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## Emerging molecular targets and therapy for cholangiocarcinoma

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### Abstract

Cholangiocarcinoma (CCA) is a rare cancer arising

from the biliary tree with a poor prognosis and limited therapeutic options. Recent large scale molecular characterisation studies have identified recurrent genetic alterations in CCA which may be amenable to therapeutic targeting. In this review we explore the genomic landscape of CCA and examine results from trials of molecularly targeted agents and immunotherapy in this disease. Challenges in CCA diagnosis, treatment and trial design are discussed and we reflect on future directions which may lead to improved outcomes for CCA patients.

**Key words:** Cholangiocarcinoma; Biliary tract cancer; Targeted therapy; Immunotherapy; Mutation; Molecular; Microenvironment; Stroma; MiRNA

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**Core tip:** Cholangiocarcinoma (CCA) is a clinically challenging malignancy; it is rare, molecularly heterogeneous and associated with a poor prognosis. Here we review recent data on the genomic landscape of CCA and highlight the results of clinical trials using targeted agents and immunotherapy. We find a number of promising therapeutic agents in development and discuss strategies to improve diagnosis and outcomes in this patient group.

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### INTRODUCTION

Cholangiocarcinoma (CCA) is a relatively infrequent malignancy arising from epithelial cells lining the biliary tree. It is associated with poor prognosis and limited

standard therapeutic options. Globally incidence varies considerably according to geographical location with significantly higher rates in South-East Asia compared to Western countries. In North-East Thailand the incidence is high at 85 per 100000<sup>[1]</sup>, whilst in the United States and United Kingdom the incidence is much lower at around 1-3 cases per 100000 population<sup>[2,3]</sup>. The typical age at diagnosis of CCA is around 70 years, with slightly higher incidence in men than women<sup>[4]</sup>. Survival depends on the stage of disease at presentation, but even in patients with localised disease, five-year survival is poor at 15% and 30% for intrahepatic (ICC) and extrahepatic (ECC) CCA respectively<sup>[5]</sup>. For unclear reasons the incidence of ICC is increasing in western countries whilst rates of ECC are falling internationally<sup>[6]</sup>.

## CLASSIFICATION

CCA is now classified according to anatomical location into ICC, perihilar and distal subtypes; the latter two are ECC tumours. Prior to this novel classification the terms intra- and extra-hepatic CCA predominated, and we will use this nomenclature for the purposes of this review. This anatomical classification is useful as in addition to guiding surgical management, it is increasingly recognised that ICC and ECC have differing molecular profiles and may arise from differing cells of origin. There is evidence to suggest that some ICCs arise from the hepatic stem cell lineage whilst other ICCs and most ECCs arise from the biliary tree stem cell lineage<sup>[7]</sup>. Understanding the differences in tumour aetiology and biology between CCA subtypes will help guide stratification of targeted therapies.

## AETIOLOGY

Parasitic infection with the liver flukes *Clonorchis sinensis* and *Opisthorchis viverrini*, which are endemic in parts of South-East Asia, are strongly associated with development of CCA. In non-Asian countries most cases occur sporadically, however conditions causing bile stasis and chronic biliary inflammation are associated with CCA development. Specific risk factors include primary sclerosing cholangitis, hepatolithiasis, choledocal cysts and Caroli's syndrome (congenital cystic dilation of intrahepatic bile ducts). All causes of liver cirrhosis potentially predispose to CCA and studies have identified viral hepatitis and alcoholic liver disease as specific risk factors. The now banned radiocontrast, thorotrast has also been associated with CCA development.

## MANAGEMENT OF CCA

### Localised disease

**Surgery and locoregional therapy:** Less than 40% of patients present with resectable disease<sup>[8]</sup> and 5-year survival rate for patients with completely resected bile duct and GBC is in the range of 20%-50%. Loco-

regional failure occurs in more than half of patients, even in absence of residual disease (R0) and provides the justification for the study of adjuvant therapy.

Previously, the role of adjuvant chemotherapy for resected patients is not clearly defined. Phase III trials in this setting had not demonstrated a survival advantage in CCA, but these studies have included a range of tumour types (including pancreatobiliary, gallbladder and ampullary carcinomas) and have lacked sufficient power to identify a survival difference specifically in CCA<sup>[9,10]</sup>. However, recently the results of the UK BILCAP study have been presented in abstract form. This large phase III randomised trial recruited patients with resected biliary cancer including 368 (plus 79 gallbladder carcinomas) cholangiocarcinoma patients and randomised between no adjuvant therapy or 6 mo of oral capecitabine. Patients treated with capecitabine had improved overall survival [53 mo vs 36 mo HR = 0.75 (95%CI: 0.58-0.97; *P* = 0.028)]. The results will lead to adjuvant capecitabine being adopted as a standard of care in resected biliary cancers.

Liver transplantation is not a standard treatment for CCA due historically high recurrence rates and donor shortage. More modern series, have reported more encouraging results<sup>[11]</sup>. Potential candidates, such as patients with poor hepatic reserve for extended hepatectomy or those with localised but unresectable perihilar CCA should be enrolled on to suitable clinical trials.

Locoregional therapies, including radiotherapy, photodynamic therapy, chemo/radio-embolisation and radiofrequency ablation may have a role in locally advanced disease or patients who are surgically unfit. There is a lack of comparative clinical trial evidence to support any of these modalities improving survival compared to standard of care chemotherapy<sup>[12]</sup>. However retrospective and phase II data suggest a promising rate of local control by adding radiotherapy in the management of ICC, and warrants further investigation<sup>[13,14]</sup>.

### Unresectable/metastatic disease

The United Kingdom phase 3 ABC-02 trial established cisplatin-gemcitabine combination therapy as standard of care for the first line treatment of advanced CCA, providing a clinically significant survival advantage compared to gemcitabine alone (median OS 11.7 mo vs 8.1 mo, HR = 0.64, 95%CI: 0.52-0.80, *P* < 0.001)<sup>[15]</sup>. There is currently no established second line treatment for advanced CCA and although a number of small phase II studies have shown disease activity, using single agent or doublet combinations of 5-FU, oxaliplatin, and gemcitabine, this has not been validated in a randomised trial<sup>[16]</sup>. Results from the ABC-06 (NCT01926236) phase III trial, investigating FOLFOX chemotherapy compared to supportive care in the second line setting are awaited.

### Pathophysiology

**Desmoplastic stroma:** The tumour microenviron-

ment plays an important role in CCA pathogenesis. CCA bile ducts are typically surrounded by a dense hypovascular desmoplastic stroma, consisting of cancer associated fibroblasts (CAF) expressing  $\alpha$ -smooth muscle actin (SMA), activated macrophages and a fibrotic collagen rich extracellular matrix<sup>[17]</sup>.  $\alpha$ -SMA positive CAFs are involved in CCA progression and tumours of patients expressing high levels of  $\alpha$ -SMA have poorer survival<sup>[18]</sup>. CAFs produce a range of factors involved in autocrine and paracrine signalling, promoting oncogenic processes such as proliferation, invasion, metastasis and apoptosis evasion. Specifically, the factors produced by CAFs include periostin, tenascin-c, thrombospondin 1, stromal cell derived factor 1 (SDF-1), hepatocyte growth factor (HGF) and Wnt-inducible signalling protein-1v (WISP1)<sup>[18]</sup>. These factors interact with CCA cells to manipulate cell-signalling pathways. For instance periostin interacts with tenascin-C, HGF and SDF-1, which bind to their respective receptors, integrin, MET and CXCR4 on CCA cells, leading to activation of the PI3K/AKT signalling pathway. Cancer associated macrophages are also important in the stromal microenvironment and appear to have prognostic significance. In one study, high numbers of CD163<sup>+</sup> macrophages in the stroma of resected ICC correlated with poor disease free survival<sup>[19]</sup>. Inflammatory macrophage infiltrates in CCA are also associated with increased WNT signalling, and abrogation of WNT signalling in preclinical models inhibits CCA growth<sup>[20]</sup>. In cholangiocarcinoma, sustained interleukin-6 (IL-6) signalling which promotes tumour growth *via* autocrine mechanisms is also associated with increasing fibrosis and dense stroma formation; it is recognised that this dense hypovascular stroma poses a challenge to cytotoxic drug delivery<sup>[18]</sup>. Therefore targeting stromal factors involved in cholangiocarcinogenesis or improving drug delivery through the desmoplastic stroma are attractive targets for novel therapeutics.

## MOLECULAR CHARACTERISATION AND POTENTIAL FOR TARGETED THERAPIES

With recent technological advances in genomic sequencing, the mutational landscape of CCA is increasingly understood. Careful evaluation is needed to determine which genetic aberrations are true drivers of CCA. This section will review recent data on key genetic abnormalities thought to be implicated in CCA pathogenesis. There are clear differences in the prevalence of known oncogenic driver mutations between ICC and ECC, implicating distinct processes of oncogenesis for these tumour subtypes (Table 1). However it is also noteworthy that the prevalence of mutations is highly variable across studies, this heterogeneity may be reflective of regional variation, small sample size, or differences in the pathological classification of ICC and ECC prior to sequencing.

**Table 1 Mutation frequency for intrahepatic and extrahepatic cholangiocarcinoma**

Genetic mutation	Frequency (%) in all tumours tested		Ref.
	Intrahepatic CC	Extrahepatic CC	
IDH 1/2	14%-36%	0%	[27,59,67,99-101]
BAP 1	9%-25%	4%-10%	[66,67,101]
KRAS	9%-24%	40%-47%	[62-64,96]
TP53	3%-38%	18%-45%	[62-64,96]
PBRM1	11%-17%	4%-11%	[63,97]
ARID1A	11%-36%	5%-16%	[62-64,95]
EGFR amplification	7%	0%	[101]
HER2	0%-2%	0%-20%	[67,101]
VEGF overexpression	42%	31%	[46,47]
PIK3CA	4%-6%	9%	[66,101]
BRAF	4%-22%	6%	[57,101-103]
FRGR translocation	6%-50%	0%-5%	[66]
MCL1 amplification	16%-21%	NR	[66]
PTEN	1%-11%	4%	[59,101]
FBXW7	1%-6%	4%-15%	[67]
CDK6	7%	NR	[66]
CDKN2A	7%	15%	[66]
BRCA 1/2	4%	NR	[66]
SMAD4	1%-4%	11%-25%	[59,67,101]
mTOR	26%	40%	[67]

HER2: Human epidermal growth factor 2; CC: Cholangiocarcinoma; NR: Not reported.

Also of interest is that liver fluke related CCA is associated with a differing pattern of genetic mutations compared to non-fluke CCA. In one of the first studies to sequence CCA, 8 liver fluke CCA were analysed, revealing novel mutations in SMAD4 (17%), MLL3 (15%), ROBO2, GNAS and RNF (9%) each and CKDN2A and PEG3 (5%) each<sup>[21]</sup>. In a subsequent follow up study of 208 CCA cases (108 caused by liver fluke *O. viverrini*), TP53 mutations were more often seen in fluke related CCA, whilst IDH1/2 and BAP1 mutations were more common in non-fluke CCA<sup>[22]</sup>. This highlights the impact of environmental risk factors on the pattern of somatic mutations. The prognostic value of several somatic mutations seen in CCA has been evaluated, however results are conflicting.

Interestingly IDH1/2 (Isocitrate dehydrogenase) mutations are seen almost exclusively in ICC. The IDH mutation results in reduced normal function of this enzyme and leads to increased production of 2-hydroxyglutarate (2-HG) from alpha-ketoglutarate. 2-HG is considered an oncometabolite and causes epigenetic changes, including histone and DNA methylation, which promotes tumour development<sup>[23]</sup>. In one study of 326 patients with resected ICC, IDH1/2 mutations were associated with improved overall survival<sup>[24]</sup>, however another whole exome sequencing study ( $n = 32$ ) suggested worse overall survival for patients with these aberrations (3 year OS 33% mutant vs 81% wildtype,  $P = 0.003$ )<sup>[25]</sup>, however in this study a higher proportion of patients with IDH mutations had advanced disease (50% vs 15%). Two other studies

**Table 2 Clinical trials of novel agents in cholangiocarcinoma**

Title	Target	Phase	Estimated sample size	Expected completion date	Trial number
CX-4945 in combination with gemcitabine and cisplatin for frontline treatment of CCA	Casein kinase 2	I/II	100	Dec 2016	NCT02128282
BGJ398 in patients with advanced CCA and <i>FGFR</i> gene fusion	<i>FGFR</i> gene fusion	II	55	Jul 2018	NCT02150967
Dasatanib in IDH-mutant advanced ICC		II	19	Sep 2022	NCT02428855
RRx-001 in second line treatment of advanced CCA prior to readministration of first line therapy	Epigenetic modifications	II	30	May 2018	NCT02452970
ASLAN001 in advanced CCA who progressed on at least 1 line of therapy	Pan-HER inhibitor	II	25	Dec 2017	NCT02609958
Regorafenib as single agent in advanced CCA who failed first line	Multi-kinase inhibitor (VEGF, KIT, PDGF, FGFR, BRAF)	II	37	Feb 2018	NCT02053376
Copanlisib in combination with gemcitabine and cisplatin in advanced CCA	PI3K inhibitor	II	25	Dec 2018	NCT02631590
LDK378 in <i>ROS1</i> /ALK overexpressed advanced CCA	<i>ROS1</i> and/or ALK	II	34	Jul 2018	NCT02374489
AG120 in advanced solid tumours with IDH1 mutation	IDH1	I	145	May 2016	NCT02073994
Study of LY2801653 in advanced cancer	MET inhibitor	I	190	Nov 2017	NCT01285037
ABC-08: Acelarin in combination with cisplatin in locally advanced/metastatic biliary tract cancers	Nucleotide analogue	I	24	Sep 2018	NCT02352765
Ramucirumab for advanced pre-treated biliary cancers	VEGFR2 antagonist	II	50	Dec 2019	NCT02520141
Keynote-158: Pembrolizumab in participants with advanced solid tumours	PD1 inhibitor	II	1100	Mar 2021	NCT02628067
Immunotherapy using TILs for patients with metastatic cancer	Adoptive T-cell therapy	II	33	Dec 2019	NCT01174121

CCA: Cholangiocarcinoma; EGFR: Epidermal growth factor receptor; FGFR: Fibroblast growth factor receptor; IDH: Isocitrate dehydrogenase; PD1: Programmed death 1; TIL: Tumour infiltrating lymphocytes; VEGF: Vascular endothelial growth factor.

examining the effect of IDH mutations in patients with resected and advanced ICC demonstrated no significant association with prognosis<sup>[26,27]</sup>. Pre-clinical data suggesting oncogenic addiction to IDH signalling can be pharmacologically abrogated resulting in control of cancer cell growth has been demonstrated in IDH mutant glioma lines<sup>[28]</sup>. Inhibitors of mutant IDH1 and IDH2 are currently in clinical trials (Table 2); early results for AG-120 which is an IDH1 inhibitor demonstrated tolerable toxicity with evidence of pharmacodynamic endpoint modulation with reduced circulating levels of 2-hydroxyglutamate were observed in most patients<sup>[29]</sup>. Of twenty CCA patients treated, one (5%) had a partial response whereas 11 (55%) had stable disease.

The genes *BAP1*, *ARID1A* and *PBRM1*, which are involved in chromatin remodelling, have been found to be frequently mutated in ICC and in one study had inactivating mutations in just under half ( $n = 15/32$ ) of ICC cases<sup>[25]</sup>. Whether these mutations can predict sensitivity to the histone deacetylase inhibitor vorinostat, which targets chromatin regulation has not yet been determined. However in a preclinical study vorinostat did show anti-cancer activity against the HuCC-T1 human CCA cell line<sup>[30]</sup>.

### **Epidermal growth factor receptor alterations as a target in CCA**

The epidermal growth factor receptor (EGFR) is abnormally activated in a number of human cancers and is

therapeutically targeted using monoclonal antibodies or tyrosine kinase inhibitors<sup>[31]</sup>. EGFR targeted agents have demonstrated clinical efficacy in non-small cell lung cancer (NSCLC), colorectal cancer and SCC of the head and neck, where they are now established standard therapies. In NSCLC *EGFR* gene mutation, predicts response to EGFR inhibition<sup>[32]</sup>. Both activating mutations and amplifications of the *EGFR* gene have been observed in CCA. *EGFR* mutations have been observed in 10%-15% of CCA<sup>[33-35]</sup>, however due to small sample numbers data are conflicting regarding whether prevalence is higher in ICC or ECC. *EGFR* overexpression appears to be more prevalent in ICC (11%-27%) compared to ECC (5%-19%)<sup>[36,37]</sup>. Prognostically, EGFR expression has been found to be a negative predictor of overall survival in CCA<sup>[37,38]</sup>, making this an attractive target for drug intervention.

There have been 3 reported phase II trials investigating anti-EGFR monoclonal antibodies (mABs) in CCA but none have demonstrated survival advantage in this patient group. In a single arm phase II trial of 30 patients with advanced biliary tract cancer (aBTC) treated with first-line GEMOX-cetuximab, Gruenberger et al reported an objective response rate (CR + PR) of 63%, mOS of 15.2 mo (9.9-20.5) and 9 patients were able to undergo potentially curative resection following systemic therapy. Whilst this trial did not have a control arm and patients were unselected for *EGFR* expression or *KRAS* status, the reported response rate was considered encouraging<sup>[39]</sup>. Subsequently in the

phase II BINGO trial, 150 patients were randomised to first-line gemcitabine and oxaliplatin (GEMOX) with or without the addition of cetuximab<sup>[40]</sup>. Median overall survival was numerically shorter in the GEMOX-cetuximab group at 11.0 mo compared to 12.4 mo in the GEMOX group indicating lack of benefit from cetuximab. Neither *EGFR* overexpression (18 of 77 cases, 23%) nor *KRAS* mutation (14 of 75 cases, 19%) was associated with patient outcome in either treatment group. More recently, Leone *et al* showed that the anti-EGFR mAb panitumumab when used in combination with GEMOX chemotherapy in patients with *KRAS*-WT aBTC produced no improvement in overall survival when compared to chemotherapy alone (9.9 mo vs 10.2 mo,  $P = 0.42$ )<sup>[41]</sup>.

The small molecule EGFR tyrosine-kinase inhibitor erlotinib has also been investigated in advanced CCA. In a randomised phase III trial of 268 patients with aBTC (CCA, gallbladder and ampullary cancer) there was no significant difference in the primary end-point, progression free survival (PFS) in patients receiving GEMOX with or without erlotinib (5.8 mo vs 4.2 mo, HR = 0.80, 95%CI: 0.61-1.03,  $P = 0.83$ )<sup>[42]</sup>. However the objective response rate (CR + PR) was higher in the erlotinib group (40 vs 21 patients,  $P = 0.005$ ) and in the subgroup of patients with CCA, PFS was longer in the erlotinib group (5.9 mo vs 3.0 mo, HR = 0.73, 95%CI: 0.53-1.0,  $P = 0.049$ ). *EGFR* overexpression was reported in 12 of 60 patients (43%) in the erlotinib group and of these there was 1 PR and 7 cases of SD. In summary trials investigating anti-EGFR therapy in CCA have to date failed to demonstrate any clinically meaningful benefit over standard of care chemotherapy. The caveat to interpretation of these trials is that the inclusion of heterogenous, non-biomarker selected groups of biliary tract cancers may obscure any real survival benefit in smaller patient subsets; biomarker selected studies might be preferred for this reason.

Human epidermal growth factor 2 (HER2) or Erb-B2 Receptor Tyrosine Kinase 2 (ERBB2) amplification is rare in ICC, but may be present in up to 20% of ECC<sup>[37,43]</sup> and this target has been successfully targeted in breast and gastroesophageal cancer patients. Currently only anecdotal reports<sup>[44]</sup> are present in the literature regarding responses to anti-HER2 therapy in CCA, however a molecularly selected trial of trastuzumab in conjunction with GEMOX is ongoing (NCT02836847), and it remains to be seen whether this approach will be beneficial.

### Angiogenesis as a target in CCA

Vascular endothelial growth factor (VEGF) has been targeted therapeutically in a number of malignancies, with anti-VEGF monoclonal antibodies demonstrating efficacy in colorectal, breast and ovarian cancers, whilst TKIs targeting the VEGF receptor are in clinical use in renal cell carcinoma and hepatocellular

carcinoma<sup>[45]</sup>.

VEGF expression is reported in around 30%-40% of CCAs and correlates with increased lymph node metastasis and poorer survival<sup>[46,47]</sup>, making angiogenesis an attractive target in CCA. Bevacizumab, a humanised monoclonal antibody against VEGF-A has been investigated in CCA. Zhu *et al* conducted a single arm phase II trial and treated aBTC patients with first-line GEMOX plus bevacizumab<sup>[48]</sup>. They reported a median PFS of 7.0 mo, mOS of 12.7 mo and ORR of 40%, which was considered favourable compared to historical controls. Similar results were found in a phase II trial combining gemcitabine and capecitabine with bevacizumab in patients with aBTC where a median PFS of 8.1 mo and OS of 11.3 mo was reported<sup>[49]</sup>. Combining VEGF and EGFR inhibition has not significantly improved outcomes. Lubner *et al* used a combination of bevacizumab and erlotinib in a phase II trial of 49 patients with aBTC<sup>[50]</sup>. This trial reported a PR in 6 (12.2%) patients in whom the median duration of response was 8.4 mo and the reported mOS was 9.9 mo. However, overall survival in these studies (10-13 mo) did not reasonably exceed expectations for a phase II population and no control arm was available for comparison.

More recently disappointing results were reported from the randomised phase II ABC-03 trial, which investigated gemcitabine-cisplatin with either cediranib or placebo as first-line treatment of patients with aBTC<sup>[51]</sup>. Cediranib, a tyrosine kinase inhibitor of VEGFR1-3 and with additional activity against PDGF and c-KIT, did not improve PFS compared to the control group (PFS 8.0 mo vs 7.4 mo, HR = 0.93, 95%CI: 0.65-1.35,  $P = 0.72$ ) or overall survival (14.1 mo vs 11.9 mo, HR = 0.86,  $P = 0.44$ ). Response rates were higher for cediranib treated patients (44% vs 19% control). An interaction between baseline PDGFbb levels and overall survival benefit from cediranib was noted; patients with PDGFbb concentrations above the median derived an overall survival benefit from cediranib ( $P$  value for interaction 0.002).

Sorafenib, a multi-targeted TKI of VEGFR-2/3, PDGFR, BRAF and CRAF which may also be considered an anti-angiogenic agent has been investigated in CCA with disappointing results. As single agent therapy no clinically meaningful benefit was observed in 2 phase II trials, with reported PFS of 2.3 (range 0-12) and 3 (95%CI: 2-4) mo respectively<sup>[52,53]</sup>. When sorafenib was added to gemcitabine and cisplatin chemotherapy, PFS and OS of 6.5 and 14.4 mo respectively was reported which was similar to historical controls using chemotherapy alone<sup>[54]</sup>. Finally a phase II trial combining sorafenib with erlotinib was closed early due to failure to meet pre-determined survival criteria and reported a disappointing PFS and OS of 2 (95%CI: 2-3) and 6 (95%CI: 3-8) mo respectively<sup>[55]</sup>.

In summary, despite encouraging early trial results, therapeutic targeting of angiogenesis has not been

**Table 3** Fibroblast growth factor receptor fusions according to reported frequency in cholangiocarcinoma

FGFR fusion partner	Frequency	Ref.
FGFR2-AHCYL	7/102 (7%)	[56]
FGFR2-BICC1	2/102 (2%)	[56]
	41/107 (38%)	[57]
	1/28 (4%)	[66]
FGFR2-PPHLN1	17/107 (16%)	[57]
FGFR2-MGEA5	1/6 (17%)	[62]
FGFR2-TACC3	1/6 (17%)	[62]
	1/28 (4%)	[66]
FGFR-KIAA1598	1/28 (4%)	[66]

FGFR: Fibroblast growth factor receptor.

successful in CCA, although using biomarkers such as PDGFbb may improve patient selection in future.

### Fibroblast growth factor receptor fusions

The *FGF* pathway is involved in a number of cellular processes including proliferation, migration and angiogenesis. Abnormalities of this pathway have been implicated as driver events in carcinogenesis. In CCA, fibroblast growth factor receptor (*FGFR*) chromosomal translocations producing *FGFR*-fusion genes have been reported in both ICC and ECC, but are much more frequent in ICC (6%-50%) than ECC (0%-6%) (Table 3). The fusion protein is constitutively activated leading to downstream signalling through mitogen-activated protein kinase (MAPK) and PI3K/mTOR pathways<sup>[56]</sup>. Sia *et al*<sup>[57]</sup> demonstrated in a cohort of 107 ICC patients that *FGFR2* translocations represented the most common actionable target detected; these occurred in 16% of patients screened; this prevalence has been confirmed in other series<sup>[58,59]</sup>. One United States study has suggested that *FGFR2* fusion in ICC is more common in females, and a Japanese study has implicated viral hepatitis infection in this pathway<sup>[56,60]</sup>, however these findings require validation. *FGFR2* translocation in CCA may confer a prognostic benefit. Cancer specific survival in one dataset for patients harbouring *FGFR2* translocations was superior to non-translocated tumours (123 mo vs 37 mo respectively)<sup>[60]</sup>. Preclinical work in cell lines and patient derived xenografts supports blockade of *FGFR2* signalling in CCA as a potential effective therapy<sup>[56,61]</sup> and early anecdotal reports of *FGFR* inhibitor therapy in *FGFR2* translocated CCA patients have been encouraging<sup>[62]</sup>. These promising results were also reflected in an interim report from a phase II clinical trial examining the efficacy of the pan-*FGFR* inhibitor BGJ398 in CCA patients with an *FGFR* abnormality (NCT02150967)<sup>[63]</sup> in which of 36 patients eligible for assessment of response, 8 (22%) had a partial response and the disease control rate was 75%. These results compare very favourably to second line chemotherapy for CCA and serve to highlight the potential benefit of targeted therapy in appropriately

selected patients.

### Other potential targets in CCA

*ROS1* gene rearrangements are seen in a number of cancers and result in a fusion protein with a constitutively activated kinase domain that promotes oncogenesis. In NSCLC, *ROS1* rearranged tumours have shown encouraging response to the *ALK/MET/ROS* inhibitor Crizotinib. In CCA the prevalence of *ROS1* rearrangement is reported to be around 8%-9%<sup>[58,64]</sup>. Larger series are needed to determine whether prevalence is higher in ICC or ECC, however Neia *et al* found that in a cohort of 65 biliary tract cancer patients *FIG-ROS1* fusions were found in 4/25 ECC, 2/14 gallbladder carcinoma, 0/26 ICC<sup>[58]</sup>. In a murine allograft model of ICC the *FIG-ROS* fusion protein was shown to promote tumorigenesis and *FIG-ROS* inactivation resulted in inhibition of tumour growth<sup>[65]</sup>. Clinical trials are in progress to assess the efficacy of targeting *ROS1* rearrangement in CCA (NCT02374489, NCT02034981).

The *RAS/RAF/MEK/ERK* MAPK pathway is a key regulator of cellular proliferation and is defective in a number of malignancies.

*KRAS* mutations are frequent in CCA and have a reported incidence of 9%-47%<sup>[59,66,67]</sup>. In mouse models, tissue specific activation of *KRAS* in the hepatic parenchyma was found to lead to development of ICC<sup>[68]</sup> and this process was accelerated by the presence of simultaneous *P53*<sup>[68]</sup> or *PTEN* loss<sup>[69]</sup>. There are currently no available drugs to directly target *KRAS*, however downstream proteins can be targeted, for instance using *MEK* inhibitors. In a phase II trial of the *MEK 1/2* inhibitor selumetinib in aBTC, 12% (3/28) had objective response and 56% (14/28) prolonged stable disease (> 16 wk) resulting in a PFS of 3.7 mo and OS of 9.8 mo<sup>[70]</sup>.

*BRAF* mutations in CCA are reported to occur at a rate of 0-22%<sup>[71,72]</sup>. In one large study, *BRAF* V600E mutation was detected in 3% (5/159) of ICC cases but in no (0/149) ECC cases<sup>[71]</sup>. *BRAF* mutation showed no correlation with prognosis in this study. Due to the infrequent occurrence of *BRAF* mutation in CC, molecularly targeted clinical trials are difficult to conduct, however a phase II trial of combination *BRAF* and *MEK* inhibition in rare cancers is in progress (NCT02034110).

### Noncoding RNA abnormalities in CCA

MicroRNAs (miRNAs) are small non-coding RNAs that act as negative regulators of gene expression at the post transcription level. They bind to the 3' untranslated region of target mRNAs causing inhibition of translation and mRNA degradation. miRNAs can regulate a number of cellular processes and their abnormal expression is recognised in human cancers, including CCA. The abnormal expression of miRNA in CCA has been found to impact on a number of cellular processes

involved in cell cycle progression, apoptosis and cell signalling. In one of the first reported studies on this topic Meng *et al* demonstrated that miR-21, miR-141, and miR-200b were highly over-expressed in malignant CCA cells, and whereas inhibition of miR-21 and miR-200b increased sensitivity to gemcitabine, inhibition of miR-141 decreased cell growth<sup>[73]</sup>. Chronic inflammation is an important risk factor for CCA. The inflammatory cytokine, IL-6 has been identified as a driver of cholangiocarcinogenesis and has been shown to alter the expression of a number of miRNAs including miRNA 7a, 370, 148a and 152<sup>[74-76]</sup>. IL-6 signalling is associated with increased expression of DNA methyltransferase (DNMT), which promotes silencing of tumour suppressor genes through DNA hypermethylation. The miRNAs 148a and 152 are believed to regulate DNMT expression, as demonstrated by decreased levels of these miRNAs in malignant cholangiocytes in in-vitro and xenograft models. Crucially in cells transfected with these miRNAs DNMT levels were shown to decrease leading to reduced cellular proliferation<sup>[74]</sup>. In another study, in a cell culture model, miRNA 29b was under-expressed in CCA cell lines compared to normal cholangiocytes, resulting in up-regulation of the anti-apoptotic protein MCL-1 and allowing tumour cells to evade apoptosis<sup>[77]</sup>. In a further study, miRNA 494 was shown to induce G1/S transition cell cycle arrest, through downregulation of cyclin dependent kinase 6. In cell-based and xenograft models of CCA, miRNA494 expression was found to be reduced and its upregulation reduced cellular proliferation<sup>[78]</sup>. miRNA 26a was shown to promote proliferation of CCA cells by lowering levels of glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ) and preventing the degradation of  $\beta$ -catenin, leading to upregulation of transcription of target genes involved in carcinogenesis<sup>[79]</sup>. Other miRNAs may be related to chemoresistance; higher levels of miR-21 and miR-200b are associated with resistance to gemcitabine in cell lines whereas the converse is true for miR-29b, miR-205 and miR-221<sup>[80]</sup>. Selaru *et al*<sup>[81]</sup> have also demonstrated that miR-21 may be oncogenic in CCA through inhibition of programmed cell death 4 (PDCD4) and tissue inhibitor of metalloproteinases 3. As pharmacological manipulation of noncoding RNAs develops into a viable therapeutic option<sup>[82]</sup>, these processes could conceivably be targeted in future to benefit CCA patients.

### **Circulating miRNAs**

Pathological diagnosis of biliary tract tumours is frequently challenging due to the limited cellularity of specimens available post ERCP and also the desmoplastic stroma associated with biliary tract cancers, making the concept of a "liquid biopsy" attractive. Differential expression of several miRNAs has been demonstrated between patients with CCA and healthy controls in both tissue and blood, miR21 is known to be expressed at higher levels in biliary tract cancers, and increasing levels are also associated with

more advanced clinical stage and fall following surgical resection<sup>[83]</sup>. Wang *et al*<sup>[84]</sup> found that miR150 was significantly elevated in the plasma of ICC patients compared to clinical controls and could be used to differentiate ICC patients from volunteers with a sensitivity of 81% and a specificity of 58%, which was enhanced when CA19.9 was used in conjunction with miRNA analysis. Other circulating miRNAs of interest include miRNA192<sup>[85]</sup> which was also linked to more advanced disease and a negative prognosis and miR106a which is downregulated in CCA compared to healthy controls and has similar prognostic value<sup>[86]</sup>.

As bile secreted by the liver through the biliary ducts is more freely available to sample than tissue this also represents a potentially useful diagnostic material for CCA. In bile, miRNAs are contained in extracellular vesicles which maintain miRNA stability. Li *et al* designed a microvesicle based miRNA panel which was able to differentiate CCA from other causes of biliary disease or obstruction with a sensitivity of 67% and specificity of 96%<sup>[87]</sup>. The panel consisted of miR-191, miR-486-3p, miR-16, and miR-484, of which the last is the most sensitive for CCA. A Japanese study evaluating a larger panel of miRNAs in bile found ten to be upregulated in biliary tract cancer compared to benign biliary disease, and selected a combination of two (miR-9 and miR-145) as a proposed diagnostic biomarker with a specificity of 100% and high sensitivity<sup>[88]</sup>. Voigtländer *et al*<sup>[89]</sup> performed a study comparing miRNA expression in both serum and bile in German patients with primary sclerosing cholangitis and CCA, in addition to the serum of healthy controls. Interestingly, distinct miRNA profiles differentiated PSC and CCA in bile and in serum. In serum, lower levels of miR-1281, miR-126, miR26a, miR30b and miR-122 were found in CCA patients compared to PSC and healthy controls, whereas in bile changes in miR-412, miR-640, miR-1537 and miR-3189 predominated. Unfortunately as paired samples from each patient were not available a predictive panel containing blood and bile biomarkers was unable to be generated, however in future such an approach could be of significant utility. Although the use of circulating miRNAs is of significant interest, these studies are relatively small and require validation before becoming clinically applicable. Furthermore consideration of the geographic region of origin of each of the above studies (impacting on CCA aetiology and biology) must be considered before generalising these findings.

### **Immunotherapy**

**Immune checkpoint inhibitors:** Immune checkpoints, which provide co-stimulatory and co-inhibitory signals to T-cells are exploited by a number of cancers to evade the host immune system and checkpoint inhibition has been used therapeutically, most notably in melanoma and non-small cell lung cancer amongst other malignancies. There may also be a role for

checkpoint inhibition in CCA. Ye *et al.*<sup>[90]</sup> studied the expression of the co-inhibitory immune checkpoint, Programmed Death Ligand 1 (PD-L1) in 31 surgically resected ICC samples from Asian patients and found PD-L1 expression to be upregulated in tumour tissue compared to adjacent tissue. Tumours with high levels of PD-L1 expression were associated with poor differentiation, higher TNM stage and higher levels of apoptotic CD8<sup>+</sup> tumour infiltrating lymphocytes (TIL). Poorer survival has also been demonstrated in Western patients with ICC with positive tumour PD-L1 expression<sup>[91,92]</sup>. Sabbatino *et al.*<sup>[92]</sup> also found that downregulation of HLA class I antigen expression by tumour cells was associated with poorer clinical outcome. These data indicate PD-L1 upregulation and HLA class I antigen downregulation may be mechanisms of immune escape in CCA and could be potential biomarkers of response to anti-PD1/PDL1 immunotherapy. Chemotherapy may also have a role in modulating the immune system *via* inducing immunogenic cell death and upregulating expression of tumour associated antigens. Koido *et al.*<sup>[93]</sup> found treatment of ICC cells with gemcitabine resulted in upregulation of the tumour antigen WT1, calreticulin (a protein that provides a pro-phagocytic signal) and PD-L1. Thus there may be a rationale for combining standard chemotherapy drugs with immune checkpoint inhibitors.

Trials investigating immune checkpoint inhibitors in CCA are in progress but early signals of efficacy have recently been reported. Keynote-028 is a multicohort phase Ib trial of pembrolizumab in PD-L1 positive pre-treated advanced solid tumours<sup>[94]</sup>. Early data from the biliary tract cohort of this trial reported an objective response rate of 17% ( $n = 4/17$ , all partial responses) and a further 17% ( $n = 4/17$ ) achieved stable disease. Responses appeared to be durable with all responding patients remaining on treatment at 40-42 wk. Le *et al.*<sup>[95]</sup> also reported data from a phase II trial of 17 patients with mismatch repair deficient non-colorectal gastrointestinal cancers treated with the PD-1 inhibitor pembrolizumab. Of the 3 patients with CCA there was one complete response, one partial response and one stable disease, with durable and ongoing responses at median follow up of 5.3 mo. More mature data with larger sample sizes are eagerly awaited but mismatch repair deficiency appears to be a promising predictive biomarker for checkpoint inhibition (although of relatively rare prevalence). The Keynote-158 phase II trial is recruiting 1100 patient with advanced solid tumours to be treated with pembrolizumab and will include a cohort of patients with biliary tract cancer (NCT02628067).

**Mutation specific adoptive T-Cell therapy:** The use of T-cells with specificity to cancer antigens is an emerging field and efficacy has been demonstrated in metastatic melanoma<sup>[96]</sup> and B cell leukaemia<sup>[97]</sup>. T-cell based therapy for epithelial malignancies, such as CCA

is under investigation.

Tran *et al.*<sup>[98]</sup> treated a female patient with metastatic CCA who had progressed on multiple lines of chemotherapy, with autologous T<sub>H</sub>1 tumour infiltrating lymphocytes (TILs) specific to a mutated antigen expressed by the patient's cancer. In this novel approach, TILs from the patient's lung metastases were retrieved and whole exome sequencing performed on tumour tissue to identify somatic mutations present. Further testing revealed that CD4<sup>+</sup> T<sub>H</sub>1 TILs recognised mutated *erbb2* interacting protein (ERBB2IP) in the tumour tissue. These mutation specific TILs were clonally expanded and the patient underwent lymphodepletive chemotherapy, before receiving 42.4 billion TILs (25% ERBB2IP-mutation reactive T cells). There was impressive reduction in size of metastatic lesions and prolonged stable disease for more than 1 year. When the disease progressed after 13 mo the patient was retreated and again achieved disease response. Whilst this demonstrated an important proof of concept for T-cell based therapy in CCA, reproducibility in further patients is needed. Furthermore the highly personalised nature of this approach has high cost implications.

## CONCLUSION

CCA is a molecularly heterogeneous malignancy with currently limited treatment options beyond first line systemic chemotherapy. Genomic profiling studies have highlighted differing patterns of mutation signatures between ICC and ECC, helping to stratify patients for targeted therapies. FGFR fusions and IDH mutations appear to be frequently mutated in ICC and hold promise as therapeutic targets. Immunotherapy also has considerable potential but requires a validated biomarker to guide selection of patients for this approach. Circulating miRNAs are of interest in improving early diagnosis and detecting disease relapse. However given the relative rarity of this cancer and the molecular heterogeneity, multi-centre collaboration is essential in order to design adequately powered clinical trials of targeted agents.

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