCC, BRIC-2
MDR3	multidrug resistance protein 3	ATP-binding cassette, sub-family B (MDR/TAP), member 4	<i>ABCB4</i>	PtC transport	hepatocyte apical membrane	PFIC-3, gallstone disease
MRP2	multidrug resistance-associated protein 2	ATP-binding cassette, sub-family C (CFTR/MRP), member 2	<i>ABCC2</i>	conjugated bilirubin and organic anion transport, O.A.	hepatocyte apical membrane	Dubin-Johnson
FIC1	familial intrahepatic cholestasis protein 1	ATPase, aminophospholipid transporter, class I, type 8B, member 1	<i>ATP8B1</i>	translocation of acidic phospholipids in cell membrane	hepatocyte and cholangiocyte apical membranes	PFIC-1/Byler's disease, BRIC-1
FXR	farnesoid X receptor/ bile acid receptor	nuclear receptor subfamily 1, group H, member 4	<i>NR1H4</i>	transcriptional regulation of ABCB4 and ABCB11	hepatocytes and cholangiocytes	cholestasis, gallstone disease

MRP2 (ABCC2) is a member of the MRP subfamily and has a known role in drug elimination and resistance. It is expressed in the canalicular apical membrane of the hepatocyte and exports numerous conjugated species into the bile. Substrates include conjugated bilirubin and other anionic-conjugated species, including many drugs and toxins.

FIC1 (ATP8B1) is a member of the P-type cation transport ATPase family and belongs to the subfamily of aminophospholipid-transporting ATPases. It is highly expressed in cholangiocytes and the canalicular membranes of hepatocytes, and is also found in the small intestine, stomach, pancreas and prostate. FIC1 transports phosphatidylserine and phosphatidylethanolamine from the outer membrane leaflet to the inner, maintaining the correct distribution of these lipids in the membrane and thereby membrane integrity. FIC1 dysfunction is associated with the disease PFIC type 1.

FXR (NR1H4) is a nuclear receptor and is expressed at high levels in the liver and intestine. Chenodeoxycholic acid and other bile acids are natural ligands for FXR. When activated, FXR translocates to the cell nucleus and binds to hormone response elements on DNA which up- or downregulate the expression of specific genes. FXR activation suppresses the production of cholesterol 7 α -hydroxylase (CYP7A1), the rate-limiting enzyme in bile acid synthesis from cholesterol. This forms a negative feedback pathway in which synthesis of bile acids is inhibited when cellular levels are already high. FXR also modifies the expression of other genes, including ABCB11 and ABCB4 (table 1).

It is highly likely that host and environmental factors interact in the development of CC. The range of proteins involved in bile formation has only been described relatively recently and the full extent of their functions and interactions has likely not been fully elucidated. However, variation in these proteins is known to modify the rate of bile formation, its flow, stability and toxicity. Genetic variation in host bile formation, physiology and flow may thereby modify susceptibility to CC.

Disclosure Statement

The authors have no conflicts of interest to declare.

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