

The challenge of cholangiocarcinoma: dissecting the molecular mechanisms of an insidious cancer

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Cholangiocarcinoma is a fatal cancer of the biliary epithelium and has an incidence that is increasing worldwide. Survival beyond a year of diagnosis is less than 5%, and therapeutic options are few. Known risk factors include biliary diseases such as primary sclerosing cholangitis and parasitic infestation of the biliary tree, but most cases are not associated with any of these underlying diseases. Numerous *in vitro* and *in vivo* models, as well as novel analytical techniques for human samples, are helping to delineate the many pathways implicated in this disease, albeit at a frustratingly slow pace. As yet, however, none of these studies has been translated into improved patient outcome and, overall, the pathophysiology of cholangiocarcinoma is still poorly understood. There remains an urgent need for new approaches and models to improve management of this insidious and devastating disease. In this review, we take a bedside-to-bench approach to discussing cholangiocarcinoma and outline research opportunities for the future in this field.

Cholangiocarcinoma: the clinical problem Epidemiology

Cholangiocarcinoma (CCA) is the second most common primary liver cancer globally, and the most common cause of death from primary liver cancer in the United Kingdom, where it kills 1500 people annually (Khan et al., 2012). At diagnosis, 65% of cases of CCA are in individuals who are over 65 years old, and the disease occurs equally in men and women. Globally, CCA incidence varies, reflecting differences in genetic and environmental risk factors. The highest incidence is in north-east Thailand (with 80-90 cases per 100,000 people), whereas Australia has the lowest incidence (with 0.4 cases per 100,000 people). Notably, the incidence of CCA is increasing worldwide for unknown reasons (Khan et al., 2012).

Classification

CCA is a primary malignancy, usually adenocarcinomatous, arising in the bile ducts that carry bile from its site of production in the liver to its site of action in the small intestine (Fig. 1). Topographically, CCA is classified as intrahepatic (IHCC), arising in the liver, or extrahepatic (EHCC), arising outside the liver. IHCC is subcategorised morphologically into mass-forming, periductular-infiltrating or intraductal, the latter being the least common but with a more favourable prognosis; however, IHCC tumours can possess a combination of these characteristics (Lim and Park, 2004). EHCC is subcategorised into perihilar CCA, involving the bifurcation of the main ducts (50% of all CCA), and distal EHCC. IHCC, distal EHCC and perihilar CCA are increasingly regarded as each having a

distinct epidemiology, pathogenesis and management requirements. However, clarifying these distinctions has been hampered by inconsistent classification, especially the inclusion of perihilar lesions as either IHCC or EHCC (Khan et al., 2012) and sometimes including gallbladder carcinoma or hepatocellular carcinoma in these classifications. Where known, relevance to subtypes will be made clear in this review.

Risk factors for CCA

In the Western world, 80% of CCA cases are sporadic and have no identifiable risk factor (Khan et al., 2012). Smoking, alcohol consumption, diabetes and obesity have not been consistently shown to increase risk, although a small contribution cannot be ruled out (Tyson and El-Serag, 2011). Risk factors that have been identified are generally associated with chronic biliary inflammation. For example, primary sclerosing cholangitis (PSC; see Box 1 for Glossary) is associated with 10% of CCA cases in the Western world (La Russo et al., 2006). In patients with PSC, the risk of developing CCA is 1% per annum over 10 years, with a cumulative lifetime risk of 9-31% – 1500-fold that of the general population (Burak et al., 2004; Claessen et al., 2009). In the management of this high-risk group, because of the often desmoplastic nature of CCA it is challenging to discriminate between malignancy and the benign strictures characteristic of PSC. Liver cirrhosis of mixed aetiology conveys a tenfold relative risk (Sorensen et al., 1998). Uncommon abnormalities of biliary anatomy, such as choledochal (bile duct) cysts, intrahepatic biliary cysts (Caroli's syndrome), biliary papillomatosis and adenomata, are associated with a high lifetime risk of CCA of 6-30% (Tyson and El-Serag, 2011). This high rate of malignant transformation warrants prophylactic resection (Tyson and El-Serag, 2011). Congenital or acquired abnormalities of pancreatic biliary-duct junctions allow pancreatic reflux and resultant chronic cholangitis, increasing risk of CCA

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Case study

The patient is a 30-year-old man with a successful business and busy family life. Although diagnosed with ulcerative colitis aged 20, his symptoms are mild and controlled by treatment with aminosalicylates. Blood tests 3 years ago showed mild derangement in liver parameters, although he was clinically asymptomatic. Investigation revealed normal perinuclear anti-neutrophil cytoplasmic antibody (pANCA) levels, and an ultrasound did not show any abnormalities in his liver. However, a magnetic resonance cholangiopancreatogram (MRCP) showed intrahepatic ducts with a beaded appearance, as well as mild narrowing of the common bile duct below the confluence of the hepatic ducts. A diagnosis of primary sclerosing cholangitis (PSC) was made, and pharmacotherapy with ursodeoxycholic acid (UDCA) was initiated. After 6 months, liver parameters had normalised and he continued to pursue business and family life as normal.

At his next review 6 months later, the patient's liver parameters were again abnormal. He felt generally fatigued, had lost 3 kg and had suffered a few instances of fever, which he attributed to work-related travel. Another MRCP showed greater narrowing of the common bile duct, with mild upstream dilatation. Carbohydrate antigen 19-9 (Ca19-9) levels were raised, at 60 U/ml. The regional multidisciplinary team were unable to rule out malignancy, and requested an endoscopic retrograde cholangiogram (ERC). The procedure was technically difficult owing to the tight stricture, and he suffered moderate pancreatitis (a complication seen in 1% of patients undergoing ERC, especially when difficult and protracted), which required a 1-week stay in hospital, intravenous fluids and antibiotics. A removable fully-covered metal stent was deployed for ongoing biliary drainage. Cytology of stricture brushings did not demonstrate malignant cells on this intervention or on two repeat procedures carried out for stent management over the following 6 months.

The patient returns for further ERC and clinical review every 3 months (stent changes and further surveillance with blood tests and imaging), and remains under review of the multidisciplinary team regarding future management. Limitations to his business activities and a growing awareness of his potentially life-limiting condition, as well as the limits of medical science in diagnosing and treating it, are proving difficult for him to adjust to. Without a more conclusive test for cholangiocarcinoma, it is difficult to advise either biliary resection (for isolated cholangiocarcinoma) or liver transplant (for PSC with dominant stricture).

(ultrasound, MRI, MRCP, CT) can detect biliary obstruction, stricture and mass lesions, but cannot confirm malignancy. Cytology from endoscopic retrograde cholangiopancreatography (ERCP) brushings fails to demonstrate malignancy in 50% of cases, owing to the desmoplastic nature of CCA (Khan et al., 2005); this can be improved to 40-70% by combining with targeted biopsies using cholangioscopy in specialist centres (Khan et al., 2012). In PSC strictures, the occurrence of polysomy [detected by fluorescent in-situ hybridisation (FISH)] is 88% specific for CCA (Bangarulingam et al., 2010), and can precede radiological and pathological changes by up to 2.7 years (Barr Fritcher et al., 2011). However, FISH is not yet part of routine workup in all centres, including those in the UK.

Treatment

Treatment options for CCA are few, and include surgery, limited pharmacotherapeutics and endobiliary therapies. Surgical resection is the only curative treatment for CCA (Friman, 2011), but a minority of cases are amenable to surgery, with most precluded by metastasis. Post-resection 5-year survival is only 20-30% (reviewed by Rosen et al., 2010). Liver transplantation was previously associated with poor survival and is hence contraindicated. However, recent data from the United States have shown that transplantation with neoadjuvant chemoradiotherapy in a carefully selected patient subgroup results in a 70% 5-year survival, and is therefore a re-emerging treatment option (Darwish Murad et al., 2012).

Because more than 50% of patients have lymph node metastases at presentation, effective chemotherapeutic regimes are vital in treating CCA. The UK NCRI ABC-01 and ABC-02 (Phase III) trials demonstrated that combination chemotherapy with cisplatin and gemcitabine improved progression-free survival and quality of life (Valle et al., 2009), although overall survival remained poor. The efficacy of adjuvant chemotherapy after resection is unproven, although ongoing trials will report soon (e.g. BILCAP, <http://public.ukcrn.org.uk>, UKCRN ID 1473).

In unresectable CCA of the main or common ducts, endoscopic stenting allows drainage, which relieves itch and sepsis. However, tumour ingrowth and obstruction frequently occur, and repeat procedures are

(Hakamada et al., 1997; Söreide et al., 2004). Chronic intraductal gallstones and hepatolithiasis are particularly linked to CCA in Asia (10% of patients with this problem develop CCA), but are a much weaker risk factor in Western countries (Khan et al., 2012). Thorotrast, a radiological contrast agent that has not been used for 50 years, increases the risk of CCA 100-fold, but it is unlikely that additional cases associated with this agent will emerge (Tyson and El-Serag, 2011).

Several different infections are also associated with CCA. In areas of south-east Asia, where the incidence of CCA is high, biliary infestation with liver flukes (including *Opisthorchis viverrini*, *Clonorchis sinensis* and *Schistosomiasis japonica*) is endemic. High faecal egg loads and high titres of fluke-specific antibodies are associated with a 14- and 27-fold increase in CCA risk, respectively, although the mechanism of cholangiocarcinogenesis is unknown (Hughes et al., 2006). The prevalence of hepatitis C (HCV) and/or hepatitis B (HBV) is several times higher in the CCA population than the general population (Kobayashi et al., 2000; Donato et al., 2001; Chuang et al., 2009; Zhou et al., 2008; Lee et al., 2009a), and infection with HCV conveys a hazard ratio

of 2.55 for IHCC (El-Serag et al., 2009). Cryptosporidiosis, a common human pathogen, has been implicated in both CCA and gastric cancer (Certad et al., 2012). Finally, chronic carriers of typhoid have a sixfold increase in CCA incidence (Abdel Wahab et al., 2007).

Presentation and diagnosis

CCA usually presents late in the course of disease, with general malaise and symptoms of biliary obstruction such as jaundice and biliary sepsis. Thereafter, the patient usually deteriorates quickly and, without treatment, death tends to occur from sequelae of obstruction. Currently, diagnosis is based on a combination of modalities, but is still sometimes only confirmed by a resection specimen. The only currently available serum biomarker, carbohydrate antigen 19-9 (Ca19-9; also known as sialylated Lewis antigen), is a poor diagnostic marker, with sensitivity of 40-70%, specificity of 50-80% and positive predictive value of 16-40% (reviewed by Khan et al., 2012). This is in part because 10% of individuals lack the Lewis antigen so cannot produce Ca19-9. Moreover, Ca19-9 can be expressed in any obstructive jaundice, and cannot therefore discriminate CCA from pancreatic or gastric malignancy. Imaging

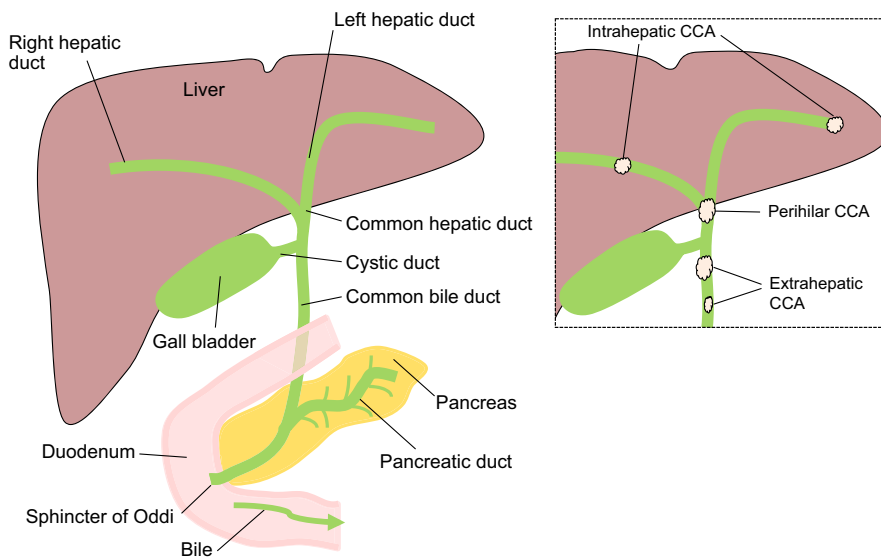


Fig. 1. Anatomy of the biliary tree showing the position of the different categories of CCA. The bile ducts carry bile from its site of production in the liver parenchyma to the duodenum. Primary malignancy of the endothelium of these ducts forms CCA. CCA is classified as intrahepatic, perihilar or extrahepatic, according to its position relative to the bifurcation of the hepatic ducts (see inset). Cellular proliferation of the duct lining causes it to become narrowed and strictured, obstructing bile flow, and causing jaundice. By this point the disease is usually advanced.

common if the patient survives beyond 6 months. Improvements in stent technology could decrease procedure frequency. Endobiliary radio-frequency ablation (RFA) coupled with stenting has been shown to be safe (Steel et al., 2011), and the results of long-term efficacy studies are eagerly awaited.

To improve the outlook for individuals with CCA, both clinical and bench science are imperative. In the rest of this article, we first review existing models of CCA, and then go on to discuss the complex and inter-related aspects of CCA pathophysiology.

Currently available models

Cell lines

Cholangiocarcinoma cell lines were first reported in 1985, when HChol-Y1 (immortalised from a patient with IHCC) was characterised and was shown to be xenotransplantable and to increase serum Ca19-9 and CEA (carcinoembryonic antigen, a marker raised in several cancers including pancreatic, hepatic and gastrointestinal) in recipient mice (Yamaguchi et al., 1985). More recently, the non-tumorigenic rat cholangiocyte line BDE1 has been transformed to constitutively express activated Neu, the rat homologue of the receptor tyrosine kinase ErbB2 (see below). Although results from tissue studies of ErbB2 expression in human tumours are

inconsistent, elevated ErbB2 is reportedly present in bile from patients suffering from CCA (Su et al., 2001), and in the ducts of high-risk patients (i.e. those with PSC, hepatolithiasis, etc.) (Endo et al., 2002). ErbB2 promotes carcinogenesis by activating p42/44 mitogen-activated protein kinase (MAPK) signalling (driving CCA cell proliferation), as well as phosphatidylinositol 3-kinase (PI3K)-Akt signalling (increasing CCA cell survival and resistance to apoptosis) (reviewed by Sirica, 2008). The BDEneu line is highly malignant and shows the molecular features of moderately differentiated CCA (Lai et al., 2005). It is also highly amenable to *in vitro* studies. The BDE1 parent line has also given rise to a less aggressive phenotype, by selection of spontaneous transformants developing after repeated cycling (Sirica et al., 2008). The availability of two malignant lines derived from one parent line offers unique opportunities for comparative studies.

Although several other CCA cell lines have been developed (see Table 1), standard cell culture methods do not reflect the molecular milieu, microenvironment and architecture inhabited by the native cholangiocyte. Thus, researchers are turning to more sophisticated methods, including tissue-engineering and 3D modelling, to study CCA. In EHCC research, bile-duct

tissue engineering has focussed on producing a functional prosthetic conduit for bile flow, rather than on modelling pathology (Miyazawa et al., 2012). In IHCC, the Sirica group recently reported a novel organotypic model using a 3D collagen gel matrix to support co-cultured clonal CCA cells and clonal α -smooth muscle actin (α -SMA)-positive stromal cancer-associated fibroblasts (CAFs). The cells used to form this model were derived from orthotopic IHCC formed by bile-duct inoculation of tumorigenic rat BDEsp transformed cholangiocytes (Campbell et al., 2012; Sirica et al., 2009). α -SMA-positive human stromal cell lines have also been extracted from patient resection specimens (Utispan et al., 2010), and establishing a similar organotypic system with human-derived tumour and stromal cells is a worthwhile aim.

Animal models

Although animal models of CCA exist, none recapitulate the human disease. In particular, few models carrying CCA-related mutations have been explored, with the exceptions being p53-deficient mice, which readily develop CCA on exposure to carbon tetrachloride (Farazi et al., 2006), and mice with liver-specific deficiency of SMAD4 and PTEN, which develop IHCC (Xu et al., 2006). Other genes known to be commonly mutated in human CCA, such as *KRAS*, have yet to be fully explored in modelling work (Hezel et al., 2010). Animal models of PSC are similarly elusive, as recently reviewed (Pollheimer et al., 2011).

In terms of modelling IHCC, xenografts created by transplanting human or cell-line material into rodents have been used extensively in assessing potential therapeutic agents; unfortunately, the results in such models correlate poorly with clinical outcome (reviewed by Sausville and Burger, 2006; Voskoglou-Nomikos et al., 2003; Fava, 2010). Earlier models of CCA relied on natural cholangiocarcinogenesis of inoculated *Opisthorchis viverrini* to induce biliary malignancy in rodents (Tesana et al., 2000), or the carcinogenic compounds furan, thioacetamide and dimethylnitrosamine (Lai and Sirica, 1999; Yeh et al., 2004; Thamavit et al., 1993). More recently, biliary inoculation of rodents with tumorigenic BDEneu or BDEsp cells has been shown to result in IHCC growth, reflecting progressive and early disease, respectively (Sirica et al., 2008). Although application of sorafenib in

Box 1. Clinical terms

Adenocarcinomas: histologically classified malignant lesions where glandular structures are visible or where the tumour is derived from glandular epithelium.

Endoscopic retrograde cholangiogram (ERC): endoscopic procedure using a flexible camera tube passed via the mouth to the small intestine, whereby smaller tubes and therapeutic devices can be inserted via the natural orifice of the biliary papilla into the biliary tree. There is a risk of complications (perforation, bleeding, pancreatitis), so this is usually reserved for cases in which imaging alone cannot suffice.

Fluorescent in-situ hybridisation (FISH): a cytogenetic technique used to detect and localize the presence or absence of specific DNA sequences on chromosomes by applying fluorescent probes that bind to specific complementary motifs. In PSC strictures, probes specific to each chromosome are used to allow chromosomal counting for polyploidy and aneuploidy (abnormal numbers of each chromosome per cell), which indicate developing malignancy.

Magnetic retrograde cholangiopancreatogram (MRCP): magnetic resonance imaging (MRI) optimised for imaging of the biliary and pancreatic duct system; has largely taken over from ERCP as a diagnostic test owing to a low incidence of complications.

Neoadjuvant chemoradiotherapy: drug and radiation treatment given before a surgical procedure with the intent of decreasing the size or spread of the tumour and improving outcome.

Perinuclear anti-neutrophil cytoplasmic antibodies (p-ANCA): used in diagnosis of inflammatory conditions such as vasculitis, arteritis and primary sclerosing cholangitis (PSC; see below). The perinuclear pattern occurs as the targeted antigens (including myeloperoxidase, lactoferrin, elastase, cathepsin) are overidically cationic at physiological pH, so are attracted to the negative charge of nuclear DNA during ethanol fixation. 80% of PSC patients are p-ANCA positive, and the test is used in combination with other modalities (imaging, biochemical tests) to support a diagnosis.

Primary sclerosing cholangitis (PSC): benign inflammatory fibrosing disease of the biliary tree; associated with other inflammatory diseases such as colitis and tends to arise in the third decade of life. Obstruction of biliary outflow by fibrosis leads to jaundice, infection and chronic liver disease.

Radiofrequency ablation (RFA): uses high-frequency alternating current to generate heat that can destroy pathological tissue. Novel technology allows endobiliary application to allow direct targeting and application to bile duct lesions.

Stent: a device for maintaining patency of a biological passageway; for biliary cases, this is deployed via ERCP to allow bile to drain; can be a plastic tube or self-expanding metal.

Stricture or stenosis: narrowing of the lumen (internal space) of a vessel, duct or hollow organ due to scarring/fibrosis of the structure's walls or compression from an external structure.

specificity of 88% for diagnosing CCA, and a sensitivity of 45% and specificity of 96% when combined with Ca19-9 (Chapman et al., 2011). In addition, mRNA and microRNA (miRNA) analyses in resected tissue and bile sediment are promising strategies for biomarker discovery (Karakatsanis et al., 2011; Shigehara et al., 2011). Successful characterisation of mRNA in biliary brushings has demonstrated CCA-specific expression profiles, with alterations in genes including various HOX genes, collagens, *PVT1*, *MUCA*, *MUC5AC* and *LEF1* (Chapman et al., 2012). Other recent advances include improvements in proteomic analysis of bile fluid by LC-MS/MS (Farina et al., 2011; Zabron et al., 2011) and capillary electrophoresis (Lankisch et al., 2011). Finally, NMR-based small-molecule analysis of urine identified diagnostic candidates for hepatocellular carcinoma (Shariff et al., 2011); a similar approach could be successful in CCA. These findings might form the basis of future biomarker panels, although further work is still required before they can be translated to the clinic.

Current understanding of CCA pathophysiology

Dissection of the underlying molecular mechanisms of CCA has begun to reveal that a background of chronic inflammation – providing a high rate of cell turnover and a rich milieu of cytokines and growth factors – allows the accumulation of mutations (Table 2) and the proliferation of mutated cells. CCA can also arise in the absence of chronic inflammation, however, through mutations that alter pathways in a manner similar to chronic inflammation. These alterations include stimulation of angiogenesis, as well as changes in various mechanisms (e.g. cell migration, interactions with stromal tissues, exposure to bile acids, cell cycle and apoptosis). Some of these inflammatory and non-inflammatory mechanisms are described below.

Angiogenesis: VEGF

CCA requires a rich vascular bed. Although minimal information is available on angiogenesis in CCA, it is known that high levels of vascular endothelial growth factor (VEGF) are expressed by various human CCA cell lines (KMC-1, KMC-2, KMBC and KMG-C) and tumour tissues (Ogasawara et al., 2001). Studies of xenografted cells have shown that endothelin 1 (ET-1) inhibits

these parallel models demonstrated therapeutic benefit and allowed mechanistic explorations (Blechacz and Gores, 2008), a recent Phase II trial of sorafenib in humans failed to demonstrate benefit and was terminated early (El-Khoueiry et al., 2012).

This trial in particular has reinforced the limited translatability of current models. The generation of new models that enable delineation of important signalling pathways in CCA, and others that emulate important CCA risk factors (such as PSC), will provide a more reliable reflection of CCA pathology and allow us to better predict the effects of new interventions.

Human samples

Despite the fact that working with human samples has 'real world' relevance, there is large inter-patient variability, and controlling for 'real world' variables is an ongoing

challenge. Nevertheless, human samples are particularly relevant for researching biomarkers. There have been several recent advances in this area by analysing various types of samples and using several techniques. Metabolomic analysis of bile by nuclear magnetic resonance (NMR) has identified differences in lipids and bile salts in CCA patients (Hashim Abdalla et al., 2011). Proteomic urinary capillary electrophoresis recently demonstrated abundant interstitial collagen fragments in CCA compared with PSC, with a 42-peptide panel giving an area under the curve (AUC) of 0.87, indicating good sensitivity and specificity (Metzger et al., 2012). However, because panel size is rather large it might be wise to await further corroboration before judging its usefulness. Recently, serum cytokeratin 19 fragment 21-1 (CYFRA 21-1) has been demonstrated to have a sensitivity of 56% and

Table 1. Cell lines derived from human and animal CCA

Cell line	Derived from	Construction	Comments	Reference
BDEsp	Rat cholangiocyte BDE1	Spontaneous transformation	Slow-growing; models early disease	Sirica et al., 2008b
BDEneu	Rat cholangiocyte BDE1	Retroviral vector Glu664-neu containing rat <i>Neu</i> oncogene	Rapid-growing; models advanced disease	Lai et al., 2005
Choi-CK	Human IHCC	Cultured	Well-differentiated CCA	Kim et al., 2001
HChol-Y1	Human resection specimen	Cultured		Yamaguchi et al., 1985
HuCC-T1	Human cholangiocarcinoma cells from malignant ascites	Cultured	Secrete Ca19-9, carbohydrate antigen 125, carcinoembryonic antigen, thrombin plasminogen activator	Miyagiwa et al., 1989
HuH-28	Human resected CCA	Cultured	Slow growing	Kusaka et al., 1988
ICBD-1	Human EHCC	Cultured	Poorly differentiated adenocarcinoma	Takiyama et al., 1998
KKU-100	Opisthorchiasis-associated human EHCC	Cultured	Poorly differentiated tubular adenocarcinoma	Sripa et al., 2005
KMBC	Human EHCC	Serial transplant to nude mouse; cultured		Yano et al., 1992
SCK	Human IHCC	Cultured	Sarcomatous	Kim et al., 2001
SK-ChA-1	Human EHCC malignant ascites	Cultured		Knuth et al., 1985
SNU-245	Human EHCC	Cultured		Ku et al., 2002
SNU-1196	Human invasive hilar EHCC	Cultured	Carries deletions in <i>p15</i> , <i>p16</i> , <i>p53</i>	
SNU-1079	Human IHCC	Cultured	Carries deletions in <i>p15</i> , <i>p16</i> ,	
TFK-1	Human EHCC, papillary adenocarcinoma and tubular adenocarcinoma	Cultured		Saijyo et al., 1995
TK	Human cholangiocarcinoma ascites	Cultured		Watanabe et al., 2000
WB-F344	Rat epithelial stem-like cells	Chemical and spontaneous transformation	Gives rise to a variety of CCA subtypes and other malignancies on transplant	Tsao et al., 1984; Hooth et al., 1998

VEGF-A and VEGF-C expression or release, which reduces cell proliferation and increases fibrosis and apoptosis in tumour tissue. The ET-1 receptor antagonist bosentan has been suggested as a possible therapeutic agent (Fava et al., 2009). It has been shown that increased VEGF might operate via an autocrine loop to increase malignant cell proliferation (Gaudio et al., 2006) in response to increased histamine production by the activation of H1HR receptors by histidine decarboxylase (HDC). It has been suggested that interrupting this autocrine loop by inhibiting HDC might offer therapeutic benefit (Francis et al., 2012).

EMT in CCA

Epithelial-mesenchymal transition (EMT) is a key process in the development of many cancers, resulting in cellular rearrangements and a motile fibroblastic phenotype that is adapted to invasion. EMT has recently been demonstrated in IHCC, and mirrors that seen in other malignancies, with cells having an aggressive, dedifferentiated phenotype and increased motility. Epithelial markers E-cadherin and α - and β -catenin are downregulated in these cells, whereas

markers such as N-cadherin, S100A4 and vimentin, are upregulated (Sirica et al., 2009; Yao et al., 2012; Seol et al., 2011). These changes correlate with increased invasiveness *in vitro*, and with metastasis and poorer prognosis *in vivo* (Sirica et al., 2009; Fabris et al., 2011; Yao et al., 2012).

Both epidermal growth factor (EGF) (see below) and transforming growth factor β (TGF β) signalling are implicated in EMT in CCA. In other cancers, receptor binding by TGF β causes SMAD2 and SMAD3 phosphorylation and their heterodimerisation with SMAD4. These heterodimers translocate to the nucleus and regulate target gene transcription, resulting in upregulated expression of *Snail*, *Slug* and *Twist*, which in turn promote EMT (reviewed by Bieri and Moses, 2006). In CCA cell lines, TGF β alters cell morphology from epithelial to mesenchymal, induces *Snail* and vimentin expression (Sato et al., 2010), and triggers a 'cadherin-switch' from E- to predominantly N-cadherin expression (Araki et al., 2011). When CCA cell lines are implanted as xenografts, TGF β treatment increases invasiveness (Sato et al., 2010). In addition, the change to N-cadherin predominance in

tissue correlates with disease progression and poor prognosis (Araki et al., 2011).

The receptor tyrosine kinase Met and its ligand, hepatocyte growth factor (HGF; also known as scatter factor), are also associated with EMT. Both of these proteins are more highly expressed in CCA than in normal tissue, and Met expression is associated with increased cell migration and invasiveness (Furge et al., 2000; Lee et al., 2009b). Met binding to HGF triggers a network of intracellular signals that accelerate tumour growth and spread (reviewed by Socoteanu et al., 2008).

MMPs in CCA

In addition to EMT, several other processes might promote cell motility and invasiveness in CCA. In particular, CCA is associated with increased levels of matrix metalloproteinases (MMPs), which break down the extracellular matrix (ECM) to allow tumour spread. Immunohistochemical analysis found that 48% and 76% of surgically resected specimens expressed MMP-9 and MMP-7, respectively (Itatsu et al., 2009; Itatsu et al., 2008), with MMP-7 being expressed by the malignant cholangiocytes themselves. Plasma MMP-7

Table 2. Selected genetic alterations associated with CCA for different cellular processes

Gene	Incidence	Functional outcome	Reference
Proliferation			
<i>KRAS</i>	50% of IHCC cases, 30% of EHCC cases, 30% of PSC cases	Gain-of-function mutations cause activation of the RAS pathway and increased proliferation	Ahrendt et al., 2000
<i>BRAF</i>	22% of CCA cases	Hot-spot substitution mutations cause activation of the MAPK pathway, increasing proliferation	Tannapfel et al., 2003
<i>KEAP1</i>	11% of gallbladder CCA cases	Mutations cause aberrant activation of NF-E2-related factor 2, and increased expression of phosphorylated p38-MAPK increases cell proliferation	Shibata et al., 2008
<i>RASSF1A</i>	65-69% of CCA cases	Promoter methylation causes activation of the RAS pathway, increasing proliferation and dysregulating cell-cycle control	Wong et al., 2002
<i>EGFR</i>	13-15% of CCA cases	Activating substitution and deletion mutations increase proliferation via multiple pathways (see main text for details)	Leone et al., 2006
Invasion and metastasis			
<i>CDH1</i>	Promoter mutations in 11% of IHCC cases	Promoter mutations are associated with invasion and metastasis	Endo et al., 2001
<i>AXIN1</i>	Substitution mutations in 41% of IHCC cases	Mutations increase invasion and metastasis	Tokumoto et al., 2005
Cell-cycle dysregulation			
<i>SMAD4</i>	45% of IHCC cases	Substitution mutations cause cell-cycle dysregulation through the TGF β pathway	Kang et al., 2002
<i>CDKNA2</i> (<i>p16INK4A</i>)	17-86% CCA cases	Loss-of-function mutation or methylation causes <i>TP53</i> inactivation and loss of cell-cycle control	Tannapfel et al., 2000a
<i>TP53</i>	37% IHCC cases	Dysregulation of cell-cycle arrest, resistance to apoptosis	Tannapfel et al., 2000b
Chronic inflammation			
<i>SOCS3</i>	60% of IHCC cases	Silencing by promoter methylation removes suppression of IL-6-STAT3 activation	Isomoto et al., 2007

was found to be increased in obstructed perihilar and intrahepatic CCA, and was suggested as a putative diagnostic biomarker with AUC 0.73 (Leelawat et al., 2009). Elevated tumour MMP-7 has also been suggested to be a marker of poor post-operative prognosis (Itatsu et al., 2008). Notably, MMP-9 requires stabilisation by binding to neutrophil gelatinase-associated lipocalin (NGAL), which is reportedly increased in bile in patients suffering from malignant pancreatobiliary obstruction (Zabron et al., 2011). NGAL knockdown in RMCCA1 cells (a CCA cell line) showed decreased invasiveness and migration *in vitro* (Nuntagowat et al., 2010).

EGFR and ErbB2

The ErbB receptor kinase family [of which epidermal growth factor receptor (EGFR) is the founding member] are potent and broad-spectrum mediators of cholangiocarcinogenesis. They interact with multiple other factors that contribute to cholangiocarcinogenesis, including COX-2, interleukin-6 (IL-6), VEGF and Met, activating not only their own signalling pathway but enhancing that of others. Although this makes understanding the part played by individual factors more challenging, it also suggests that

manipulation of ErbB activity with novel therapies could have a wide-reaching therapeutic benefit.

The four members of the ErbB family each have an extracellular ligand-binding domain, a transmembrane domain and an intracellular kinase domain. Ligand binding induces homo- or heterodimerisation, leading to transphosphorylation of the cytoplasmic tail, which regulates downstream signalling molecules. EGFR (ErbB1) binds several soluble ligands, including EGF, transforming growth factor- α (TGF α) and amphiregulin. In CCA, EGF operates through EGFR to activate p42/44 MAPK and increase CCA growth (Yoon et al., 2004). In 32% of CCA patient samples, an increase in EGFR staining was found (Nonomura et al., 1988), resulting from its increased expression or defective downregulation (Nonomura et al., 1988). Mutations in the tyrosine kinase domain of EGFR have been identified in CCA tissue in 15% of patient samples; these mutations correlate with increased phosphorylation of downstream MAPK or Akt (Leone et al., 2006).

In contrast to EGFR, ErbB2 cannot bind soluble ligands, but has a high basal kinase activity, and heterodimerises independently

of ligand binding. Overexpression of the ErbB2 homologue Neu in the rat cell line BDeneu results in highly tumorigenic cells, confirming the role of this receptor in cholangiocarcinogenesis (Lai et al., 2005). ErbB2-ErbB3 heterodimers are potent stimulators of mitogenesis and neoplasia owing to their ability to activate the p42/44 MAPK, extracellular regulated kinase (ERK) and PI3K-Akt pathways (thereby stimulating cellular proliferation), as well as to promote cell survival and inhibit apoptosis (reviewed by Sirica, 2008). As found for EGFR, immunohistochemistry was used to demonstrate increased expression of ErbB2 in CCA tissue and bile fluid (Endo et al., 2002; Su et al., 2001). Expression of ErbB2 is also increased in tissues of patients with PSC and hepatolithiasis (risk factors for CCA) (Endo et al., 2002).

Stromal factors

One of the characteristic histological findings in CCA is a dense desmoplastic stroma surrounding malignant glandular structures. This stroma is mostly made up of CAFs, which are histologically identified by immunoreactivity for α -SMA. In IHCC, increased numbers of α -SMA-positive CAFs

correlate with larger tumours and shorter patient survival, and conditioned medium from α -SMA-expressing CAFs stimulates greater proliferation of CCA cell lines than conditioned medium from other stromal cell populations (Campbell et al., 2012). Recently, it has been recognised that the stroma can directly influence the progression of CCA. A detailed review on this topic has recently been published (Sirica, 2012), so we limit our discussion below to the key points.

CAFs from CCA are known to produce various factors that can promote tumour progression. For example, periostin promotes the survival and invasion of CCA cell lines *in vitro*, and expression of periostin in resected tumours is a poor prognostic factor (Utispan et al., 2010; Sirica et al., 2009). Tenascin expression is also correlated with poor IHCC prognosis after resection, although its mechanism in IHCC is not clear (Sirica et al., 2009). Thrombospondin-1 overexpression in CAFs correlates with hypovascularity and metastasis (Tang et al., 2006), whereas galectin-1 expression is associated with dedifferentiation (Shimonishi et al., 2001). More is known about the mechanism of stromal-cell-derived factor-1, which is overexpressed at the CCA invasion front. Here, it activates the MEK1/2 and Akt pathways in IHCC cells via its cognate receptor C-X-C chemokine receptor 4 (CXCR4) (Leelawat et al., 2007). In addition, it has been shown that the mechanism whereby CAF-derived HGF promotes CCA cell motility and invasion *in vitro* involves E-cadherin dislocation and activation of the PI3K-Akt pathway (Menakongka and Suthiphongchai, 2010). WNT1-inducible-signalling pathway protein 1 (WISP1) is overexpressed in 49% of IHCC cases, and *in situ* hybridisation has shown *WISP1* mRNA expression in CAFs rather than in the malignant cells (Tanaka et al., 2003). This overexpression correlated with lymphatic and perineural invasion, and with poor prognosis and survival. Moreover, WISP1-conditioned medium was shown to stimulate migration of HuCCT1 CCA cells *in vitro* (Tanaka et al., 2003). It has been suggested that targeting these stromal factors in combination with CCA cell factors could improve therapeutic efficacy (Sirica, 2012).

Bile acids

Bile acids, which are produced by hepatocytes from cholesterol, are transported to the small intestine in the bile,

via the biliary tree, constantly bathing the biliary endothelium. In obstruction and cholestasis, which are associated with several risk factors for CCA, bile acids accumulate abnormally. Although bile acids have long been known to assist in nutrient absorption, their active roles in the regulation (and pathology) of the biliary endothelium in health and disease are the topic of recent and ongoing research. In particular, the bile-acid component deoxycholic acid activates EGFR (via TGF α) and induces COX-2 expression, activating p42/44 and p38 MAPK, as well as c-jun-N-terminal kinase (JNK) (Yoon et al., 2004); as discussed, activation of these pathways contributes to CCA. Deoxycholic acid also prolongs the activity of myeloid-cell leukaemia-1 (Mcl-1; a member of the pro-survival Bcl-2 protein family), promotes cellular proliferation and conveys resistance to Fas-induced apoptosis (Werneburg et al., 2003). However, not all bile acids are carcinogenic; for example, tauroursodeoxycholate (TUDCA) inhibits human CCA growth *in vitro* (Alpini et al., 2004). Further understanding of the mechanisms by which bile acids contribute to CCA might reveal targets for therapy.

Apoptosis and cell survival

Suppression of the normal mechanisms that eliminate cells that accumulate DNA damage is a crucial step in carcinogenesis. In CCA, this suppression generally involves mutation or dysregulation of proteins such as p53 and the anti-apoptotic protein Bcl-2. In CCA cell lines, Bcl-2 is overexpressed (Tadlock and Patel, 2001), preventing caspase-3 activation and conferring resistance to apoptosis. p53 mutation is seen in 20-61% of CCA cases (Khan et al., 2003; Nault and Zucman-Rossi, 2011), dysregulating cell-cycle arrest, allowing Bcl-2 expression and suppressing the normal apoptotic response. Tumour-necrosis-factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis is reduced in CCA via mechanisms involving Notch-1 and cyclooxygenase-2 (COX-2) (see below) (Kobayashi et al., 2005). TGF β regulates cell proliferation positively or negatively, mediated by down- or upregulation of the cyclin-dependent kinase inhibitor p21 (also known as Waf1), respectively (Miyazaki et al., 1998). Promoter methylation, which reduces transcription, and loss-of-function mutations in the cell-cycle regulators p14ARF and p16INK4a, and in β -catenin, occur less frequently in CCA

than in other cancers, but when present are thought to promote cell survival (Tannapfel et al., 2002).

Mechanisms associated with inflammation

An inflammatory milieu contributes to the development of CCA. High concentrations of inflammatory mediators cause or allow the accumulation of mutations in proto-oncogenes, DNA mismatch-repair genes and tumour suppressor genes, and directly promote cell proliferation. In particular, expression of activation-induced cytidine deaminase (AID), a member of the DNA-RNA editing family, is induced by pro-inflammatory cytokines via the nuclear factor- κ B (NF κ B) pathway, and AID is present at high levels in PSC and CCA biopsies. Aberrant AID expression in biliary cells results in somatic mutations in *p53*, *MYC* and *p16INK4A*, potentiating malignant transformation (Komori et al., 2008). There is also evidence that cell-mediated immune surveillance might be deficient in CCA. Analysis of single nucleotide polymorphisms (SNPs) in CCA in patients with underlying PSC identified two polymorphisms in *NKG2D* (natural killer group 2D-receptor), which is expressed in NK cells and T lymphocytes: carriers of either of these SNPs have odds ratios above 2 for developing CCA (Melum et al., 2008). Such polymorphisms remain to be identified in sporadic or fluke-related CCA.

In the sections below, we review the role of some specific inflammatory mediators and pathways in CCA.

IL-6

The cytokine IL-6 is dramatically upregulated in cultured CCA cells compared with normal biliary tract cells, and is regarded as a growth and survival factor (Sugawara et al., 1998; Isomoto et al., 2005). IL-6 has multiple effects in promoting CCA (Fig. 2). In normal cholangiocytes, activation of the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway by IL-6 enhances transcription of suppressor of cytokine signalling 3 (SOCS3), which mediates a classic negative feedback loop for IL-6 production. In CCA, SOCS3 expression is decreased (Isomoto et al., 2007). Methylation of the *SOCS3* promoter at CpG islands is increased in tumour tissues and in human CCA cell lines (Mz-ChA-1 and CCLP1), which correlates with decreased transcription-factor binding and SOCS3

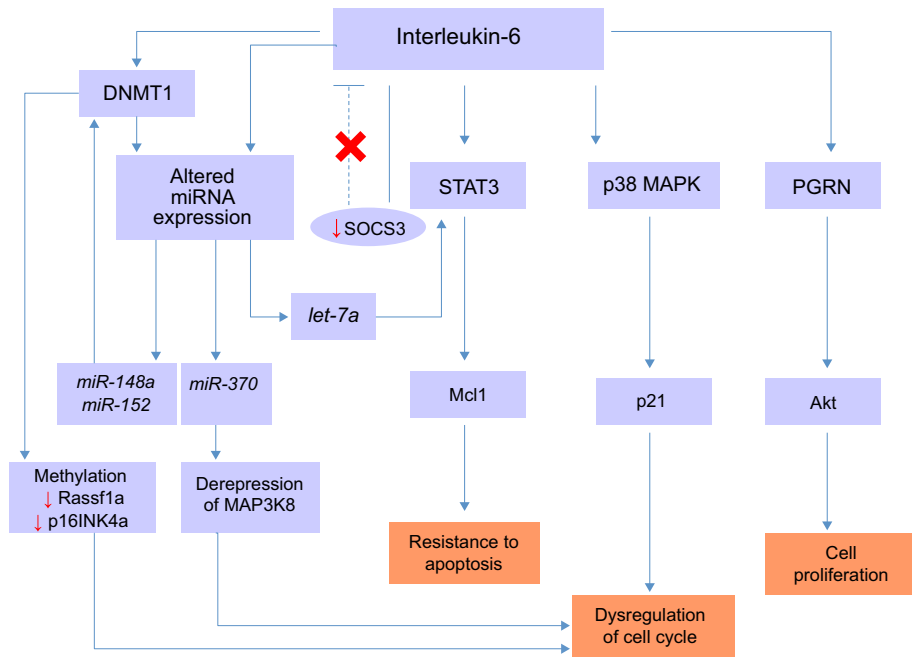


Fig. 2. IL-6 contributes to CCA through multiple pathways, linking chronic inflammation with the development of malignancy. IL-6-mediated activation of STAT-3 confers resistance to apoptosis via upregulating Mcl-1. SOCS3, a negative regulator of IL-6 signalling, seems to be downregulated in CCA. IL-6-mediated upregulation of p38 MAPK leads to dysregulation of the cell cycle, whereas increased PGRN increases cellular proliferation. IL-6 also alters the expression and activity of various miRNAs (only some are shown here), at least in part by upregulating DNMT1 and increasing miRNA methylation. These changes lead to downregulation of Rassf1a and p16INK4a, and derepression of MAP3K8, causing the dysregulation of cell-cycle control.

transcription (Isomoto et al., 2007). *In vitro*, demethylation re-establishes SOCS3 expression and decreases IL-6-dependent STAT3 phosphorylation (Isomoto et al., 2007). IL-6 is known to induce DNA methyltransferase-1 (DNMT1) expression in CCA cell lines via miRNAs (see below), and DNMT1 causes epigenetic modification of several oncogenes (Braconi et al., 2010). It has been demonstrated in colon cancer arising in ulcerative colitis (another inflammation-associated malignancy) that SOCS3 is silenced by DNMT1 (Li et al., 2012); it would be interesting to investigate whether this is also the case in CCA.

IL-6-induced phosphorylation and activation of STAT3 causes transcriptional upregulation of Mcl-1 (Kobayashi et al., 2005), which desensitises CCA cells to TRAIL-mediated apoptosis, an effect that is reversed when autonomous IL-6 signalling is interrupted by SOCS3 re-expression (Isomoto et al., 2007). Disrupting this pathway is a promising therapeutic strategy: indeed, a multikinase inhibitor, sorafenib, which induces STAT3 dephosphorylation, resensitised CCA cells to TRAIL-mediated

apoptosis both *in vitro* and in an orthotopic model (Blechacz et al., 2011). However, this agent did not have the same effect in early human trials (El-Khoueiry et al., 2012).

IL-6 induces increased expression of progranulin (PGRN) in CCA compared with non-malignant cholangiocytes (Frampton et al., 2012). Increased PGRN expression is seen in several tumour types, and correlates with high tumorigenicity and in some cases poor prognosis [e.g. epithelial ovarian cancer (Han et al., 2011)]. In CCA cell lines, increased PGRN expression is independent of the JAK-STAT pathway, and instead occurs in response to IL-6-mediated phosphorylation of ERK1 or ERK2 and subsequent activation of ribosomal S6 kinase-1 (RSK-1). Activated RSK-1 phosphorylates the transcription factor C/ERPβ, which binds the PGRN promoter (Frampton et al., 2012). Furthermore, recombinant PGRN increased proliferation of the same CCA cell lines through an Akt-dependent pathway. Notably, Akt is also involved in several pathways promoting CCA tumorigenesis that are independent of PGRN. For example, prostaglandin E2 (derived from COX-2

activation during inflammation) promotes CCA cell growth and invasion through prostaglandin E2 receptor EP1-mediated activation of Akt and EGF (Han and Wu, 2005). Furthermore, liver-specific disruption of the tumour suppressors SMAD4 (also known as DPC4) and PTEN leads to increased Akt phosphorylation and the development of CCA (Xu et al., 2006).

In addition to its anti-apoptotic and pro-proliferative roles, increased IL-6 production in CCA also dysregulates cell-cycle control through activating p38 MAPK, which decreases expression of the cell-cycle regulator p21 and is mitogenic (Tadlock and Patel, 2001).

NO and COX-2

Several lines of evidence support a role for COX-2 in cholangiocarcinogenesis. COX-2 is a common inflammatory mediator, whose expression is induced by cytokines and lipopolysaccharide, resulting in the synthesis of inflammatory prostaglandins. COX-2 directly links cholestasis and inflammation, because oxysterols (derived from cholesterol and present at high levels in cholestatic bile) stabilise COX-2 mRNA in human cholangiocytes (Yoon et al., 2004). Cholangiocytes in PSC, where risk of developing CCA is high, have high levels of COX-2 compared with another chronic inflammatory biliary disease, primary biliary cirrhosis (PBC), where CCA risk is normal (Sirica, 2005). Overexpression of COX-2 or treatment with prostaglandin E2 (a downstream mediator of COX-2) enhances the growth of human CCA cells, an effect that is reversed by COX-2 antisense depletion (Han et al., 2004). In CCA cell lines, oxysterol-induced COX-2 accumulation results in cellular proliferation and inhibition of apoptosis, whereas COX-2 inhibitors (e.g. celecoxib) induce apoptosis and decrease Akt phosphorylation (Yoon et al., 2004; Zhang et al., 2004). Celecoxib also inhibits proliferation in CCA cells via activation of the cyclin-dependent kinase inhibitors p21 and p27 (KIP1), arresting cells at G1-S (Han et al., 2004).

In vitro experiments with a mouse CCA cell line indicate that cholangiocyte COX-2 expression is regulated in part via inducible nitric oxide synthase (iNOS) and nitric oxide (NO) (Ishimura et al., 2004). iNOS is expressed in response to inflammatory cytokines. Indeed, *Opisthorchis viverrini* expresses iNOS, although whether this promotes CCA remains to be seen

(Prakobwong et al., 2012). As well as upregulating COX-2 expression, iNOS acts independently of COX-2, by increasing NO production. NO counteracts effective DNA repair and allows the accumulation of mutations (Jaiswal et al., 2001). iNOS and NO also upregulate Notch-1, which interacts with COX-2 to reduce the susceptibility of cholangiocytes to TRAIL-mediated apoptosis (Ishimura et al., 2005). Aberrant expression of Notch receptors is seen in EHCC and IHCC, although the mechanism by which this promotes cholangiocarcinogenesis remains to be elucidated (Yoon et al., 2011; Sekiya and Suzuki, 2012).

MicroRNAs

Information on the role of miRNAs in CCA – mechanistically, and as diagnostic markers and therapeutic targets – has recently been reviewed (Papaconstantinou et al., 2012). Several miRNAs are upregulated (*miR-141*, *miR-200b*, *miR-21*, *let-7a*) (Meng et al., 2006; Meng et al., 2007) or downregulated [*miR-370*, *miR-29b* (Meng et al., 2008; Mott et al., 2007), *miR-148a*, *miR-152* and *miR-301* (Braconi et al., 2010)] in CCA. Nascent mechanistic understanding suggests that these changes in miRNA expression increase CCA proliferation and survival: for example, *miR-141* decreases *CLOCK* expression, which disinhibits cell proliferation (Meng et al., 2006). Cell line transfection studies showed that *miR-200b* causes increased expression of protein tyrosine phosphatase nonreceptor type 12 (PTPN12), which deactivates Ras signalling and promotes survival (Meng et al., 2006). *miR-21* transfection decreases expression of PTEN, disinhibiting PI3K signalling and desensitising cells to death signals (Meng et al., 2006). Expression of *let-7a* miRNA occurs in response to IL-6 and suppresses neurofibromatosis-2 (NF2) expression, resulting in increased STAT3 phosphorylation (Meng et al., 2007). IL-6-mediated downregulation of *miR-148a* and *miR-152* increases DNMT1 activity, thereby decreasing the expression of the methylation-sensitive tumour-suppressor genes *Rassfla* and *p16INK4a* (Braconi et al., 2010). Finally, IL-6-dependent downregulation of *miR-370* results in de-repression of mitogen-activated protein kinase kinase kinase 8 (MAP3K8) expression (Meng et al., 2008).

Clinical and basic research opportunities

- To develop animal models with mutations in key genes implicated in cholangiocarcinogenesis.
- To apply new developments in materials science and nanotechnology to develop better *in vitro* 3D multilineage models of IHCC and EHCC.
- To explore the relationship between the liver fluke and its endobiliary habitat, and identify factors that trigger malignant transformation in the host.
- To develop *in vivo* and *in vitro* models of PSC that reflect both the benign inflammatory condition and its contribution malignant transformation.
- To combine biostatistics with multiple biological, biochemical and biophysical techniques to analyse human samples of bile, blood and protein and develop accurate diagnostic tests.
- To apply expanding knowledge of cholangiocarcinogenesis to drug development for CCA.

The future

Recent years have seen an expansion in basic research on CCA, meaning that pathways of cholangiocarcinogenesis are now beginning to be delineated. However, we are still unable to provide an early and definitive diagnosis of CCA, to predict which patients with high-risk conditions will go on to develop the disease, or to provide effective targeted therapy for any patient group. Thus, more work is urgently needed to identify diagnostic biomarkers and to design effective pharmacotherapies using existing and new models. A multidisciplinary approach that brings together biologists and engineers, clinicians and statisticians, parasitologists and materials scientists, among others, is required to tackle this insidious disease.

In analysing human samples, multi-modal analysis of multiple sample types, together with application of systems biology and bioinformatics approaches, is most likely to identify the next generation of biomarkers and diagnostic tests. Although various *in vivo* and *in vitro* models have been developed, they have thus far fallen short in translating useful ideas to the clinic. New animal models carrying specific mutations will provide important insights, but humanised *in vitro* models that mirror the molecular ecosystem of cancer-stroma interactions in a 3D matrix will probably provide the most significant advances. In terms of treatment strategies, our rapidly expanding understanding of genetic mechanisms will hopefully be translated into therapeutic siRNA- or miRNA-based drugs, possibly allowing a local application and avoiding systemic side effects.

The insidious and complex nature of CCA will continue to challenge us in understanding its pathophysiology, and the need for new models, innovative approaches and talented researchers is immense.

COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS

A.Z. drafted the original manuscript with help from R.J.E. S.A.K. provided the structure and overall management of the manuscript.

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REFERENCES

- Abdel Wahab, M., Mostafa, M., Salah, T., Fouad, A., Kandeel, T., Elshobary, M., Abd Allah, O. F., Elghawalby, N., Sultan, A. and Ezzat, F. (2007). Epidemiology of hilar cholangiocarcinoma in Egypt: single center study. *Hepatogastroenterology* **54**, 1626-1631.
- Ahrendt, S. A., Rashid, A., Chow, J. T., Eisenberger, C. F., Pitt, H. A. and Sidransky, D. (2000). p53 overexpression and K-ras gene mutations in primary sclerosing cholangitis-associated biliary tract cancer. *J. Hepatobiliary Pancreat. Surg.* **7**, 426-431.
- Alpini, G., Kanno, N., Phinizz, J. L., Glaser, S., Francis, H., Taffetani, S. and LeSage, G. (2004). Tauroursodeoxycholate inhibits human cholangiocarcinoma growth via Ca2+, PKC-, and MAPK-dependent pathways. *Am. J. Physiol. Gastrointest. Liver Physiol.* **286**, G973-G982.
- Araki, K., Shimura, T., Suzuki, H., Tsutsumi, S., Wada, W., Yajima, T., Kobayashi, T., Kubo, N. and Kuwano, H. (2011). E/N-cadherin switch mediates cancer progression via TGF- β -induced epithelial-to-mesenchymal transition in extrahepatic cholangiocarcinoma. *Br. J. Cancer* **105**, 1885-1893.
- Bangarulingam, S. Y., Bjornsson, E., Enders, F., Barr Fritcher, E. G., Gores, G., Halling, K. C. and Lindor, K. D. (2010). Long-term outcomes of positive fluorescence in situ hybridization tests in primary sclerosing cholangitis. *Hepatology* **51**, 174-180.
- Barr Fritcher, E. G., Kipp, B. R., Voss, J. S., Clayton, A. C., Lindor, K. D., Halling, K. C. and Gores, G. J. (2011). Primary sclerosing cholangitis patients with serial polysomy fluorescence in situ hybridization results are at increased risk of cholangiocarcinoma. *Am. J. Gastroenterol.* **106**, 2023-2028.
- Bierie, B. and Moses, H. L. (2006). Tumour microenvironment: TGF β : the molecular Jekyll and Hyde of cancer. *Nat. Rev. Cancer* **6**, 506-520.
- Blechacz, B. and Gores, G. J. (2008). Cholangiocarcinoma: advances in pathogenesis, diagnosis, and treatment. *Hepatology* **48**, 308-321.
- Blechacz, B., Komuta, M., Roskams, T. and Gores, G. J. (2011). Clinical diagnosis and staging of cholangiocarcinoma. *Nat. Rev. Gastroenterol. Hepatol.* **8**, 512-522.
- Braconi, C., Huang, N. and Patel, T. (2010). MicroRNA-dependent regulation of DNA methyltransferase-1 and tumor suppressor gene expression by interleukin-

- 6 in human malignant cholangiocytes. *Hepatology* **51**, 881-890.
- Burak, K., Angulo, P., Pasha, T. M., Egan, K., Petz, J. and Lindor, K. D.** (2004). Incidence and risk factors for cholangiocarcinoma in primary sclerosing cholangitis. *Am. J. Gastroenterol.* **99**, 523-526.
- Campbell, D. J., Dumur, C. I., Lamour, N. F., Dewitt, J. L. and Sirica, A. E.** (2012). Novel organotypic culture model of cholangiocarcinoma progression. *Hepatol. Res.* **42**, 1119-1130.
- Certad, G., Benamrouz, S., Guyot, K., Mouray, A., Chassat, T., Flament, N., Delhaes, L., Coiteux, V., Delaire, B., Praet, M. et al.** (2012). Fulminant cryptosporidiosis after near-drowning: a human *Cryptosporidium parvum* strain implicated in invasive gastrointestinal adenocarcinoma and cholangiocarcinoma in an experimental model. *Appl. Environ. Microbiol.* **78**, 1746-1751.
- Chapman, M. H., Sandanayake, N. S., Andreola, F., Dhar, D. K., Webster, G. J., Dooley, J. S. and Pereira, S. P.** (2011). Circulating CYFRA 21-1 is a specific diagnostic and prognostic biomarker in biliary tract cancer. *J. Clin. Exp. Hepatol.* **1**, 6-12.
- Chapman, M. H., Tidswell, R., Dooley, J. S., Sandanayake, N. S., Cerec, V., Deheragoda, M., Lee, A. J., Swanton, C., Andreola, F. and Pereira, S. P.** (2012). Whole genome RNA expression profiling of endoscopic biliary brushings provides data suitable for biomarker discovery in cholangiocarcinoma. *J. Hepatol.* **56**, 877-885.
- Chuang, S. C., La Vecchia, C. and Boffetta, P.** (2009). Liver cancer: descriptive epidemiology and risk factors other than HBV and HCV infection. *Cancer Lett.* **286**, 9-14.
- Claessen, M. M., Vleggaar, F. P., Tytgat, K. M., Siersema, P. D. and van Buuren, H. R.** (2009). High lifetime risk of cancer in primary sclerosing cholangitis. *J. Hepatol.* **50**, 158-164.
- Darwish Murad, S., Kim, W. R., Harnois, D. M., Douglas, D. D., Burton, J., Kulik, L. M., Botha, J. F., Mezrich, J. D., Chapman, W. C., Schwartz, J. J. et al.** (2012). Efficacy of neoadjuvant chemoradiation, followed by liver transplantation, for perihilar cholangiocarcinoma at 12 US centers. *Gastroenterology* **143**, 88-98, e3, quiz e14.
- Donato, F., Gelatti, U., Tagger, A., Favret, M., Ribero, M. L., Callea, F., Martelli, C., Savio, A., Trevisi, P. and Nardi, G.** (2001). Intrahepatic cholangiocarcinoma and hepatitis C and B virus infection, alcohol intake, and hepatolithiasis: a case-control study in Italy. *Cancer Causes Control* **12**, 959-964.
- El-Khoueiry, A. B., Rankin, C. J., Ben-Josef, E., Lenz, H. J., Gold, P. J., Hamilton, R. D., Govindarajan, R., Eng, C. and Blanke, C. D.** (2012). SWOG 0514: a phase II study of sorafenib in patients with unresectable or metastatic gallbladder carcinoma and cholangiocarcinoma. *Invest. New Drugs* **30**, 1646-1651.
- El-Serag, H. B., Engels, E. A., Landgren, O., Chiao, E., Henderson, L., Amaratunge, H. C. and Giordano, T. P.** (2009). Risk of hepatobiliary and pancreatic cancers after hepatitis C virus infection: A population-based study of U.S. veterans. *Hepatology* **49**, 116-123.
- Endo, K., Ashida, K., Miyake, N. and Terada, T.** (2001). E-cadherin gene mutations in human intrahepatic cholangiocarcinoma. *J. Pathol.* **193**, 310-317.
- Endo, K., Yoon, B. I., Pairojku, C., Demetris, A. J. and Sirica, A. E.** (2002). ERBB-2 overexpression and cyclooxygenase-2 up-regulation in human cholangiocarcinoma and risk conditions. *Hepatology* **36**, 439-450.
- Fabris, L., Cadamuro, M., Moserle, L., Dziura, J., Cong, X., Sambado, L., Nardo, G., Sonzogni, A., Colledan, M., Furlanetto, A. et al.** (2011). Nuclear expression of S100A4 calcium-binding protein increases cholangiocarcinoma invasiveness and metastasization. *Hepatology* **54**, 890-899.
- Farazi, P. A., Zeisberg, M., Glickman, J., Zhang, Y., Kalluri, R. and DePinho, R. A.** (2006). Chronic bile duct injury associated with fibrotic matrix microenvironment provokes cholangiocarcinoma in p53-deficient mice. *Cancer Res.* **66**, 6622-6627.
- Farina, A., Dumonceau, J. M., Delhay, M., Frossard, J. L., Hadengue, A., Hochstrasser, D. F. and Lescuyer, P.** (2011). A step further in the analysis of human bile proteome. *J. Proteome Res.* **10**, 2047-2063.
- Fava, G.** (2010). Molecular mechanisms of cholangiocarcinoma. *World J. Gastrointest. Pathophysiol.* **1**, 12-22.
- Fava, G., Demorrow, S., Gaudio, E., Franchitto, A., Onori, P., Carpino, G., Glaser, S., Francis, H., Coufal, M., Marucci, L. et al.** (2009). Endothelin inhibits cholangiocarcinoma growth by a decrease in the vascular endothelial growth factor expression. *Liver Int.* **29**, 1031-1042.
- Frampton, G., Invernizzi, P., Bernuzzi, F., Pae, H. Y., Quinn, M., Horvat, D., Galindo, C., Huang, L., McMillin, M., Cooper, B. et al.** (2012). Interleukin-6-driven progranulin expression increases cholangiocarcinoma growth by an Akt-dependent mechanism. *Gut* **61**, 268-277.
- Francis, H., DeMorrow, S., Venter, J., Onori, P., White, M., Gaudio, E., Francis, T., Greene, J. F., Jr, Tran, S., Meiningner, C. J. et al.** (2012). Inhibition of histidine decarboxylase ablates the autocrine tumorigenic effects of histamine in human cholangiocarcinoma. *Gut* **61**, 753-764.
- Friman, S.** (2011). Cholangiocarcinoma – current treatment options. *Scand. J. Surg.* **100**, 30-34.
- Furge, K. A., Zhang, Y. W. and Vande Woude, G. F.** (2000). Met receptor tyrosine kinase: enhanced signaling through adapter proteins. *Oncogene* **19**, 5582-5589.
- Gaudio, E., Barbaro, B., Alvaro, D., Glaser, S., Francis, H., Ueno, Y., Meiningner, C. J., Franchitto, A., Onori, P., Marziani, M. et al.** (2006). Vascular endothelial growth factor stimulates rat cholangiocyte proliferation via an autocrine mechanism. *Gastroenterology* **130**, 1270-1282.
- Hakamada, K., Sasaki, M., Endoh, M., Itoh, T., Morita, T. and Konn, M.** (1997). Late development of bile duct cancer after sphincteroplasty: a ten- to twenty-two-year follow-up study. *Surgery* **121**, 488-492.
- Han, C. and Wu, T.** (2005). Cyclooxygenase-2-derived prostaglandin E2 promotes human cholangiocarcinoma cell growth and invasion through EP1 receptor-mediated activation of the epidermal growth factor receptor and Akt. *J. Biol. Chem.* **280**, 24053-24063.
- Han, C., Leng, J., Demetris, A. J. and Wu, T.** (2004). Cyclooxygenase-2 promotes human cholangiocarcinoma growth: evidence for cyclooxygenase-2-independent mechanism in celecoxib-mediated induction of p21waf1/cip1 and p27kip1 and cell cycle arrest. *Cancer Res.* **64**, 1369-1376.
- Han, J. J., Yu, M., Houston, N., Steinberg, S. M. and Kohn, E. C.** (2011). Progranulin is a potential prognostic biomarker in advanced epithelial ovarian cancers. *Gynecol. Oncol.* **120**, 5-10.
- Hashim Abdalla, M. S., Taylor-Robinson, S. D., Sharif, A. W., Williams, H. R., Crossey, M. M., Badra, G. A., Thillainayagam, A. V., Bansil, D. S., Thomas, H. C., Waked, I. A. et al.** (2011). Differences in phosphatidylcholine and bile acids in bile from Egyptian and UK patients with and without cholangiocarcinoma. *HPB (Oxford)* **13**, 385-390.
- Hezel, A. F., Deshpande, V. and Zhu, A. X.** (2010). Genetics of biliary tract cancers and emerging targeted therapies. *J. Clin. Oncol.* **28**, 3531-3540.
- Hooth, M. J., Coleman, W. B., Presnell, S. C., Borchert, K. M., Grisham, J. W. and Smith, G. J.** (1998). Spontaneous neoplastic transformation of WB-F344 rat liver epithelial cells. *Am. J. Pathol.* **153**, 1913-1921.
- Hughes, N. R., Pairojku, C., Royce, S. G., Clouston, A. and Bhatl, P. S.** (2006). Liver fluke-associated and sporadic cholangiocarcinoma: an immunohistochemical study of bile duct, peribiliary gland and tumour cell phenotypes. *J. Clin. Pathol.* **59**, 1073-1078.
- Ishimura, N., Bronk, S. F. and Gores, G. J.** (2004). Inducible nitric oxide synthase upregulates cyclooxygenase-2 in mouse cholangiocytes promoting cell growth. *Am. J. Physiol. Gastrointest. Liver Physiol.* **287**, G88-G95.
- Ishimura, N., Bronk, S. F. and Gores, G. J.** (2005). Inducible nitric oxide synthase up-regulates Notch-1 in mouse cholangiocytes: implications for carcinogenesis. *Gastroenterology* **128**, 1354-1368.
- Isomoto, H., Kobayashi, S., Werneburg, N. W., Bronk, S. F., Guicciardi, M. E., Frank, D. A. and Gores, G. J.** (2005). Interleukin 6 upregulates myeloid cell leukemia-1 expression through a STAT3 pathway in cholangiocarcinoma cells. *Hepatology* **42**, 1329-1338.
- Isomoto, H., Mott, J. L., Kobayashi, S., Werneburg, N. W., Bronk, S. F., Haan, S. and Gores, G. J.** (2007). Sustained IL-6/STAT-3 signaling in cholangiocarcinoma cells due to SOCS-3 epigenetic silencing. *Gastroenterology* **132**, 384-396.
- Itatsu, K., Zen, Y., Yamaguchi, J., Ohira, S., Ishikawa, A., Ikeda, H., Sato, Y., Harada, K., Sasaki, M., Sasaki, M. et al.** (2008). Expression of matrix metalloproteinase 7 is an unfavorable postoperative prognostic factor in cholangiocarcinoma of the perihilar, hilar, and extrahepatic bile ducts. *Hum. Pathol.* **39**, 710-719.
- Itatsu, K., Sasaki, M., Yamaguchi, J., Ohira, S., Ishikawa, A., Ikeda, H., Sato, Y., Harada, K., Zen, Y., Sato, H. et al.** (2009). Cyclooxygenase-2 is involved in the up-regulation of matrix metalloproteinase-9 in cholangiocarcinoma induced by tumor necrosis factor- α . *Am. J. Pathol.* **174**, 829-841.
- Jaiswal, M., LaRusso, N. F., Shapiro, R. A., Billiar, T. R. and Gores, G. J.** (2001). Nitric oxide-mediated inhibition of DNA repair potentiates oxidative DNA damage in cholangiocytes. *Gastroenterology* **120**, 190-199.
- Kang, Y. K., Kim, W. H. and Jang, J. J.** (2002). Expression of G1-S modulators (p53, p16, p27, cyclin D1, Rb) and Smad4/Dpc4 in intrahepatic cholangiocarcinoma. *Hum. Pathol.* **33**, 877-883.
- Karakatsanis, A., Papaconstantinou, I., Gazouli, M., Lyberopoulou, A., Polymeneas, G. and Voros, D.** (2011). Expression of microRNAs, miR-21, miR-31, miR-122, miR-145, miR-146a, miR-200c, miR-221, miR-222, and miR-223 in patients with hepatocellular carcinoma or intrahepatic cholangiocarcinoma and its prognostic significance. *Mol. Carcinog.* (Epub ahead of print).
- Khan, S. A., Carmichael, P. L., Taylor-Robinson, S. D., Habib, N. and Thomas, H. C.** (2003). DNA adducts, detected by 32P postlabelling, in human cholangiocarcinoma. *Gut* **52**, 586-591.
- Khan, S. A., Thomas, H. C., Davidson, B. R. and Taylor-Robinson, S. D.** (2005). Cholangiocarcinoma. *Lancet* **366**, 1303-1314.
- Khan, S. A., Emadossady, S., Ladep, N. G., Thomas, H. C., Elliott, P., Taylor-Robinson, S. D. and Toledano, M. B.** (2012). Rising trends in cholangiocarcinoma: is the ICD classification system misleading us? *J. Hepatol.* **56**, 848-854.

- Kim, D. G., Park, S. Y., You, K. R., Lee, G. B., Kim, H., Moon, W. S., Chun, Y. H. and Park, S. H. (2001). Establishment and characterization of chromosomal aberrations in human cholangiocarcinoma cell lines by cross-species color banding. *Genes Chromosomes Cancer* **30**, 48-56.
- Knuth, A., Gabbert, H., Dippold, W., Klein, O., Sachsse, W., Bitter-Suermann, D., Prellwitz, W. and Meyer zum Büschel, K. H. (1985). Biliary adenocarcinoma. Characterisation of three new human tumor cell lines. *J. Hepatol.* **1**, 579-596.
- Kobayashi, M., Ikeda, K., Saitoh, S., Suzuki, F., Tsubota, A., Suzuki, Y., Arase, Y., Murashima, N., Chayama, K. and Kumada, H. (2000). Incidence of primary cholangiocellular carcinoma of the liver in Japanese patients with hepatitis C virus-related cirrhosis. *Cancer* **88**, 2471-2477.
- Kobayashi, S., Werneburg, N. W., Bronk, S. F., Kaufmann, S. H. and Gores, G. J. (2005). Interleukin-6 contributes to Mcl-1 up-regulation and TRAIL resistance via an Akt-signaling pathway in cholangiocarcinoma cells. *Gastroenterology* **128**, 2054-2065.
- Komori, J., Marusawa, H., Machimoto, T., Endo, Y., Kinoshita, K., Kou, T., Haga, H., Imai, I., Uemoto, S. and Chiba, T. (2008). Activation-induced cytidine deaminase links bile duct inflammation to human cholangiocarcinoma. *Hepatology* **47**, 888-896.
- Ku, J. L., Yoon, K. A., Kim, I. J., Kim, W. H., Jang, J. Y., Suh, K. S., Kim, S. W., Park, Y. H., Hwang, J. H., Yoon, Y. B. et al. (2002). Establishment and characterization of six human biliary tract cancer cell lines. *Br. J. Cancer* **87**, 187-193.
- Kusaka, Y., Tokiwa, T. and Sato, J. (1988). Establishment and characterization of a cell line from a human cholangiocellular carcinoma. *Res. Exp. Med. (Berl.)* **188**, 367-375.
- Lai, G. H. and Sirica, A. E. (1999). Establishment of a novel rat cholangiocarcinoma cell culture model. *Carcinogenesis* **20**, 2335-2340.
- Lai, G. H., Zhang, Z., Shen, X. N., Ward, D. J., Dewitt, J. L., Holt, S. E., Rozich, R. A., Hixson, D. C. and Sirica, A. E. (2005). erbB-2/neu transformed rat cholangiocytes recapitulate key cellular and molecular features of human bile duct cancer. *Gastroenterology* **129**, 2047-2057.
- Lankisch, T. O., Metzger, J., Negm, A. A., Vosskuhl, K., Schiffer, E., Siwy, J., Weismüller, T. J., Schneider, A. S., Thedieck, K., Baumeister, R. et al. (2011). Bile proteomic profiles differentiate cholangiocarcinoma from primary sclerosing cholangitis and choledocholithiasis. *Hepatology* **53**, 875-884.
- LaRusso, N. F., Shneider, B. L., Black, D., Gores, G. J., James, S. P., Doo, E. and Hoofnagle, J. H. (2006). Primary sclerosing cholangitis: summary of a workshop. *Hepatology* **44**, 746-764.
- Lee, C. H., Chang, C. J., Lin, Y. J., Yeh, C. N., Chen, M. F. and Hsieh, S. Y. (2009a). Viral hepatitis-associated intrahepatic cholangiocarcinoma shares common disease processes with hepatocellular carcinoma. *Br. J. Cancer* **100**, 1765-1770.
- Lee, M. J., Yu, G. R., Yoo, H. J., Kim, J. H., Yoon, B. I., Choi, Y. K. and Kim, D. G. (2009b). ANXA8 downregulation by EGF-FOXO4 signaling is involved in cell scattering and tumor metastasis of cholangiocarcinoma. *Gastroenterology* **137**, 1138-1150, e1-e9.
- Leelawat, K., Leelawat, S., Narong, S. and Hongeng, S. (2007). Roles of the MEK1/2 and AKT pathways in CXCL12/CXCR4 induced cholangiocarcinoma cell invasion. *World J. Gastroenterol.* **13**, 1561-1568.
- Leelawat, K., Sakchinabut, S., Narong, S. and Wannaprasert, J. (2009). Detection of serum MMP-7 and MMP-9 in cholangiocarcinoma patients: evaluation of diagnostic accuracy. *BMC Gastroenterol.* **9**, 30.
- Leone, F., Cavalloni, G., Pignochino, Y., Sarotto, I., Ferraris, R., Piacibello, W., Venesio, T., Capussotti, L., Risio, M. and Aglietta, M. (2006). Somatic mutations of epidermal growth factor receptor in bile duct and gallbladder carcinoma. *Clin. Cancer Res.* **12**, 1680-1685.
- Li, Y., Deuring, J., Peppelenbosch, M. P., Kuipers, E. J., de Haar, C. and van der Woude, C. J. (2012). IL-6-induced DNMT1 activity mediates SOCS3 promoter hypermethylation in ulcerative colitis-related colorectal cancer. *Carcinogenesis* **33**, 1889-1896.
- Lim, J. H. and Park, C. K. (2004). Pathology of cholangiocarcinoma. *Abdom. Imaging* **29**, 540-547.
- Melum, E., Karlsen, T. H., Schrupf, E., Bergquist, A., Thorsby, E., Boberg, K. M. and Lie, B. A. (2008). Cholangiocarcinoma in primary sclerosing cholangitis is associated with NKG2D polymorphisms. *Hepatology* **47**, 90-96.
- Menakongka, A. and Suthiphongchai, T. (2010). Involvement of PI3K and ERK1/2 pathways in hepatocyte growth factor-induced cholangiocarcinoma cell invasion. *World J. Gastroenterol.* **16**, 713-722.
- Meng, F., Henson, R., Lang, M., Wehbe, H., Maheshwari, S., Mendell, J. T., Jiang, J., Schmittgen, T. D. and Patel, T. (2006). Involvement of human micro-RNA in growth and response to chemotherapy in human cholangiocarcinoma cell lines. *Gastroenterology* **130**, 2113-2129.
- Meng, F., Henson, R., Wehbe-Janeck, H., Smith, H., Ueno, Y. and Patel, T. (2007). The MicroRNA let-7a modulates interleukin-6-dependent STAT-3 survival signaling in malignant human cholangiocytes. *J. Biol. Chem.* **282**, 8256-8264.
- Meng, F., Wehbe-Janeck, H., Henson, R., Smith, H. and Patel, T. (2008). Epigenetic regulation of microRNA-370 by interleukin-6 in malignant human cholangiocytes. *Oncogene* **27**, 378-386.
- Metzger, J., Negm, A. A., Plentz, R. R., Weismüller, T. J., Wedemeyer, J., Karlsen, T. H., Dakna, M., Mullen, W., Mischak, H., Manns, M. P. et al. (2012). Urine proteomic analysis differentiates cholangiocarcinoma from primary sclerosing cholangitis and other benign biliary disorders. *Gut* **62**, 122-130.
- Miyagiwa, M., Ichida, T., Tokiwa, T., Sato, J. and Sasaki, H. (1989). A new human cholangiocellular carcinoma cell line (HuCC-T1) producing carbohydrate antigen 19/9 in serum-free medium. *In Vitro Cell. Dev. Biol.* **25**, 503-510.
- Miyazaki, M., Ohashi, R., Tsuji, T., Mihara, K., Gohda, E. and Namba, M. (1998). Transforming growth factor-beta 1 stimulates or inhibits cell growth via down- or up-regulation of p21/Waf1. *Biochem. Biophys. Res. Commun.* **246**, 873-880.
- Miyazawa, M., Aikawa, M., Okada, K., Toshimitsu, Y., Okamoto, K., Koyama, I. and Ikada, Y. (2012). Regeneration of extrahepatic bile ducts by tissue engineering with a bioabsorbable polymer. *J. Artif. Organs* **15**, 26-31.
- Mott, J. L., Kobayashi, S., Bronk, S. F. and Gores, G. J. (2007). mir-29 regulates Mcl-1 protein expression and apoptosis. *Oncogene* **26**, 6133-6140.
- Nault, J. C. and Zucman-Rossi, J. (2011). Genetics of hepatobiliary carcinogenesis. *Semin. Liver Dis.* **31**, 173-187.
- Nonomura, A., Ohta, G., Nakanuma, Y., Izumi, R., Mizukami, Y., Matsubara, F., Hayashi, M., Watanabe, K. and Takayanagi, N. (1988). Simultaneous detection of epidermal growth factor receptor (EGF-R), epidermal growth factor (EGF) and ras p21 in cholangiocarcinoma by an immunocytochemical method. *Liver* **8**, 157-166.
- Nuntagawat, C., Leelawat, K. and Tohtong, R. (2010). NGAL knockdown by siRNA in human cholangiocarcinoma cells suppressed invasion by reducing NGAL/MMP-9 complex formation. *Clin. Exp. Metastasis* **27**, 295-305.
- Ogasawara, S., Yano, H., Higaki, K., Takayama, A., Akiba, J., Shiota, K. and Kojiro, M. (2001). Expression of angiogenic factors, basic fibroblast growth factor and vascular endothelial growth factor, in human biliary tract carcinoma cell lines. *Hepato. Res.* **20**, 97-113.
- Papaconstantinou, I., Karakatsanis, A., Gazouli, M., Polymenestis, G. and Voros, D. (2012). The role of microRNAs in liver cancer. *Eur. J. Gastroenterol. Hepatol.* **24**, 223-228.
- Pollheimer, M. J., Trauner, M. and Fickert, P. (2011). Will we ever model PSC? – "It's hard to be a PSC model!". *Clin. Res. Hepatol. Gastroenterol.* **35**, 792-804.
- Prakobwong, S., Pinlaor, P., Charoensuk, L., Khoontawad, J., Yongvanit, P., Hiraku, Y. and Pinlaor, S. (2012). The liver fluke *Opisthorchis viverrini* expresses nitric oxide synthase but not gelatinases. *Parasitol. Int.* **61**, 112-117.
- Rosen, C. B., Heimbach, J. K. and Gores, G. J. (2010). Liver transplantation for cholangiocarcinoma. *Transpl. Int.* **23**, 692-697.
- Saijyo, S., Kudo, T., Suzuki, M., Katayose, Y., Shinoda, M., Muto, T., Fukuhara, K., Suzuki, T. and Matsuno, S. (1995). Establishment of a new extrahepatic bile duct carcinoma cell line, TFK-1. *Tohoku J. Exp. Med.* **177**, 61-71.
- Sato, Y., Harada, K., Itatsu, K., Ikeda, H., Kakuda, Y., Shimomura, S., Shan Ren, X., Yoneda, N., Sasaki, M. and Nakanuma, Y. (2010). Epithelial-mesenchymal transition induced by transforming growth factor-beta1/Snai1 activation aggravates invasive growth of cholangiocarcinoma. *Am. J. Pathol.* **177**, 141-152.
- Sausville, E. A. and Burger, A. M. (2006). Contributions of human tumor xenografts to anticancer drug development. *Cancer Res.* **66**, 3351-3354, discussion 3354.
- Sekiya, S. and Suzuki, A. (2012). Intrahepatic cholangiocarcinoma can arise from Notch-mediated conversion of hepatocytes. *J. Clin. Invest.* **122**, 3914-3918.
- Seol, M. A., Chu, I. S., Lee, M. J., Yu, G. R., Cui, X. D., Cho, B. H., Ahn, E. K., Leem, S. H., Kim, I. H. and Kim, D. G. (2011). Genome-wide expression patterns associated with oncogenesis and sarcomatous transdifferentiation of cholangiocarcinoma. *BMC Cancer* **11**, 78.
- Shariff, M. I., Gomaa, A. I., Cox, I. J., Patel, M., Williams, H. R., Crosse, M. M., Thillainayagam, A. V., Thomas, H. C., Waked, I., Khan, S. A. et al. (2011). Urinary metabolic biomarkers of hepatocellular carcinoma in an Egyptian population: a validation study. *J. Proteome Res.* **10**, 1828-1836.
- Shibata, T., Kokubu, A., Gotoh, M., Ojima, H., Ohta, T., Yamamoto, M. and Hirohashi, S. (2008). Genetic alteration of Keap1 confers constitutive Nrf2 activation and resistance to chemotherapy in gallbladder cancer. *Gastroenterology* **135**, 1358-1368, e1-e4.
- Shigehara, K., Yokomuro, S., Ishibashi, O., Mizuguchi, Y., Arima, Y., Kawahigashi, Y., Kanda, T., Akagi, I., Tajiri, T., Yoshida, H. et al. (2011). Real-time PCR-based analysis of the human bile microRNAome identifies miR-9 as a potential diagnostic biomarker for biliary tract cancer. *PLoS ONE* **6**, e23584.
- Shimonishi, T., Miyazaki, K., Kono, N., Sabit, H., Tuneyama, K., Harada, K., Hirabayashi, J., Kasai, K. and Nakanuma, Y. (2001). Expression of endogenous galectin-1 and galectin-3 in intrahepatic cholangiocarcinoma. *Hum. Pathol.* **32**, 302-310.

- Sirica, A. E.** (2005). Cholangiocarcinoma: molecular targeting strategies for chemoprevention and therapy. *Hepatology* **41**, 5-15.
- Sirica, A. E.** (2008). Role of ErbB family receptor tyrosine kinases in intrahepatic cholangiocarcinoma. *World J. Gastroenterol.* **14**, 7033-7058.
- Sirica, A. E.** (2012). The role of cancer-associated myofibroblasts in intrahepatic cholangiocarcinoma. *Nat. Rev. Gastroenterol. Hepatol.* **9**, 44-54.
- Sirica, A. E., Zhang, Z., Lai, G. H., Asano, T., Shen, X. N., Ward, D. J., Mahatme, A. and Dewitt, J. L.** (2008). A novel "patient-like" model of cholangiocarcinoma progression based on bile duct inoculation of tumorigenic rat cholangiocyte cell lines. *Hepatology* **47**, 1178-1190.
- Sirica, A. E., Dumur, C. I., Campbell, D. J. W., Almenara, J. A., Ogunwobi, O. O. and Dewitt, J. L.** (2009). Intrahepatic cholangiocarcinoma progression: prognostic factors and basic mechanisms. *Clin. Gastroenterol. Hepatol.* **7 Suppl.**, S68-S78.
- Socoteanu, M. P., Mott, F., Alpini, G. and Frankel, A. E.** (2008). c-Met targeted therapy of cholangiocarcinoma. *World J. Gastroenterol.* **14**, 2990-2994.
- Søreide, K., Körner, H., Havnen, J. and Søreide, J. A.** (2004). Bile duct cysts in adults. *Br. J. Surg.* **91**, 1538-1548.
- Sorensen, H. T., Friis, S., Olsen, J. H., Thulstrup, A. M., Mellemkjaer, L., Linet, M., Trichopoulos, D., Vilstrup, H. and Olsen, J.** (1998). Risk of liver and other types of cancer in patients with cirrhosis: a nationwide cohort study in Denmark. *Hepatology* **28**, 921-925.
- Sripa, B., Leungwattananant, S., Nitta, T., Wongkham, C., Bhudhisawasdi, V., Puapairoj, A., Sripa, C. and Miwa, M.** (2005). Establishment and characterization of an opisthorchiasis-associated cholangiocarcinoma cell line (KKU-100). *World J. Gastroenterol.* **11**, 3392-3397.
- Steel, A. W., Postgate, A. J., Khorsandi, S., Nicholls, J., Jiao, L., Vlavianos, P., Habib, N. and Westaby, D.** (2011). Endoscopically applied radiofrequency ablation appears to be safe in the treatment of malignant biliary obstruction. *Gastrointest. Endosc.* **73**, 149-153.
- Su, W. C., Shiesh, S. C., Liu, H. S., Chen, C. Y., Chow, N. H. and Lin, X. Z.** (2001). Expression of oncogene products HER2/Neu and Ras and fibrosis-related growth factors bFGF, TGF-beta, and PDGF in bile from biliary malignancies and inflammatory disorders. *Dig. Dis. Sci.* **46**, 1387-1392.
- Sugawara, H., Yasoshima, M., Katayanagi, K., Kono, N., Watanabe, Y., Harada, K. and Nakanuma, Y.** (1998). Relationship between interleukin-6 and proliferation and differentiation in cholangiocarcinoma. *Histopathology* **33**, 145-153.
- Tadlock, L. and Patel, T.** (2001). Involvement of p38 mitogen-activated protein kinase signaling in transformed growth of a cholangiocarcinoma cell line. *Hepatology* **33**, 43-51.
- Takiyama, I., Terashima, M., Ikeda, K., Kawamura, H., Kashiwaba, M., Tamura, G., Suto, T., Nakashima, F., Sasaki, R. and Saito, K.** (1998). Establishment and characterization of a new human extrahepatic bile duct carcinoma cell line (ICBD-1). *Oncol. Rep.* **5**, 463-467.
- Tanaka, S., Sugimachi, K., Kameyama, T., Maehara, S., Shirabe, K., Shimada, M., Wands, J. R. and Maehara, Y.** (2003). Human WISP1v, a member of the CCN family, is associated with invasive cholangiocarcinoma. *Hepatology* **37**, 1122-1129.
- Tang, D., Nagano, H., Yamamoto, H., Wada, H., Nakamura, M., Kondo, M., Ota, H., Yoshioka, S., Kato, H., Damdinsuren, B. et al.** (2006). Angiogenesis in cholangiocellular carcinoma: expression of vascular endothelial growth factor, angiotensin-1/2, thrombospondin-1 and clinicopathological significance. *Oncol. Rep.* **15**, 525-532.
- Tannapfel, A., Benicke, M., Katalinic, A., Uhlmann, D., Köckerling, F., Hauss, J. and Wittekind, C.** (2000a). Frequency of p16(INK4A) alterations and K-ras mutations in intrahepatic cholangiocarcinoma of the liver. *Gut* **47**, 721-727.
- Tannapfel, A., Weinans, L., Geissler, F., Schütz, A., Katalinic, A., Köckerling, F., Hauss, J. and Wittekind, C.** (2000b). Mutations of p53 tumor suppressor gene, apoptosis, and proliferation in intrahepatic cholangiocellular carcinoma of the liver. *Dig. Dis. Sci.* **45**, 317-324.
- Tannapfel, A., Sommerer, F., Benicke, M., Weinans, L., Katalinic, A., Geissler, F., Uhlmann, D., Hauss, J. and Wittekind, C.** (2002). Genetic and epigenetic alterations of the INK4a-ARF pathway in cholangiocarcinoma. *J. Pathol.* **197**, 624-631.
- Tannapfel, A., Sommerer, F., Benicke, M., Katalinic, A., Uhlmann, D., Witzigmann, H., Hauss, J. and Wittekind, C.** (2003). Mutations of the BRAF gene in cholangiocarcinoma but not in hepatocellular carcinoma. *Gut* **52**, 706-712.
- Tesana, S., Takahashi, Y., Sithithaworn, P., Ando, K., Sakakura, T., Yutanawiboonchai, W., Pairojkul, C. and Ruangjirachuporn, W.** (2000). Ultrastructural and immunohistochemical analysis of cholangiocarcinoma in immunized Syrian golden hamsters infected with *Opisthorchis viverrini* and administered with dimethylnitrosamine. *Parasitol. Int.* **49**, 239-251.
- Thamavit, W., Pairojkul, C., Tiwawech, D., Itoh, M., Shirai, T. and Ito, N.** (1993). Promotion of cholangiocarcinogenesis in the hamster liver by bile duct ligation after dimethylnitrosamine initiation. *Carcinogenesis* **14**, 2415-2417.
- Tokumoto, N., Ikeda, S., Ishizaki, Y., Kurihara, T., Ozaki, S., Iseki, M., Shimizu, Y., Itamoto, T., Arihiro, K., Okajima, M. et al.** (2005). Immunohistochemical and mutational analyses of Wnt signaling components and target genes in intrahepatic cholangiocarcinomas. *Int. J. Oncol.* **27**, 973-980.
- Tsao, M. S., Smith, J. D., Nelson, K. G. and Grisham, J. W.** (1984). A diploid epithelial cell line from normal adult rat liver with phenotypic properties of 'oval' cells. *Exp. Cell Res.* **154**, 38-52.
- Tyson, G. L. and El-Serag, H. B.** (2011). Risk factors for cholangiocarcinoma. *Hepatology* **54**, 173-184.
- Utispan, K., Thuwajit, P., Abiko, Y., Charnkaew, K., Paupairoj, A., Chau-in, S. and Thuwajit, C.** (2010). Gene expression profiling of cholangiocarcinoma-derived fibroblast reveals alterations related to tumor progression and indicates periostin as a poor prognostic marker. *Mol. Cancer* **9**, 13.
- Valle, J. W., Wasan, H., Johnson, P., Jones, E., Dixon, L., Swindell, R., Baka, S., Maraveyas, A., Corrie, P., Falk, S. et al.** (2009). Gemcitabine alone or in combination with cisplatin in patients with advanced or metastatic cholangiocarcinomas or other biliary tract tumours: a multicentre randomised phase II study - The UK ABC-01 Study. *Br. J. Cancer* **101**, 621-627.
- Voskoglou-Nomikos, T., Pater, J. L. and Seymour, L.** (2003). Clinical predictive value of the in vitro cell line, human xenograft, and mouse allograft preclinical cancer models. *Clin. Cancer Res.* **9**, 4227-4239.
- Watanabe, M., Chigusa, M., Takahashi, H., Nakamura, J., Tanaka, H. and Ohno, T.** (2000). High level of CA19-9, CA50, and CEA-producible human cholangiocarcinoma cell line changes in the secretion ratios in vitro or in vivo. *In Vitro Cell. Dev. Biol. Anim.* **36**, 104-109.
- Werneburg, N. W., Yoon, J. H., Higuchi, H. and Gores, G. J.** (2003). Bile acids activate EGF receptor via a TGF-alpha-dependent mechanism in human cholangiocyte cell lines. *Am. J. Physiol. Gastrointest. Liver Physiol.* **285**, G31-G36.
- Wong, N., Li, L., Tsang, K., Lai, P. B., To, K. F. and Johnson, P. J.** (2002). Frequent loss of chromosome 3p and hypermethylation of RASSF1A in cholangiocarcinoma. *J. Hepatol.* **37**, 633-639.
- Xu, X., Kobayashi, S., Qiao, W., Li, C., Xiao, C., Radaeva, S., Stiles, B., Wang, R. H., Ohara, N., Yoshino, T. et al.** (2006). Induction of intrahepatic cholangiocellular carcinoma by liver-specific disruption of Smad4 and Pten in mice. *J. Clin. Invest.* **116**, 1843-1852.
- Yamaguchi, N., Morioka, H., Ohkura, H., Hirohashi, S. and Kawai, K.** (1985). Establishment and characterization of the human cholangiocarcinoma cell line HChol-Y1 in a serum-free, chemically defined medium. *J. Natl. Cancer Inst.* **75**, 29-35.
- Yano, H., Maruiwa, M., Iemura, A., Mizoguchi, A. and Kojiro, M.** (1992). Establishment and characterization of a new human extrahepatic bile duct carcinoma cell line (KMBC). *Cancer* **69**, 1664-1673.
- Yao, X., Wang, X., Wang, Z., Dai, L., Zhang, G., Yan, Q. and Zhou, W.** (2012). Clinicopathological and prognostic significance of epithelial mesenchymal transition-related protein expression in intrahepatic cholangiocarcinoma. *Oncotargets Ther.* **5**, 255-261.
- Yeh, C. N., Maitra, A., Lee, K. F., Jan, Y. Y. and Chen, M. F.** (2004). Thioacetamide-induced intestinal-type cholangiocarcinoma in rat: an animal model recapitulating the multi-stage progression of human cholangiocarcinoma. *Carcinogenesis* **25**, 631-636.
- Yoon, J. H., Canbay, A. E., Werneburg, N. W., Lee, S. P. and Gores, G. J.** (2004). Oxysterols induce cyclooxygenase-2 expression in cholangiocytes: implications for biliary tract carcinogenesis. *Hepatology* **39**, 732-738.
- Yoon, H. A., Noh, M. H., Kim, B. G., Han, J. S., Jang, J. S., Choi, S. R., Jeong, J. S. and Chun, J. H.** (2011). Clinicopathological significance of altered Notch signaling in extrahepatic cholangiocarcinoma and gallbladder carcinoma. *World J. Gastroenterol.* **17**, 4023-4030.
- Zabron, A. A., Horneffer-van der Sluis, V. M., Wadsworth, C. A., Laird, F., Gierula, M., Thillainayagam, A. V., Vlavianos, P., Westaby, D., Taylor-Robinson, S. D., Edwards, R. J. et al.** (2011). Elevated levels of neutrophil gelatinase-associated lipocalin in bile from patients with malignant pancreatobiliary disease. *Am. J. Gastroenterol.* **106**, 1711-1717.
- Zhang, Z., Lai, G. H. and Sirica, A. E.** (2004). Celecoxib-induced apoptosis in rat cholangiocarcinoma cells mediated by Akt inactivation and Bax translocation. *Hepatology* **39**, 1028-1037.
- Zhou, Y. M., Yin, Z. F., Yang, J. M., Li, B., Shao, W. Y., Xu, F., Wang, Y. L. and Li, D. Q.** (2008). Risk factors for intrahepatic cholangiocarcinoma: a case-control study in China. *World J. Gastroenterol.* **14**, 632-635.