Young *et al. Hepatoma Res* 2023;9:34
DOI: 10.20517/2394-5079.2023.37

Hepatoma Research

Review

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Genomic alterations in intrahepatic cholangiocarcinoma

Sara E. Young[1,2], Ramja Sritharan[1,2], Daniela Sia[2]

[1]Graduate School of Biomedical Sciences, Icahn School of Medicine at Mount Sinai, New York, NY 11029, USA.
[2]Division of Liver Diseases, Department of Medicine, Tisch Cancer Institute, Liver Cancer Program, Icahn School of Medicine at Mount Sinai, New York, NY 11029, USA.

Correspondence to: Prof. Daniela Sia, Division of Liver Diseases, Department of Medicine, Tisch Cancer Institute, Liver Cancer Program, 1425 Madison Avenue, New York, NY 11029, USA. E-mail: daniela.sia@mssm.edu

How to cite this article: Young SE, Sritharan R, Sia D. Genomic alterations in intrahepatic cholangiocarcinoma. *Hepatoma Res* 2023;9:34. https://dx.doi.org/10.20517/2394-5079.2023.37

Received: 24 Apr 2023 **First Decision:** 19 Jun 2023 **Revised:** 11 Jul 2023 **Accepted:** 18 Jul 2023 **Published:** 28 Jul 2023

Academic Editor: Luca Fabris **Copy Editor:** Yanbing Bai **Production Editor:** Yanbing Bai

Abstract

Intrahepatic cholangiocarcinoma (iCCA) is an aggressive and heterogeneous biliary cancer with a poor prognosis and limited treatment options. The molecular pathogenesis of iCCA involves a highly complex process entailing multiple genetic alterations and dysregulation of signaling pathways. Recent advancements in our understanding of the genetic landscape of iCCA have opened new opportunities for therapeutic interventions. Technologies such as next-generation sequencing (NGS) have contributed to elucidating the genetic heterogeneity of iCCA, leading to the identification of numerous potentially actionable genetic alterations. Despite these advances, the prognosis of iCCA patients remains dismal. In this review, we provide an extensive summary of the current knowledge on genetic alterations in iCCA, their biological impact on patients, potential therapeutic targets, approved targeted therapies, and ongoing clinical trials with targeted agents. Furthermore, we discuss the main technologies available for studying genetic alterations and their advantages and limitations. Finally, we highlight future directions in studying genetic alterations and the development of new targeted therapies and personalized medicine approaches.

Keywords: Cholangiocarcinoma, iCCA, cancer, genetic alterations, targeted therapy, precision medicine

INTRODUCTION

Cholangiocarcinoma (CCA) is a rare but fatal malignancy arising in the epithelial cells of the biliary

tract[1,2]. Second only to hepatocellular carcinoma (HCC), CCA is one of the most prevalent primary liver cancers, occurring in 15%-20% of patients[1]. Based on anatomical origin, CCAs are classified into intrahepatic (iCCA) and extrahepatic cholangiocarcinoma (eCCA), with the latter being further subtyped into perihilar (pCCA) and distal (dCCA) CCA. These subtypes not only differ in the anatomical location and cell of origin (as extensively reviewed by Moeini *et al.* 2021[2]), but also present with distinct risk factors, clinical presentations and management, as well as different molecular pathogenesis[1,2]. In this review, we will focus on iCCA.

Risk factors for iCCA vary and include obesity, liver fluke infestation, primary sclerosing cholangitis, and chronic liver diseases, such as viral hepatitis and non-alcoholic fatty liver disease[1,3]. However, most of iCCA cases are sporadic and do not have identifiable risk factors[1]. Age-standardized incidence rates have been steadily increasing in both males and females[3]. The highest mortality rates are still in Asian countries, but mortality rates in European and other Western nations have been on the rise over the last decade[1,3,4].

Diagnosis of iCCA is typically made at advanced stages because of the lack of symptoms and effective biomarkers[3]. Over 70% of patients diagnosed with iCCA have unresectable tumors or extrahepatic spread, lowering the median overall survival to less than 12 months[1]. Traditionally, the diagnosis of CCA is based on the anatomical location of tumors and the evaluation of histological features obtained from tumor biopsies[5,6]. With an examination of gross morphology, iCCA is categorized into either mass-forming, periductal-infiltrating, or mixed-type iCCA[7,8]. At the microscopic level, iCCAs can be divided into small-duct and large-duct types, both of which have distinct features[7-9]. These classifications are useful for initial diagnosis, staging, and determination of first-line therapy options, but are not effective for devising treatment strategies using targeted therapies.

For over a decade, the standard of care treatment for iCCA and other biliary tract cancers has been a regimen of gemcitabine *plus* cisplatin (GemCis)[10-14]. Since results with GemCis are only modest (median overall survival: 11.7 months), it has been challenging to improve patient survival in iCCA patients[10-14], leaving an unmet need for more effective first- and second-line therapies[1]. In an effort to meet this need, clinical trials have been designed to explore adding a third agent or using immune checkpoint inhibitors to enhance the effectiveness of the standard of care therapy, as extensively reviewed by Rizzo and Brandi[15]. Notably, while immune checkpoint inhibitors as single agents have been unsuccessful, the recent TOPAZ 1 trial has shown that the combination of cisplatin and gemcitabine with durvalumab, an immune checkpoint inhibitor (ICI) targeting PD-L1, has led to greater overall survival (median overall survival: 12.8 months *vs.* 11.5 months for placebo)[16]. Following the conclusion of the TOPAZ 1 trial, in September 2022, the Food and Drug Administration (FDA) granted approval to update the standard of care treatment for iCCA patients to include durvalumab in combination with GemCis. Pembrolizumab, an immune checkpoint inhibitor targeting PD-1, showed promising results when combined with GemCis in the KEYNOTE-966 clinical trial (NCT04003636)[17]. The recently published results show that patients had significantly improved median progression-free survival (6.5months *vs.* 5.6 months) and median overall survival (12.7 months *vs.* 10.9 months) with the combination treatment versus GemCis alone[17], further demonstrating the added benefit of immune checkpoint inhibitors in combination with the standard of care regimen for patients with iCCA.

In addition, over the past few years, next-generation sequencing (NGS) technologies have shed light on the molecular landscape of iCCA[18-21], and unveiled various oncogenic drivers and potential therapeutic targets[18,22-24]. These discoveries have spurred the development of targeted therapies tailored to iCCA patients with specific molecular profiles. For instance, the first FGFR inhibitors have received FDA approval

for patients harboring *FGFR2* fusions[25,26]. Moreover, comprehensive genomic profiling of iCCA patients has identified the presence of biomarkers such as tumor mutational burden and microsatellite instability[27]. These results suggest that deepening our understanding of genetic alterations (GAs) and molecular profiling in iCCA is crucial for advancing personalized therapeutic strategies for iCCA.

In this review, we first summarize current knowledge on GAs in iCCA and their biological impact. Next, we discuss the various molecular classification systems used to stratify patients for treatment strategies and the clinical application of different testing procedures to identify GAs harbored by each iCCA patient. Finally, we discuss the knowledge gaps and the approaches being used to close those gaps and develop more effective therapeutics and treatment strategies for patients with strategies for this disease.

GENETIC ALTERATIONS IN ICCA

In recent years, thanks to the identification of recurrent and "druggable" GAs, iCCA has emerged as a model for precision cancer treatment within gastrointestinal malignancies, with more than 45% of patients harboring at least one clinically actionable genetic alteration according to the results of the FIGHT-202 trial[28]. Among the identified GAs, those in *isocitrate dehydrogenase (IDH)* and *fibroblast growth factor receptor 2 (FGFR2)* genes account for the most frequent and clinically-relevant alterations. In addition, despite occurring at lower frequencies in patients, GA's in *B-raf proto-oncogene (BRAF), erb-b2 receptor tyrosine kinase 2 (ERBB2), neurotrophic tyrosine receptor kinase (NRTK), BReast CAncer gene 1 (BRCA1),* and *Kirsten rat sarcoma virus (KRAS),* among others, are prime targets for new and developing therapeutic options. In the following paragraphs, we will provide an overview of the GAs occurring in iCCA [Figure 1] with particular emphasis on the most clinically relevant alterations. Ongoing clinical trials investigating therapeutic agents targeting the identified GAs are summarized in Table 1.

Currently actionable GAs

IDH1/2 mutations

In iCCA, *IDH1/2* mutations occur in up to ~10%-15% of patients[29]. Isocitrate dehydrogenase (IDH) 1 and 2 are integral enzymes for cellular respiration. IDH1, localized to the cytoplasm, reduces NADPH to NADP+ during the conversion of isocitrate to -ketoglutarate in the citric acid cycle, while IDH2 performs the same function within the mitochondria [Figure 2]. Mutations in *IDH1* are most often a single nucleotide variation (SNV) at R132, while in *IDH2*, the SNVs occur more frequently at residues R140 or R170[27,30]. Mutations in *IDH1/2* alter their enzymatic activity and promote the production of the oncometabolite 2-hydroxyglutarate (2-HG)[31-34]. Over-production of 2-HG inhibits -ketoglutarate-dependent dioxygenase enzymes, causing hypermethylation of histones and DNA, ultimately altering the chromatin landscape and reducing expression of known chromatin modifier genes[35,36]. Downregulation of the chromatin modifier genes was found to anticorrelate with mitochondrial gene sets, not only in iCCA but also across other cancers[35], suggesting a crucial link between these two pathways and a key role of *IDH* mutations in its regulation. In addition, an abundance of 2-HG results in a dysregulation of the mTOR pathway through increased VEGFR activation[37]. More importantly, Saha *et al.* demonstrated that increased levels of 2-HG impair the progression of hepatocyte differentiation by inhibiting the hepatocyte-specific transcriptional regulator, HNF-4, without affecting biliary differentiation[38]. Interestingly, *IDH1/2* alterations are frequently accompanied by alterations in AT-rich interactive domain-containing protein 1A (*ARID1A)*, cyclin-dependent kinase inhibitor 2A and 2B (*CDKN2A/B)*, polybromo-1 (*PBRM1)*, and BRCA1-associated protein-1 (*BAP1)*[27,39].

In August of 2021, the FDA approved the use of ivosidenib, an IDH1 inhibitor, for the treatment of patients with *IDH1*-mutated iCCAs based on the results of the phase III clinical trial, ClarIDHy (NCT02989857),

Young *et al. Hepatoma Res* 2023;9:34 | https://dx.doi.org/10.20517/2394-5079.2023.37

Table 1. Current clinical trials investigating target therapies for actionable driver mutations in iCCA

Target gene	Drug	Combination therapies included	Drug class	FDA approval	Ongoing clinical trials	Conditions	Status
IDH1 mutations (20%-25%)	Ivosidenib	-	IDH1 inhibitor	For advanced cholangiocarcinoma with IDH mutations	NCT02989857	Advanced, metastatic cholangiocarcinoma	Completed
	Ivosidenib	In combination with Gemcitabine and Cisplatin	IDH1 inhibitor	For advanced cholangiocarcinoma with IDH mutations	NCT04088188	Unresectable or metastatic cholangiocarcinoma	Recruiting
	Olaparib	-	PARP inhibitor	For breast, ovarian, and pancreatic cancers	NCT03212274	Advanced Glioma, Cholangiocarcinoma, or Solid Tumors with IDH1 or IDH2 Mutations	Recruiting
	Olaparib	In combination with Ceralasertib (AZD6738)	PARP inhibitor	For breast, ovarian, and pancreatic cancers	NCT03878095	IDH1 and IDH2 mutant cholangiocarcinoma or solid tumors	Recruiting
	Olaparib	In combination with Durvalumab	PARP inhibitor	For breast, ovarian, and pancreatic cancers	NCT03991832	IDH1-Mutated solid tumors	Recruiting
	AB-218	-	IDH1 inhibitor	No	NCT05814536	Advanced IDH1 mutant cholangiocarcinoma	Not yet recruiting
	LY3410738	In combination with Gemcitabine, Cisplatin, and Durvalumab	covalent mutant IDH inhibitor	No	NCT04521686	Advanced solid tumors with IDH1 or IDH2 mutations	Recruiting
	HMPL-306	-	dual IDH1/2 inhibitor	No	NCT04762602	Advanced solid tumors with IDH mutations	Recruiting
	IDH305	-	mutant-selective IDH1 inhibitor	No	NCT02381886	Advanced Malignancies that Harbor IDH1R132 Mutations	Active, not recruiting
FGFR2 fusions/alterations (10%-20%)	Infigratinib	-	selective ATP-competitive inhibitor	For metastatic cholangiocarcinoma with FGFR2 fusion/rearrangement	NCT04233567	Advanced or metastatic solid tumors with FGFR Gene Mutations	Recruiting
	Infigratinib	In combination with Nab-paclitaxel, Gemcitabine, and Cisplatin	selective ATP-competitive inhibitor	For metastatic cholangiocarcinoma with FGFR2 fusion/rearrangement	NCT05514912	Resectable Intrahepatic Cholangiocarcinoma	Not yet recruiting
	Infigratinib	In combination with Atezolizumab and Bevacizumab	selective ATP-competitive inhibitor	For metastatic cholangiocarcinoma with FGFR2 fusion/rearrangement	NCT05510427	Advanced Cholangiocarcinoma with FGFR2 Fusion/Amplification	Not yet recruiting
	Pemigatinib	-	tyrosine kinase inhibitor	For metastatic cholangiocarcinoma with FGFR2 fusion/rearrangement	NCT04256980	Advanced/Metastatic cholangiocarcinoma including FGFR2 rearrangement	Active, not recruiting
	Pemigatinib	Versus Gemcitabine and Cisplatin	Tyrosine kinase inhibitor	For metastatic cholangiocarcinoma with FGFR2 fusion/rearrangement	NCT03656536	Unresectable or Metastatic Cholangiocarcinoma	Recruiting
	Pemigatinib	In combination with Gemcitabine and Cisplatin	tyrosine kinase inhibitor	For metastatic cholangiocarcinoma with FGFR2 fusion/rearrangement	NCT04088188	Unresectable or metastatic cholangiocarcinoma	Recruiting
	Pemigatinib	After SBRT	tyrosine kinase inhibitor	For metastatic cholangiocarcinoma with FGFR2 fusion/rearrangement	NCT05565794	Advanced iCCA with FGFR Fusion/Rearrangement	Not yet recruiting

	Futibatinib (Tas-120)	Versus Gemcitabine and Cisplatin	Irreversible FGFR 1-4 inhibitor	For metastatic cholangiocarcinoma with FGFR2 fusion/rearrangement	NCT04093362	Advanced cholangiocarcinoma with FGFR2 Gene Rearrangements	Active, not recruiting
	Futibatinib (Tas-120)	-	irreversible FGFR 1-4 inhibitor	For metastatic cholangiocarcinoma with FGFR2 fusion/rearrangement	NCT05727176	Advanced cholangiocarcinoma with FGFR2 fusion or rearrangement	Not yet recruiting
	Derazantinib	In combination with Atezolizumab	FGFR 1-3 kinase inhibitor	No	NCT05174650	Advanced iCCA with FGFR Fusion/Rearrangement	Recruiting
	E7090	-	selective FGFR inhibitor	No	NCT04238715	Advanced or metastatic cholangiocarcinoma with FGFR2 Gene Fusion	Active, not recruiting
	3D185	-	selective FGFR 1-3 inhibitor	No	NCT05039892	Locally advanced or metastatic cholangiocarcinoma	Not yet recruiting
	Gunagratinib (ICP-192)	-	selective irreversible FGFR inhibitor	No	NCT05678270	FGFR2-rearranged unresectable or metastatic intrahepatic cholangiocarcinoma	Recruiting
	Gunagratinib (ICP-192)	-	selective irreversible FGFR inhibitor	No	NCT04565275	Advanced solid tumors with FGFR gene alterations	Recruiting
	Tinengotinib (TT-00420)	-	multi-kinase inhibitor	No	NCT04919642	Advanced cholangiocarcinoma with FGFR alterations	Recruiting
	HMPL-453 Tartrate	-	selective FGFR 1-3 inhibitor	No	NCT04353375	Advanced intrahepatic cholangiocarcinoma with FGFR2 Fusion	Not yet recruiting
	RLY-4008	-	selective irreversible FGFR2 inhibitor	No	NCT04526106	ICC and other advanced solid tumors	Recruiting
	Erdafitinib	-	pan-FGFR tyrosine kinase inhibitor	For metastatic urothelial carcinoma	NCT02699606	Advanced Non-Small-Cell Lung Cancer, Urothelial Cancer, Esophageal Cancer Or Cholangiocarcinoma	Active, not recruiting
	KIN-3248	-	irreversible pan-FGFR inhibitor	No	NCT05242822	Advanced tumors harboring FGFR2 and/or FGFR3 gene alterations	Recruiting
	CPL304110	-	selective FGFR 1-3 inhibitor	No	NCT04149691	Advanced Solid Malignancies, including cholangiocarcinoma	Recruiting
KRAS (8%-20%)	Sotorasib	in combination with Anti-PD-1/L1 or Midazolam	KRAS G12C inhibitor	For advanced non-small cell lung cancer (NSCLC)	NCT03600883	Solid Tumors with KRASG12C Mutations	Active, not recruiting
	Adagrasib	in combination with Cetuximab, Afatinib, or Cetuximab	KRAS G12C inhibitor	For advanced non-small cell lung cancer (NSCLC)	NCT03785249	Advanced metastatic cancer	Recruiting
	Trametinib	In combination with Hydroxychloroquine (HCQ)	MEK inhibitor	For NSCLC, anaplastic thyroid cancer, and metastatic solid tumors with BRAFv600E mutations	NCT04566133	Refractory bile tract carcinoma with KRAS mutation	Recruiting
PI3K/AKT/mTOR	Everolimus	-	mTOR inhibitor	For non-functional neuroendocrine tumors (NET) of GI or lung originFor advanced renal cell carcinoma	NCT00973713	Advanced cholangiocarcinoma	Completed

BRAFV600E (2%-7%)	Vemurafenib	in combination with Cobimetinib	BRAF inhibitor	For metastatic solid tumors with BRAFV600E mutation	NCT02091141	Biliary Tract Cancers	Active, not recruiting
	ABM-1310	In combination with Cobimetinib	BRAF inhibitor	No	NCT04190628	Advanced solid tumors, including Cholangiocarcinoma	Recruiting
	ABM-1311	-	BRAF inhibitor	No	NCT05501912	BRAF V600-Mutant Advanced Solid Tumors	Recruiting
HER2 (2%-6%)	Zanidatamab	-	HER2-targeted antibody	No	NCT04466891	HER2-amplified Biliary Tract Cancers	Active, not recruiting
	Zanidatamab	In combination with established chemotherapy regimens	HER2-targeted antibody	No	NCT03929666	HER2-expressing GI Cancers, including Biliary Tract Cancer	Recruiting
	Trastuzumab deruxtecan	-	HER2-targeted antibody-drug conjugate	For HER2-positive breast cancer and HER2-positive gastric adenocarcinomas	NCT04482309	Open-Label Study including Biliary Tract Cancer	Active, not recruiting
	MRG002	-	HER2-targeted antibody-drug conjugate	No	NCT04837508	Advanced or Metastatic Biliary Tract Cancer	Recruiting
	IMM2902	-	HER2/SIRPa bispecific mAb	No	NCT05805956	Advanced solid tumors expressing HER2	Recruiting
NTRK (1%-4%)	Larotrectinib	-	pan-NTRK inhibitor	For solid tumors with NTRK gene fusion	NCT02576431	Solid tumors, including Biliary Tract Cancer	Recruiting
	Entrectinib	-	NTRK inhibitor	For solid tumors with NTRK gene fusion	NCT02568267	Basket trial including Cholangiocarcinoma	Active, not recruiting
EGFR (0%-8%)	Erlotinib	In combination with Pemetrexed	EGFR inhibitor	For non-small cell lung cancer (NSCLC)	NCT03110484	Biliary Tract Cancers	Recruiting
BRCA1/2 (1%-3%)	Olaparib	In combination with Pembrolizumab	PARP inhibitor	For breast, ovarian, and pancreatic cancers	NCT04306367	Advanced cholangiocarcinoma	Active, not recruiting
	Olaparib	In combination with Ceralasertib (AZD6738)	PARP inhibitor	For breast, ovarian, and pancreatic cancers	NCT04298021	Bile Duct Cancers	Recruiting
	Rucaparib	In combination with Nivolumab	PARP inhibitor	No	NCT03639935	Biliary Tract Cancers	Active, not recruiting

where a significant improvement in progression-free survival was observed compared to placebo (median of 2·7 months *vs.* 1·4 months), despite a non-significant overall survival[40]. Considering the cytostatic mechanism of action of ivosidenib, the modest clinical benefit is consistent with those reported with other targeted non-cytotoxic drugs. To further explain modest clinical responses, the first mechanisms of acquired resistance have emerged, with two patients enrolled in a phase I trial of ivosidenib as monotherapy (NCT02073994) developing secondary *IDH1* mutations, R172K and D279N, at disease progression[41]. These acquired mutations are thought to disrupt the binding of ivosidenib to the *IDH1* mutant, rendering the drug ineffective and enabling disease progression[41]. In addition, a third patient enrolled in the clinical trial exhibited persistence of a pre-treatment *IDH1* R132C mutation and acquired a novel

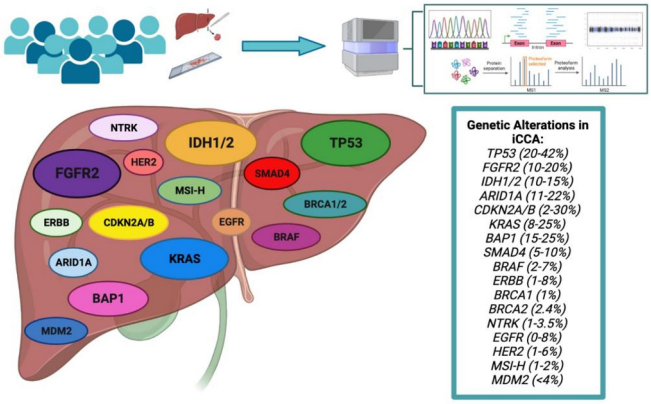

Figure 1. Genetic alterations in iCCA. Multi-omic analyses of large patient cohorts have revealed the most prevalent alterations in iCCA. *FGFR2* fusions and mutations in *IDH1/2* are the most prevalent, while alterations in *KRAS*, *ARID1A*, *CDKN2A/B*, and *BAP1* occur at slightly lower frequencies. Other alterations occur in iCCA at low frequencies but are still considered actionable therapeutic targets are herein depicted. Created with https://www.biorender.com/ .

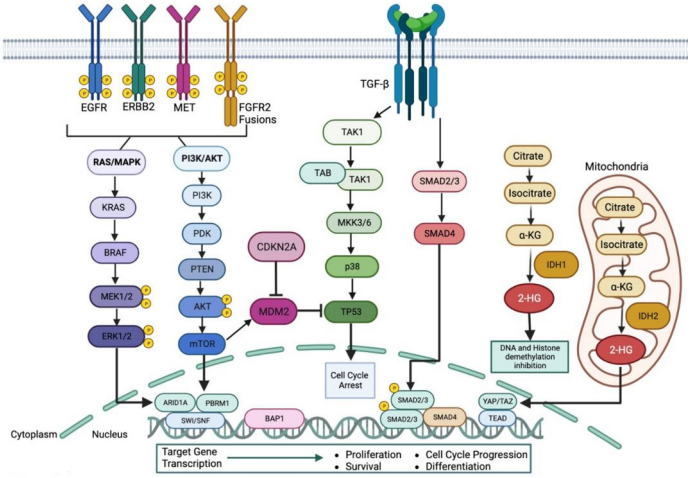

Figure 2. Key signaling pathways in intrahepatic cholangiocarcinoma. A simplified view of pathways activated by genetic alterations identified in iCCA. Mutations in receptor tyrosine kinases like EGFR, ERBB, MET, and FGFR2 fusions dysregulate signaling of the RAS/MAPK and PI3K/AKT pathways. Uncontrolled TGF-β signaling is defined by loss-of-function mutations in SMAD4 and is linked to dysregulated signaling through the TAK1 pathway. Mutations in *IDH1/2* lead to increased levels of 2-HG and increased YAP/TAZ-directed transcription. Created with https://www.biorender.com/.

IDH2 R172V mutation[42]. Future studies further investigating these, and other mechanisms of resistance are needed.

In addition to the downregulation of chromatin modifier genes, an increase in the 2-HG oncometabolite seen in IDH1/2 mutated cancers inhibits proteins associated with DNA damage response, such as lysine-specific demethylase 4A/B (KDM4A/B) and ataxia-telangiectasia mutated (ATM), as well as the DNA repair enzyme alkB homolog (ALKBH)[43,44]. Decreased function in these proteins causes an increase in DNA double-strand breaks and increased DNA damage. This effect, similar to the homologous recombination deficiency (HRD) that is exhibited in *BRCA1/2* mutated cancers (referred to as the "BRCAness" phenotype)[45], confers sensitivity to inhibition of DNA repair mechanisms, such as poly(ADP-ribose) polymerase (PARP) inhibition. As *IDH1/2* mutations are among the most frequent mutations in iCCA, the PARP inhibitor, Olaparib, is currently being tested in phase II studies (NCT03212274; NCT03878095) to

evaluate its efficacy in patients with IDH1/2 mutant solid tumors including CCA[46].

FGFR2 alterations

Fibroblast growth factor receptor 2 (FGFR2) is a member of the family of receptor tyrosine kinases (RTKs) which, upon ligand binding, dimerizes and signals activation of downstream pathways including RAS/MAPK, PI3K/AKT, and JAK/STAT. These pathways play a role in cell proliferation, differentiation, and apoptosis, and when dysregulated, contribute to increased proliferation, tumorigenesis, and metastasis[47-49]. GAs in the *FGFR2* gene encompass mutations, gene fusions and in-frame deletions[25,28,40,50-54]. Nearly 80% of all *FGFR2* GAs are fusions involving exons 1-18 of the *FGFR2* with the kinase domain intact, fused in-frame to diverse gene partners[28,53-56]. This fusion partner leaves FGFR2 without an important proline-rich sequence that allows GRB2 to bind and deactivate downstream signaling[57]. The most prevalent fusion partners are *bicaudal C homolog 1 (BICC1), Periphilin 1 (PPHLN1), adenosylhomocysteinase like 1 (AHCYL1), Transforming Acidic Coiled-Coil Containing Protein 3 (TACC3), and meningioma expressed antigen 5 (MGEA5)*, although over 60 fusion partners have been catalogued[28,50,53-55,58]. Regardless of the partner identity, fusions result in constitutive kinase activity [Figure 2][50,53,54]. Transforming capabilities of FGFR2 fusions have been demonstrated and can be suppressed both *in vitro* and *in vivo* with FGFR1-3 inhibitors[50,53], providing the rationale for the first biomarker-driven clinical trials. It has been recently described that while *FGFR2* fusions tend to co-occur with alterations in *BAP1* and *CDKN2A/B*, they are usually mutually exclusive with mutations in *KRAS*[27,39]. Although rare, patients harboring *FGFR* alterations may present with concomitant alterations which could contribute to resistance mechanisms[56] or, when targetable itself, such as the presence of *IDH* mutations, make treatment choices difficult. In the latter circumstances, discussion of each clinical case at designated molecular tumor boards should be actively pursued. In this regard, increasing efforts are ongoing to implement local, regional or digital multidisciplinary liver tumor boards to share, discuss and harness the challenges brought by precision oncology[59]. Additional GAs in *FGFR2* occur in the form of point mutations or short variants[56], with the most common including gain-of-function mutations (S372, Y375, C382, N549 and F276) that stabilize the active dimerized conformation of FGFR2[27,39]. Extracellular in-frame deletions in the *FGFR2* gene resulting in structural and conformational changes that can activate FGFR2 have also been recently documented[51]. These alterations possess transforming capabilities and seem to predict sensitivity to FGFR inhibitors[51].

Based on the results of recent phase 2 clinical trials, three FGFR inhibitors (e.g., infigratinib, pemigatinib, and futibatinib) have received accelerated FDA approval as a treatment option for patients with iCCA harboring a *FGFR2* fusion or other alterations[40,60,61]. In particular, infigratinib (BGJ398) and pemigatinib - two reversible ATP-competitive FGFR1-4 inhibitors - showed an objective response rate of 23%[61] and 36%[40], respectively, in the setting of a manageable adverse event profile. As a result, pemigatinib is currently being investigated in a phase III clinical trial as monotherapy versus the standard of care treatment (NCT0365653), whereas infigratinib is no longer being pursued and has been discontinued for commercial reasons. Clinical trials using infigratinib in combination with GemCis and immune checkpoint inhibitors are still registered [NCT05510427, NCT05514912, Table 1]; however, whether these trials will be discontinued has not yet been reported to date.

Despite an initial response to these two FGFR inhibitors, the clinical benefit remains modest and short-lived since resistance inevitably emerges, as elegantly described in 4 patients enrolled in a trial for treatment with infigratinib (NCT02150967) where the evaluation of tumor biopsies and plasma at the time of disease progression revealed the onset of secondary mutations in the *FGFR2* gene[55,62]. Notably, 3 of the 4 patients developed a mutation in the gatekeeper residue V564, leading to structural change in the *FGFR2* binding pocket, ultimately preventing infigratinib from binding. Additionally, these patients acquired mutations in

residues V549, E565, K641, and K659, all of which led to kinase activation and increased PI3K/AKT/mTOR signaling, and a mutation in L617 that weakens the stability of an Asp-Phe-Gly conformation that favors inhibitor binding[55,62]. Similarly, patients evaluated after treatment with pemigatinib also acquired secondary mutations in additional residues (N549, E565, K659, L617, and K641), indicating that these mutations confer resistance to multiple FGFR inhibitors[28,63]. In this context, in a phase I clinical trial (NCT02052778), Futibatinib (TAS-120), an irreversible ATP-competitive FGFR1-4 inhibitor, demonstrated efficacy in 4 patients with *FGFR2* fusion-positive iCCA who developed resistance to infigratinib or Debio 1347[64]. The application of molecular profiling in circulating tumor DNA (ctDNA) combined with *in vitro* studies using patient-derived iCCA cells further confirmed that TAS-120 was active against multiple *FGFR2* mutations conferring resistance to BGJ398 or Debio 1347, while mutations in the gatekeeper V565 residue continued to show resistance to FGFR inhibition[64]. These results suggest that serial molecular testing could inform sequential treatment with FGFR inhibitors and prolong responses to these agents. However, unlike other GAs, there are limitations to using ctDNA to detect FGFR2 fusions. Berchuck *et al.* report that *FGR2* fusions were detected in serial circulating free DNA (cfDNA) at lower frequencies than in tissues biopsies (1.4% *vs.* 4.3%), revealing that concordance between them was only 18% in their patient cohort (n = 67 patients with *FGFR2* fusions)[65]. Additionally, the sensitivity in their detection assays was higher for the *FGFR2-BICC1* fusion versus other fusion partners (58% *vs.* 2%, respectively), a trend that was not improved by repeated serial sampling[65]. They suggest this is not due to a lack of cfDNA in blood samples, but rather because of the promiscuity of the *FGFR2* gene[65]. The abundance and variety of fusion partners in iCCA patients mean that more probes are required to detect those fusions, but limitations on the panel size for cfDNA assays mean increasing the number of probes targeted towards fusion partners is not always feasible[65]. Additionally, Goyal *et al.* emphasize that while molecular profiling of ctDNA is a useful tool in monitoring a patient's response to therapy, it is a technique best used in combination with genetic sequencing of tumor biopsies at the multiple stages of treatment and disease progression[64]. Finally, validation with larger clinical cohorts is needed to elucidate whether patterns in resistance mutations can inform treatment strategies[64].

The successful completion of the multinational, open-label, phase 2 study (NCT02052778) confirmed the clinical benefit elicited by futibatinib with a 42% objective response rate[60]. Notably, the use of futibatinib resulted in durable responses and survival that surpassed those indicated by historical data with chemotherapy in patients with refractory iCCA[60]. This agent is being investigated in a phase III clinical trial (NCT04093362) versus the standard of care treatment gemcitabine plus cisplatin. At the same time, several efforts are currently underway to shed light on additional resistance mechanisms to FGFR inhibitors and improve therapeutic responses. In this regard, Wu *et al.* recently demonstrated that feedback activation of EGFR signaling limits the effectiveness of FGFR inhibitor therapy and drives adaptive resistance in patient-derived models of iCCA carrying *FGFR2* fusions, suggesting that the combination of FGFR and EGFR inhibitors could improve responses in this molecularly-defined subgroup of patients[66]. Additionally, Subbiah *et al.* have reported promising preliminary results for RLY-4008, a selective and irreversible inhibitor of FGFR2, in the ongoing phase I/II ReFocus clinical trial (NCT04526106)[67]. Preclinical models show that RLY-4008 is effective at inhibiting tumor growth, including tumors generated with a gatekeeper V564I resistance mutation[67]. Patients in the ReFocus clinical trial all had a partial response to RLY-4008 and marked tumor reduction without the same adverse effects that other pan-FGFR inhibitors confer such as elevated serum phosphate levels and diarrhea[67]. To improve the treatment of iCCA, FGFR inhibitors are also being tested in combination with ICIs [NCT05510427, NCT05174650, Table 1]. However, considering the poorly understood immunoregulatory role of *FGFR2* fusions and the paucity of immunocompetent *FGFR2*-driven iCCA models, the design of such clinical trials is currently not guided by preclinical data.

Alterations in KRAS and the MAPK pathway

Kirsten rat sarcoma virus (KRAS) belongs to a group of small GTPases called the Ras family[68]. The protein encoded by *KRAS* is involved in the regulation of cell growth, differentiation and survival through the RAS/MAPK signaling cascade, and mutations lead to uncontrolled proliferation of cells and tumor growth[69,70] [Figure 2]. Mutations in the *KRAS* gene are among the most frequent alterations in iCCA (8%-25%)[19,23,27,71-78], and other malignancies[79], with missense activating mutations most frequently occurring at codons 12 and 13[27,80,81]. Of these activating mutations, G12D is the most frequent *KRAS* variant, occurring in 43.3% of patients[82]. Other variants include G12V (19.7%), G12C (7.1%), and G13D (6.3%)[82-84]. Additionally, the variants G12A, G12S, G12R, G13C have been reported in iCCA, although these are more rare[82-84]. GAs in genes coding for other components of the MAPK pathway have also been reported in iCCA, although at a lower frequency, and include mostly somatic mutations in *MEK1/2* (*MAP2K1*), *ERK1/2* (*MAP3K1*), *NF1* (6%-7%), *ARAF* (11%)[35,53,77,85]. In addition to *KRAS*, mutations in other RAS family proteins, such as *HRAS* and *NRAS*, are rarely reported in iCCA (4%-10%)[27,74,86]. Recent studies have shown that alterations in *TP53* and *CDKN2A/B* are most frequently co-occurring in *KRAS* mutant iCCA. Other frequent co-mutations include those in *ARID1A*, *SMAD4*, *BAP1*, and *PBRM1* genes[27,72,87]. Although their biological impact is not fully understood, *KRAS* mutations have been associated with poor prognosis and reduced survival in iCCA[21,23,71,88-90]. The presence of co-occurring *KRAS* and *TP53* mutations has also been found to be associated with poor prognosis in biliary tract cancers[87].

Despite *KRAS* long being considered "undruggable", recent landmark studies have led to the FDA approval of sotorasib (AMG 510), the first small molecule inhibitor targeting specifically $KRAS^{G12C}$ mutation, for advanced non-small cell lung cancer (NSCLC) based on the results of the CodeBreaK 100 trial (NCT03600883)[91]. This study included one biliary cancer patient who achieved disease control[92]. In addition, in December 2022, the FDA granted accelerated approval to adagrasib (MRTX849)[93], another RAS GTPase inhibitor, based on the results of the KRYSTAL-1 study, a multicenter, single-arm, open-label clinical trial (NCT03785249) which included patients with locally advanced or metastatic NSCLC with $KRAS^{G12C}$ mutations[94]. An expansion cohort of this study is currently ongoing in patients with advanced tumors, including CCA [Table 1]. Furthermore, inhibitors targeting mutations other than G12C are in the early stages of development. MRTX1133, the $KRAS^{G12D}$ inhibitor[95,96], is set to enter a Phase 1 clinical evaluation in 2023 for $KRAS^{G12D}$-driven cancers, including pancreatic, colorectal, lung, and others (NCT05737706). Despite these promising results, acquired resistance is a common pitfall of targeted therapies and KRAS inhibitors are no exception. Acquired resistance to KRAS inhibitors has been recently demonstrated by the identification of multiple treatment-emergent alterations in 27 out of 43 patients with distinct solid cancers (the majority being NSCLC) treated with sotorasib, including secondary alterations in *KRAS*, *NRAS*, *BRAF*, *EGFR*, *FGFR2*, *MET*, *MYC* and other genes[97-99]. Because of the intense crosstalk between signaling pathways downstream of *KRAS* and the activation of feedback loops due to treatment-emergent mutations, combining inhibitors targeting multiple redundant signaling pathways seems most promising. For example, genetic or pharmacological targeting of ERK signaling has been shown to enhance the antiproliferative effect of $KRAS^{G12C}$ inhibition models with acquired *RAS* or *BRAF* mutations[99]. In conclusion, targeting the KRAS pathway holds great promise as a therapeutic approach, but further research is needed to optimize treatment strategies, properly design combination strategies to overcome potential resistance and improve outcomes for iCCA patients.

BRAF

B-raf proto-oncogene (*BRAF*) is a serine/threonine protein kinase that acts as a downstream effector of KRAS via the ERK/MAPK pathway. Mutations in *BRAF* result in its constant dimerization, leading to uncontrolled cell proliferation, differentiation, and survival[100-102]. $BRAF^{V600E}$ is the most common variant

implicated in many cancers including iCCA where it has been detected in 2%-7% of patients[27,71,74,78,103-106]. Interestingly, in the FoundationCORE database, $BRAF^{V600E}$ and $BRAF^{non\text{-}V600E}$ alterations occurred at similar frequencies but featured a strikingly different mutational spectrum with $BRAF^{V600E}$ mutations showing a predominance of *CDKN2A/B* deletions over *TP53* alterations[27]. In contrast, patients with $BRAF^{non\text{-}V600E}$ alterations had a lower frequency of *KRAS*, *FGFR2* and TERT promoter alterations and an enrichment in *ARID1A* Gas[27].

While monotherapies against the $BRAF^{V600E}$ mutation have had discouraging results[107], the phase II Rare Oncology Agnostic study (ROAR trial; NCT02034110), including 43 patients with advanced $BRAF^{V600E}$ mutated BTC (iCCA patients, n = 39), showed promising results for the combination of the trametinib MEK 1/2 inhibitor and the BRAF inhibitor, dabrafenib[108]. Based on these results, a combination of dabrafenib and trametinib was approved as a tumor-agnostic therapy for all solid metastatic cancers with $BRAF^{V600E}$ mutations[109,110]. In addition, the BRAF inhibitor ABM-1310 is currently in phase I study (NCT04190628) evaluating its efficacy alone or in combination with cobimetinib for solid tumors with $BRAF^{V600E}$ mutation, including iCCA.

Targeting the ERBB family

The ERBB family encompasses four receptor tyrosine kinases: *EGFR* (*ERBB1*), *HER2/NEU* (*ERBB2*), *ERBB3*, and *ERBB4*[111]. These receptors play a vital role in regulating cellular proliferation, differentiation, and migration by activating MAPK and PI3K/AKT signaling pathways, among others [Figure 2]. Alterations in the ERBB family have been reported in iCCA patients, with *EGFR* and *ERBB2* being the most frequent[73]. *EGFR* overexpression and *HER2* amplification have been reported in iCCA with a frequency of 0%-8% and 1%-6%, respectively, and represent appealing therapeutic targets[19,23,27,58,71,89,112-119].

Various EGFR and HER2 inhibitors have been investigated in early phase trials, including lapatinib, erlotinib, pertuzumab, and trastuzumab[119-122]. However, in various clinical trials, EGFR inhibitors have shown limited benefit. For advanced BTC patients, cetuximab and panitumumab did not show survival benefits when combined with chemotherapy. Erlotinib, an oral EGFR inhibitor, was combined with oxaliplatin and gemcitabine in the phase III trial for advanced, untreated BTC patients, but it did not provide a survival benefit[123]. Currently, Erlotinib is being evaluated in combination with pemetrexed in phase II single-arm prospective study (NCT03110484) in metastatic biliary tract cancer patients.

A few studies are currently ongoing to evaluate HER2-directed therapies in mutant biliary tract cancers. Zanidatamab, a HER2-targeted bispecific antibody, received an FDA breakthrough therapy designation based on an ongoing phase 2b clinical trial (NCT04466891) testing this agent as treatment of patients with locally advanced and/or metastatic HER2-expressing tumors, including biliary tract cancer[124]. In addition, Zanidatamab is being investigated in combination with chemotherapy as first-line treatment in a phase II clinical trial (NCT03929666) for HER2-expressing gastrointestinal cancers, with overall promising preliminary results including a confirmed objective response of 79% and a disease control rate of 92%[125]. The Phase 3 randomized trial HERIZON-GEA-01 (NCT05152147) is ongoing and its successful completion will support the approval for zanidatamab in combination with chemotherapy as an effective treatment option for this molecularly-defined subgroup. In addition, promising results were recently reported in a phase I study testing trastuzumab deruxtecan (T-DXd), a novel HER2-targeted antibody-drug conjugate linked to a topoisomerase I inhibitor, in various solid tumors, including biliary tract cancers[122]. Reported preliminary results for the HERB trial (NCCH1805; JMA-IIA00423) show that HER2 positive iCCA patients (n = 30) treated with T-DXd had longer median PFS *vs.* the control group (6.1 months *vs.* 5.1 months) and longer median OS (10.8 months *vs.* 7.1 months)[126,127]. These results will need to be further evaluated in a

larger cohort of patients. Currently, T-DXd is also being tested in an open-label study (NCT04482309) in patients with selected HER2-expressing tumors. Another phase II (NCT04837508) study is currently testing MRG002, a HER2-targeted antibody-drug conjugated to the microtubule-disrupting cytotoxic agent monomethyl auristatin E (MMAE), for advanced or metastatic biliary tract cancer.

NTRK fusions

The *neurotrophic tyrosine receptor kinase* (*NTRK)* gene family consists of *NTRK1*, *NTRK2*, and *NTRK3*, which encode tropomyosin receptor kinases (TRK) A, B, and C, respectively. *NTRK* gene fusions are rare, with a reported frequency of 1%-3.5% in iCCA[21,121]. Oncogenic fusions of the *NTRK* gene result in a chimeric protein with the N-terminus of the fusion partner joined to the C-terminus of the TRK protein with the catalytic tyrosine kinase domain. These oncogenic fusions lead to constitutive activation of MAPK cascade and PI3K pathway, promoting cell proliferation and survival[128].

Despite being rare, two potent NTRK inhibitors, entrectinib and larotrectinib, have recently emerged as novel therapeutic options in *NTRK* fusion-positive malignancies, including CCA. In this regard, larotrectinib, which has previously shown promising results in patients expressing *TRK* fusions, including 2 CCA patients[129], is currently being evaluated in the NAVIGATE phase 2 basket trial (NCT02576431)[130]. Chromosomal rearrangement of *ROS1* represents an additional rare oncogenic driver mutation in iCCA (0%-9%)[114,131] and a phase 2 basket study (NCT02568267) is currently evaluating entrectinib in patients with solid tumors including CCA with fusions of the *NTRK1/ 2*, *ROS1* or *ALK* gene. Notably, both inhibitors - entrectinib and larotrectinib - have been approved for patients with *NTRK* fusion-positive cancer, independent of their histology[129,132].

DNA mismatch repair and microsatellite instability

DNA mismatch repair (dMMR) is an important system for maintaining genomic integrity through the recognition and repair of DNA replication errors[133]. Mutations in dMMR lead to the accumulation of frameshift mutations in microsatellite-coding genes, leading to microsatellite instability (MSI)[134,135]. Overall, the frequency of MSI is low in iCCA, with MSI-high being reported in about 1%-2% of patients[27,136]. Recent studies have shown that MSI-high iCCA patients exhibit concomitant alterations in genes such as *TP53*, *ARID1A*, *RNF43*, *PBRM1*, and *CDKN2A*. In addition, enrichment of alterations related to activation of the WNT signaling pathway and mutations in *MLH1* and *MSH6* were reported in this subgroup of patients in the FoundationCORE database[27].

Based on the findings from the phase II KEYNOTE-158 trial which demonstrated clinical benefit in MSI-H and dMMR tumors treated with immune checkpoint inhibitors (ICIs)[137], MSI-high status is currently regarded as the best predictive biomarker of ICI responses across all tumor histologies. Despite MSI-H and dMMR being rare events in iCCA, patients in the KEYNOTE-158 trial responded well to pembrolizumab (median PFS 4.2 months, median OS 24.3 months), therefore making these two events a potentially useful biomarker for predicting ICI response in iCCA patients[137].

BRCA1/2 mutations and the potential benefit of PARP inhibitors

Breast Cancer gene 1 (BRCA1) and *Breast Cancer gene 2* (*BRCA2*) are tumor suppressor genes that play a vital role in DNA damage response (DDR) mediated by the homologous recombination pathway. Mutations in *BRCA1/2* lead to the accumulation of DNA double-stranded breaks, resulting in genomic instability and tumor growth[138-142]. *BRCA1* and *BRCA2* mutations have been reported in 1% and 2.4% of iCCA patients, respectively[27,143-145]. iCCA patients with these mutations are concurrently mutated for *TP53*, *CDKN2A/B*, *KRAS*, *ARID1A*, and *PBRM1* amongst others[27]. Studies have shown that poly adenosine

diphosphate-ribose polymerase (PARP) inhibitors may be effective for CCA patients with mutations in the DDR genes, including *BRCA1/2* alterations[146-148]. Several clinical trials are currently in progress to evaluate the efficacy and safety of PARP inhibitors, either as monotherapy or in combination with other therapies in cholangiocarcinoma. For example, an umbrella study (NCT04298021) is currently evaluating the efficacy of the PARP inhibitor, Olaparib, alone or in combination with durvalumab in advanced biliary tract cancers. A single-arm phase II study (NCT04306367) is testing the safety and efficacy of the combination of pembrolizumab and olaparib to improve the response rate of second-line systemic therapy in patients with advanced CCA who cannot tolerate gemcitabine-based therapy. Another PARP inhibitor, rucaparib, is currently being evaluated in phase II study (NCT03639935) in combination with nivolumab in patients with advanced or metastatic biliary tract cancer following platinum therapy. Additional clinical trials involving more iCCA patients are required to provide definitive evidence of the therapeutic potential of PARP inhibitors for treating iCCA patients.

Other "currently undruggable" alterations

TP53

The *tumor suppressor gene tumor protein P53* (*TP53*) is involved in DNA damage repair, cell cycle arrest, apoptosis, and metabolism[149,150]. Mutations in *TP53* have been found in most cancers, including NSCLC, breast cancer, pancreatic ductal cancer, and primary liver cancers (both HCC and iCCA)[27,87,149,151,152]. Typically, mutations in *TP53* lead to a loss of function, resulting in increased cell proliferation, metabolic pathway activity, and tumorigenesis[39]. In iCCA, *TP53* mutations are the most common alterations occurring in ~20%-42% of patients[27,72,153]. Amplifications of the *MDM2* gene, one of the main *TP53* negative regulators through ubiquitination and proteasomal degradation, have also been described across many cancers, although only in less than ~4% of iCCA[27]. *TP53* mutations frequently co-occur with alterations in *KRAS*, *BRCA1/2*, and *ERBB2*, but are mutually exclusive with *FGFR2* fusions[27,39].

Alterations in chromatin remodeling genes

AT-rich interactive domain-containing protein 1A (ARID1A) encodes a key component of the SWI/SNF chromatin-remodeling complex. Loss-of-function mutations in *ARID1A* frequently occur in iCCA (11%-22%)[27,74,89,106,114,154,155], and lead to aberrant chromatin remodeling, genomic instability, and altered DNA repair, ultimately contributing to CCA progression[156]. Other chromatin remodeling genes such as polybromo-1 (*PBRM1)* and BRCA1-associated protein 1 (*BAP1*) are also found mutated in 11%-17%[19,27,86,89] and ~15%-25% of iCCA patients[154,157], respectively, with at least one of the three genes being mutated in 47% of iCCA cases[154]. In particular, loss of function in *BAP1* occurs through either chromosomal deletions or inactivating mutations, and leads to tumor formation and progression[158,159]. Germline alterations in *BAP1* have also been linked to an increased risk of iCCA and other malignancies[157].

SMAD4 and the TGF-b signaling pathway

Mothers against decapentaplegic homolog 4 (SMAD4) is a regulator of the transforming growth factor- (TGF-) signaling pathway, which controls cell proliferation, differentiation, and other cellular functions[160,161]. In its tumor suppressive role, SMAD4 loss leads to increased TGF- signaling and an increase in transcription of downstream targets[160]. *SMAD4* inactivation mutations through chromosomal deletion or point mutations are found in ~5%-10% of iCCA patients[27,72,121], and recent studies have shown that patients with lower S*MAD4* expression have poor cell differentiation and more metastatic disease[160]. GAs in other components of the pathway, including both receptors and ligands, have been described at a lower frequency (2%-3%)[83,162].

CDKN2A/B

Deletions in *cyclin-dependent kinase inhibitor 2A and 2B (CDKN2A/B)* have been reported between 2%-30% for iCCA patients[72,121,163,164]. *CDKN2A* is a tumor suppressor gene that encodes $p16^{INK4A}$ and $p14^{ARF}$, while *CDKN2B* encodes $p15^{INK4B}$[150,163]. Both $p16^{INK4A}$ and $p15^{INK4B}$ are regulators of the cyclin-dependent kinases CDK4 and CDK6 and play an integral role in inhibiting cell cycle progression through G1 phase[163,165]. The protein p14ARF activates and stabilizes TP53 to activate cell cycle arrest and apoptosis[165,166]. When *CDKN2A/B* genes are lost, CDK4/6 activity is left unregulated and cell proliferation is promoted[163,165].

TERT

The TERT gene encodes for the catalytic subunit of telomerase enzyme. Telomerase plays a vital role in maintaining genomic integrity and telomere length at the end of the chromosomes, which is vital for cellular replication and stability. TERT acts as a gatekeeper for cellular immortalization while maintaining chromosomal integrity. Unlike HCC, promoter mutations in TERT are found in iCCA at low frequencies (6%)[27,35,85].

MOLECULAR CLASSIFICATIONS OF ICCA

Several classifications have been proposed to date for iCCA patients, building molecular profiles based on transcriptomics, proteogenomics, and genetic characteristics[72,162,167,168]. The overarching goal of these classifications is to ultimately identify groups of patients with similar genomic profiles and use this information to determine a course of treatment. However, despite numerous efforts to establish an effective classification system for iCCA patients that includes genomic, molecular, and immune profiles for subtypes, there is still no consensus on which system to use, mostly due to the lack of validation in prospective clinical trials as well as the difficulties in routinely accessing tissue biopsies. In this paragraph, we will discuss how genetic alterations have contributed to defining distinct molecular and immune subgroups of iCCA. Molecular classifications based on transcriptomics and other -omics have been extensively reviewed elsewhere[169].

Genetic alterations-driven subtypes of iCCA

As the role of massive parallel sequencing has expanded over the past few years, several molecular classes with distinct mutational profiles have emerged[35,72,162,167,168,170-173]. Using genomic and epigenomic data from 489 CCA patients, Jusakul *et al.* identified four distinct clusters with separate genetic and epigenetic features. Cluster 1 showed enrichment of *ARID1A* and *BRCA1/2* mutations as well as DNA hypermethylation. Both cluster 1 and cluster 2 showed enrichment of *TP53* and *ERBB2* mutations, but cluster 2 was also enriched with *CTNNB1*, *WNT5B*, and *AKT1* mutations. In contrast, cluster 3 showed high levels of somatic copy number alterations and upregulation in immune checkpoint genes like *PD-1* and *PD-L2*. Finally, cluster 4 exhibited enrichment in *BAP1*, *IDH1/2*, and *FGFR* alterations. In addition, these clusters were correlated with clinical outcomes, with clusters 3 and 4 having better overall survival compared to clusters 1 and 2[171]. Similarly, Bagante *et al.* classified patients into two genetic groups after reviewing previously published clustering based on gene mutations[35,170,171]. Their *KRAS/TP53* group included patients with mutations in *KRAS*, *NRAS*, *TP53*, and *ARID1A*, while patients with *IDH1/2*, *BAP1*, and *PBRM1* were included in the second group. Analysis of pathological characteristics showed that the *KRAS/TP53* group had more periductal infiltration while the *IDH1-2/BAP1/PBRM1* group exhibited larger tumor sizes. Additionally, they were able to associate the *IDH1-2/BAP1/PBRM1* group with longer overall survival versus the *KRAS/TP53* group[170]. While both studies had cohorts containing multiple subtypes of CCA (iCCA, eCCA and gallbladder cancer), Nepal *et al.* proposed a stratification of *"only"* iCCA patients using the most recurrent mutations identified in a dataset of 496 patients analyzed by whole-exome and targeted exome sequencing[172]. Their subgroups (*IDH1*, *KRAS*, *TP53*, and 'Undetermined') exhibited

distinct co-mutation profiles as well as unique pathway enrichments. For example, patients in the *IDH1* group did not co-occur with *TP53* or *KRAS* mutations but were enriched in *BAP1* and *BCLAF1* gene alterations and showed enrichment in metabolic pathways. In contrast, patients in the *TP53* group had co-occurrences with *KRAS* mutations, enrichment in alterations in *PTEN*, *RB1* and *LATS2*, and upregulated *MAPK*, *WNT*, and *p53* signaling. Consistent with previous studies, Nepal *et al.* associated their gene mutation groups with patient overall survival, showing that the *KRAS* and *TP53* groups had worse overall survival versus the *IDH1* and 'Undetermined' groups within the cohort[172]. More recently, Wang *et al.* expanded existing efforts to analyze a cohort of 1481 iCCAs derived from several publicly available datasets. They identified three clusters based on the most prevalent mutations, with cluster 1 including patients with mutations in *KRAS*, *TP53*, and *SMAD4*, while cluster 2 included patients with *IDH1/2* and *BAP1* mutations and *FGFR2* fusions. Their third cluster, "Wild-Type", included all other patients with enrichment in *ARID1A*, *PBRM1*, *CDKN2A*, *PIK3CA*, and *EPHA2* gene mutations, but exhibiting wild-type genes for those in the other 2 clusters as well as patients with no detectable genetic mutations[173]. These clusters were associated with distinct gene expression patterns, histological features, and clinical prognosis and outcomes. For example, patients in cluster 2 exhibited small-duct type iCCAs with lower tumor burden, better prognosis, and higher expression of growth factors and receptors. In contrast, patients in cluster 1, with *KRAS*, *TP53*, and *SMAD4* mutations, had higher tumor burden, worse prognosis, and increased expression of genes such as *COX-2*, *MUC1*, and *IL-6*, which are associated with disease progression[173]. Overall, great consistency exists across these studies, with patients with *KRAS* and *TP53* mutations clustering separately from patients with *IDH1/2* mutations and *FGFR2* fusions. Additionally, a worse prognosis was reported for the subgroups harboring *KRAS* or *TP53* alterations compared to the subgroups including *IDH1/2*, *BAP1*, and *FGFR2* fusions, indicating that clustering along these lines could provide beneficial information in the clinical setting.

Genotype-immunophenotype correlations in iCCA

With the recent success of immunotherapeutic strategies across several solid cancers, including iCCA, the identification of immune-related subgroups of patients with prognostic/predictive value has gained significant traction[72,174,175]. However, only a few of these studies have paid particular attention to elucidating how the tumor genotype of iCCA affects the immunophenotype and *vice versa*. Evidence from other solid tumors clearly indicates that tumor-intrinsic alterations, such as oncogenic pathways (i.e., PI3K, MYC) and mutations in driver genes (i.e., gain-of-function mutations in the *KRAS*), contribute to the immunosuppressive tumor microenvironment that supports cancer growth through various mechanisms and potentially influence responses to ICIs[176-178]. The recent pan-cancer immunogenomic analysis of the intratumoral immune landscape of over 20 solid cancers has further reinforced the concept that tumor-intrinsic genomic alterations determine the tumor immunophenotype and dictate distinct escape mechanisms which are therefore predetermined based on the intra-tumor genetic composition[179]. As an example, the Pan Cancer Immunome ATLAS study concluded that *BRAF*-mutated tumors may utilize distinct evasion mechanisms compared to *KRAS*-mutated tumors. Specifically, *BRAF*-mutated tumors were found to be enriched in immunosuppressive cells, whereas *RAS* tumors showed downregulation of MHC molecules and immunomodulatory molecules. Nonetheless, whether these associations and underlying mechanisms of immunoevasion differ from one solid tumor to another according to the tissue-specific co-occurring genomic alterations and/or other oncogenic pathways dysregulation remains still unresolved. This is particularly relevant for iCCA, an understudied malignancy whose immune milieu remains poorly understood. Of note, no CCA was included in the Pan Cancer Immunome ATLAS, underlying the dire need to conduct immunogenomic analysis specifically in this disease. A step forward in elucidating the genotype-immunophenotype relationships in iCCA is represented by the recent STIM classification proposed by Martin-Serrano *et al.*, who, through the multi-omics analysis of about 900 iCCA samples, identified five distinct classes, each characterized by a distinct tumor genotype associated with a unique

stromal and immune composition[72]. Notably, this immunogenomic analysis suggests that mutations in driver genes such as *KRAS* and *TP53* may play a key role in dictating the immune milieu of iCCA with *KRAS*-mutant and *TP53*-mutant tumors being enriched in the inflamed classes of iCCAs, and alterations in *FGFR2*, *IDH1/2* and chromatin remodeling genes being enriched in the non-inflamed classes. Interestingly co-occurrence of *TP53* and *KRAS* mutations seemed to dictate a distinct immune milieu compared to *KRAS* mutant iCCAs (in absence of co-occurring TP53 mutations) with the former being associated with accumulation of Tregs in poorly inflamed tumors and the latter being enriched in myeloid cells, dysfunctional T cells, myofibroblastic cancer-associated fibroblasts (CAFs) and TGF- signaling in both murine and human iCCAs, all features associated with resistance to immunotherapy. Consistently, Martin-Serrano *et al* demonstrated that selective KRAS inhibitors can sensitize *KRAS*-mutant murine iCCAs to ICIs[72]. Clinical trials testing the combination of the newly FDA-approved $KRAS^{G12C}$ inhibitor with ICIs with or without ICIs are currently ongoing in lung (NCT04720976, NCT04613596, NCT05472623) and colorectal cancer (NCT04793958). Similar clinical studies are anxiously awaited in iCCA. Consistent results supporting the unique genotype-immunophenotype relationship in iCCA have been recently reported by Lin *et al*., who applied whole exome sequencing, T-cell receptor sequencing and multiplexed immunofluorescence on 207 tumor regions from 45 patients with iCCA[180]. In this study, highly immune infiltrated subgroups correspond with alterations in *KRAS* and *TP53*, while immune "cold" groups correspond with alterations in *FGFR2* and *BAP1*[72,153,180]. Interestingly, while Lin *et al.* found that patients with *IDH1/2* mutations displayed more T-cell infiltration[180], a recent study by Wu *et al.* has reported that *IDH1* mutations could shape a “cold” immune microenvironment in iCCA[181]. Interestingly, in this study, pharmacologic inhibition of IDH1 mutant induced profound immune changes, including $CD8^+$ T-cell recruitment and promotion of TET2-dependent induction of IFNγ response genes in tumor cells, and sensitized murine iCCAs to ICI targeting CTLA4[181]. Overall, these exciting emerging data suggest that a better understanding of the genotype-immunophenotype relationships may provide the rationale for combination immunotherapies to be tested in future clinical trials in molecular-defined subgroups of iCCA patients.

CLINICAL ASSESSMENT OF ACTIONABLE TARGETS

As discussed in the paragraphs above, genetic profiling enables the identification of actionable genetic alterations for targeted therapies. Therefore, both the European Society for Medical Oncology (ESMO) and United States National Comprehensive Cancer Network (NCCN) guidelines recommend molecular testing in all iCCA patients with advanced disease suitable for systemic treatments[182,183]. However, the different nature of GAs, the type of tissue available and the well-known technical challenges of tissue biopsies (i.e., poor quality and/or insufficient material) represent major pitfalls to the routine implementation of molecular testing.

Different testing methods exist due to the different nature of targetable GAs. Conventional methods, which include immunohistochemistry (IHC), fluorescence in situ hybridization and PCR-based DNA or RNA sequence amplification, can be readily and easily applied to detect the presence of ERBB2 amplifications, FGFR2 fusions and $BRAF^{V600}$ mutations[103,184]. MSI status can also be inferred via IHC to detect the expression of MMR proteins which consist of hMLH1, hPMS2, hMSH2 and hMSH6, or by fragment PCR[185], whereas FGFR2 fusions can be detected by break-apart FISH[186]. However, despite being valuable and relatively inexpensive tools, these methods are limited in scope, lack sensitivity and specificity, do not allow the interrogation of multiple genes, and require prior knowledge of the targeted GAs, ultimately hindering a comprehensive understanding of the iCCA genetic landscape in the clinical setting.

NGS-based technologies allow parallel sequencing of several genes and, thus, should represent the preferred testing modality. NGS approaches effectively detect genetic changes, rearrangements, and copy number alterations in multiple genes in a single analysis. Both WES and RNA-seq allow physicians to perform a comprehensive unbiased molecular screening of individual patients for informed treatment decisions, with RNA-seq being the preferred method for the detection of gene fusions[187,188]. However, major barriers to the routine implementation of these approaches exist, including financial (i.e., high costs and subsequent reimbursement by healthcare payers), turnaround time, and technical challenges (i.e., standardized tissue collection, protocols, *etc.*). In this regard, recent advances in the field have allowed large gene panels to be assayed with high accuracy, sensitivity, and specificity and at a reduced cost. NGS-based targeted panels such as MSK-IMPACT[187] and FoundationOne CDX offer a high coverage of tumor-specific genes, requiring less input material, easier data analysis and shorter turnaround time. Amplicon-based and anchored multiplex PCR approaches further expand the capabilities of these tests by allowing the identification of gene fusions from known (Oncomine™ Dx Target Test) as well as unknown fusion partners (Archer FusionPlex Solid Tumor Panel). However, iCCA tissue biopsies are often difficult to obtain, or even when a biopsy is feasible, it may yield insufficient material. In this regard, liquid biopsies represent a valid complementary strategy for molecular testing. Liquid biopsy methodologies are noninvasive and encompass NGS, droplet digital PCR, and digital PCR. Liquid biopsies can be used to detect biomarkers circulating tumor cells, circulating tumor DNA/RNA, exosomes, and microRNAs[189]. The clinical applications of liquid biopsies have been recently demonstrated in seminal studies of iCCA where cfDNA sampling successfully informed the sequence of FGFR inhibitors at the time of progression[55]. Nonetheless, the low sensitivity of cfDNA for the detection of FGFR2 fusions, with only 18% concordance between cfDNA and tissue biopsies, requires further optimization before being routinely implemented in clinical practice[65].

FUTURE DIRECTIONS

In summary, significant progress has been made in identifying recurrent and targetable genetic alterations in iCCA, with some being targeted by FDA-approved selective inhibitors while others are being tested in clinical trials. Nonetheless, the prognosis of this disease remains dismal, highlighting the dire need for additional research efforts aimed at identifying new therapeutic targets, understanding how genetic alterations orchestrate the tumor immune microenvironment, and ultimately translating these findings into improved patient outcomes.

The recent application of single cell-based technologies has shed light on the intra-tumor and inter-tumor heterogeneity of iCCA[190,191], although, thus far, only few iCCA samples have been analyzed due to the high costs. It is expected that, in the near future, a more widespread application of these technologies in large human cohorts will allow researchers to identify additional molecular drivers and understand the impact of specific genetic alterations on tumor composition and growth. Within each genetic molecular subgroup of patients (i.e., *KRAS*-, *IDH1*-mutant, *etc.*), it will also be crucial to understand how the specific co-mutational network impacts tumor progression and therapeutic responses to enhance the efficacy of precision medicine.

In a malignancy where 40%-50% of patients harbor at least a targetable alteration, the application of multi-omics (i.e., genomics, proteomics, transcriptomics, *etc.*) to tissue biopsies before the start of treatment could shed light on mechanisms of resistance to current chemotherapy and targeted therapies. These studies are anxiously awaited since they may identify predictive biomarkers for (1) better patient stratification and (2) informed decisions on sequential treatments when multiple therapeutic options will be available [upon completion of the ongoing phase 3 trials shown in Table 1]. To achieve this goal, education of all medical professionals will play a major role in ensuring universal access to molecular testing for all iCCA patients at

diagnosis and collection of serial tumor and blood samples.

An alternative approach to further improve responses to targeted therapies, including FGFR inhibitors, requires the rational design of effective combination strategies. Based on the recent results of the TOPAZ-1 (anti-PD-L1 blockade *plus* chemotherapy) and KEYNOTE-966 trials (anti-PD1 blockade *plus* chemotherapy), ICI combinations hold great promise for this malignancy but remain significantly underexplored. To achieve these clinical goals, the development of novel preclinical models that better represent the genetic heterogeneity as well as the tumor-immune interactions of iCCA is urgently needed. Significant efforts should also be devoted to establishing and characterizing precision mouse models, while cross-species analyses should be conducted to assert their clinical relevance. Furthermore, more advanced co-culture models of 3D organoids with other microenvironment components (i.e., immune cells, CAFs, *etc.*)[192] could help to better understand the genotype-immunophenotype relationships and underlying mechanisms, and thus not only improve our understanding of the tumor biology but also accelerate the development of more effective combinations targeting both the tumor and its host.

In conclusion, while a lot more remains to be done, the recent progress may indicate that we are on the right path to improve the outcome for iCCA patients.

DECLARATIONS

Authors' contributions

Conceived the structure of this manuscript, researched the literature and wrote the manuscript: Young SE, Sritharan R, Sia D

Availability of data and materials

Not applicable.

Financial support and sponsorship

DS is supported by the Tisch Cancer Institute (Icahn School of Medicine at Mount Sinai), the F. Klion Award, i3 Genesis Award and the TCI Dev. Funds Award 2023.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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