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Intrahepatic cholangiocarcinoma: morpho-molecular pathology, tumor reactive microenvironment, and malignant progression

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Abstract

Intrahepatic cholangiocarcinoma (iCCA) is a relatively rare, but highly lethal and biologically complex primary biliary epithelial cancer arising within liver. After hepatocellular carcinoma, iCCA is the second most common primary liver cancer, accounting for approximately 10–20% of all primary hepatic malignancies. Over the last 10–20 years, iCCA has become the focus of increasing concern largely due to its rising incidence and high mortality rates in various part of the world, including the USA. The challenges posed by iCCA are daunting and despite recent progress in the standard of care and management options for iCCA, the prognosis for this cancer continues to be dismal. In an effort to provide a framework for advancing our understanding of iCCA malignant aggressiveness and therapy resistance, this review will highlight key etiological, biological, molecular, and microenvironmental factors hindering more effective management of this hepatobiliary cancer. Particular focus will be on critically reviewing the cell origins and morpho-molecular heterogeneity of iCCAs, providing mechanistic insights into high risk fibroinflammatory cholangiopathies associated with iCCA development, and notably discussing the deleterious role played by the tumor reactive desmoplastic stroma in regulating iCCA malignant progression, lymphangiogenesis, and tumor immunobiology.

Keywords

morpho-molecular classification; fibroinflammatory risk conditions; tumor reactive microenvironment; cancer-associated fibroblasts; extracellular matrix; transforming growth factor-β; periostin; immune milieu; M2 macrophages; therapeutic targeting

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1. Introduction

Intrahepatic cholangiocarcinoma (iCCA) denotes a rare heterogeneous class of epithelial cancers arising within liver, which exhibits characteristics of cholangiocyte differentiation (Sirica *et al.*, 2009). After hepatocellular carcinoma (HCC), iCCA is the second most common primary liver cancer, accounting for approximately 3.0% all gastrointestinal malignancies (Khan *et al.*, 2019; Banales *et al.*, 2020) and comprising around 10–20% hepatobiliary cancers (Blechacz, 2017; Gupta & Dixson, *et al.*, 2017; Massarweh *et al.*, 2017; Banales *et al.*, 2020) and an estimated 8–20% of total biliary tract cholangiocarcinomas (Gupta & Dixson, *et al.*, 2017; Vijgen *et al.*, 2017; Rizvi *et al.*, 2018; Khan *et al.*, 2019).

Over the past few decades, iCCA has become the focus of heightened interest and concern, largely due to its increasing incidence in various countries around the world (Saha *et al.*, 2016; Banales *et al.*, 2016; Cardinale *et al.*, 2018), together with its globally high mortality rates also rising in several areas of the world (Bertuccio *et al.*, 2019), including the USA (Yao *et al.*, 2016; Beal *et al.*, 2017). Specific high risk factors for CCA include liver fluke infection with *Opisthorchis viverrini* (Thailand) or *Clonorchis sinensis* (Korea), primary sclerosing cholangitis (PSC), hepatolithiasis, and fibropolycystic liver diseases, such as Caroli disease (Massarweh & El-Serag, 2017; Khan *et al.*, 2019). The highest rates of iCCA are reported in Southeast and East Asia, particularly where parasite infection with liver flukes is endemic (Sripa *et al.*, 2018), whereas PSC with or without ulcerative colitis has been established as having the strongest association with CCA development in Western populations (Kirstein & Vogel, 2016; Khan *et al.*, 2019). Hepatitis B (HBV), hepatitis C, and cirrhosis have also been identified as strong risk factors for iCCA (Kirstein & Vogel, 2016; Gupta & Dixon, 2017; Clements *et al.*, 2020) and may account in part for the rising incidence of iCCA observed in different countries of the world.

In Western countries, most iCCAs (i.e., 50–70%) arise sporadically without any identifiable risk factors except for advancing age (Kirsten & Vogel, 2016; Khan *et al.*, 2019). Furthermore, in contrast to HCC, iCCAs usually develop in non-cirrhotic liver (Lee & Lee, 2017), but it is likely that the presence of liver cirrhosis in iCCA may have been underestimated (Jesper *et al*, 2018). It is also becoming increasingly evident that existing global trends in iCCA incidence rates associated with geographically variable risk factors need to be interpreted with caution based on recently reported concerns about historical under-reporting, country-based variations in data recording, and notably, iCCA misclassification in cancer registries (Khan *et al.*, 2019; Cardinale, 2019; Labib *et al.* 2019).

The vast majority of iCCAs are diagnosed at an advanced incurable stage [multicentric disease within the liver, lymph node and/or peritoneal metastasis] (Sirica *et al.*, 2019), accounting for the dismally high mortality rates for this hepatobiliary cancer. Moreover, the indolence of the early disease, the malignant aggressiveness and therapeutic refractoriness of the advanced disease, and the high reported rates (> 60%) of iCCA recurrence following curative-intent surgical resection (Chan *et al.*, 2018; Zhang *et al.*, 2018; Wang *et al.*, 2019; Hu *et al.*, 2019) all underscore the importance of advancing our current understanding of interactive cellular and molecular mechanisms that drive the development

and progression of iCCA, with the ultimate aim of devising more effective biomarker-driven targeted approaches for managing and/or preventing this challenging and most often fatal hepatobiliary cancer.

In sharp contrast to conventional hepatocellular carcinomas (HCC), iCCAs are typically characterized by a prominent desmoplastic and usually hypovascularized tumor stroma (Sirica & Gores, 2014; Bösmüller *et al.*, 2018), which in many cases represents the dominant histological feature of the tumor. This complex tumor reactive stroma is largely composed of a dense collagen-fiber enriched extracellular matrix (ECM) together with matricellular proteins and proteinases, and containing an abundance of activated cancer-associated fibroblasts (CAFs), and to a lesser extent tumor associated macrophages (TAMs) and varying numbers of vascular and innate immune cells (Sirica & Gores, 2014; Brivio *et al.*, 2017; Affo *et al.*, 2017). The cellular and ECM components of the desmoplastic stroma of iCCA are not structurally nor functionally static, but should be viewed as providing an evolving, interactive and mutable microenvironment that plays a preeminent role in promoting iCCA progression and invasive growth, cancer cell survival, and resistance to chemotherapeutic drugs, targeted agents, and immunotherapies (Cadamuro *et al.*, 2017; Gentilini *et al.*, 2018; Sirica *et al.*, 2019; Loeuillard *et al.*, 2019; Fabris *et al.*, 2019a)

This review will highlight timely research findings aimed at providing mechanistic insights into the biological and translational significance of the desmoplastic microenvironment in iCCA. Our focus will be on (**1**) iCCA pathological and molecular classification and cell origins, (**2**) fibroinflammatory cholangiopathies and mechanisms, (**3**) the desmoplastic reaction and iCCA progression, (**4**) the immune milieu of iCCA and immune resistance, and (**5**) clinical implications and challenges.

2. iCCA Classification, Cell Origins, and Molecular Subtypes

2.1 Macroscopic and Microscopic Classification

iCCAs are macroscopically and microscopically diverse. Classically, iCCA can arise from both small intrahepatic bile ducts (septal & interlobular BD) and large intrahepatic bile ducts (segmental and area BD) within liver (Nakanuma *et al.*, 2010; Vijgen *et al.*, 2017; Kendall *et al.*, 2019). Peribiliary glands associated with large intrahepatic BD may also give rise to iCCA (Nakanuma *et al.*, 2010; Nakagawa *et al.* (2018).

Based on macroscopic growth patterns, iCCAs have been grossly classified as mass-forming (MF), periductular infiltrating (PI), intraductal growing (IG) and MF + PI types (Shimada *et al.*, 2007; Guglielmi *et al.* 2009; Nakanuma *et al.*, 2010; Vijgen *et al.*, 2017; Kendall *et al.*, 2019). The MF growth pattern is the most common form of the macroscopic types, accounting for about 65% of all iCCAs (Guglielmi *et al.*, 2009; Vijgen *et al.* 2017; Kendall *et al.*, 2019). The PI and IG types are much less common, comprising 6.0%−14%% and 4.0%, respectively, of all iCCAs (Guglielmi *et al.*, 2009; Tsukahara *et al.*, 2016; Vijgen *et al.*, 2017; Kendall *et al.*, 2019). Mixed MF + PI iCCAs, on the other hand, account for about 25% of CCA in liver (Guglielmi *et al.*, 2009; Vijgen *et al.*, 2017; Kendall *et al.*, 2019). The MF + PI type has the worst prognosis with higher rates of early recurrence following

curative-intent surgical resection when compared with other types of iCCA (Shimada *et al*; 2007; Guglielmi *et al*. 2009; Blechaz *et al*, 2011; Vijgen *et al*., 2017; Bagante *et al*., 2018).

The MF type of iCCA is generally thought to preferentially arise from small intrahepatic bile ducts (Cardinale *et al*., 2018; Kendall *et al*., 2019). This type commonly presents as a singular solid nodular mass that is gray to gray-white in color, firm and solid, non-encapsulated, and polylobulated with no macroscopically discernable connection with a bile duct (Nakanuma *et al*., 2010; Vijgen *et al*. 2017). MF-iCCAs can be quite large, often ranging between 5 and10 cm in diameter at the time of diagnosis (Buettner *et al*., 2017). Advanced MF type of iCCA may also exhibit satellite or multifocal tumor growth within liver, consisting of various sized nodules that may coalesce. In comparison, iCCAs arising within the large intrahepatic bile ducts are usually of the PI, IG, or the more frequent MF+PI type (Vijgen *et a*l., 2017). The PI type of iCCA does not form a nodular mass, but rather grows longitudinally along the wall of the large intrahepatic bile ducts and spreads along the portal tracts, resulting in strictures of the affected bile ducts and dilatation of the smaller proximal bile ducts (Nakanuma *et al*., 2010; Vijgen *et al*., 2017; Kendall *et al*., 2019). The IG type of iCCA typically presents as a slow growing polypoid or papillary tumor growing within a dilated bile duct lumen (Nakanuma *et al*., 2010; Vijgen *et al*. 2017; Kendall *et al*., 2019). IG-iCCA can be divided into two groups, those with tumors showing a predominantly papillary growth pattern and those whose tumors exhibited a predominantly tubular growth pattern (Chung *et al*., 2009; Tsukahara *et al*., 2016). Both the papillary and tubular subtypes of IG-iCCA have been found to have similar favorable survival outcomes, although tumors with the tubular growth pattern showed increased incidences of lymphatic and venous invasion and a higher rate of liver metastasis than those with the papillary growth pattern. Bagante *et al*. (2018) have also reported that while patients who had undergone curative-intent surgery for either MF or MF + PI types of iCCA were more likely to experience early recurrence, IG-iCCA patients were more at risk for late recurrence after 5-years from surgery.

Obstructive jaundice may be manifested in patients with the PI and IG types (Vijgen *et al*., 2017), but is significantly less common in MF type of iCCA originating at the periphery of the biliary tree (Liau *et al*., 2014; Akita *et al*., 2018). Jaundice is significantly associated with the more advanced MF + PI type (Shimada *et a*l., 2007). The MF type of iCCA tends to invade the hepatic parenchyma via the portal vein system and at advanced stages via the lymphatics, whereas the PI type has a tendency to spread along the Glisson sheath via lymphatics (Yang & Yan, 2008; Blechacz *et al*., 2012; Meng *et al*., 2017).

Histologically, 90–95% of iCCAs are classified as adenocarcinomas (typically well- to-moderately differentiated) displaying variable architectural patterns [i.e., tubular, acinar, papillary, tubular-papillary, anastomosing glandular] and characterized by varying degrees (usually prominent) of desmoplasia, which is a hallmark feature of iCCA (Sirica *et al*., 2009; Nakanuma *et al*., 2010; Sirica, 2013; Akita *et al*., 2017; Blechacz, 2017; Kendall *et al*., 2019). In a classification scheme proposed by Nakanuma *et al*. (2010) classic or conventional iCCA is subclassified according to histological features and anatomic location into two main types: small bile duct type and large bile duct type. Grossly, the small bile duct type of iCCA is almost exclusively of the MF type and is often associated with chronic

non-bile duct liver diseases, such as viral hepatitis (B & C) and cirrhosis (Cardinale *et al*; 2018; Alita *et al.*, 2019; Kendall *et al.*, 2019), whereas the large bile duct type typically characterizes the PI and PI + MF types of iCCAs, such as those associated with predisposing high risk fibroinflammatory cholangiopathies like primary sclerosing cholangitis (PSC), hepatolithiasis, liver-fluke infestation, and Caroli's disease. Small bile duct type iCCAs, also described as "mixed type" (Cardinale *et al.*, 2018) or "cholangiolar type" (Liau *et al.*, 2014), display no or low mucin production, while large duct type iCCAs and those arising from associated peribiliary glands are mucin-producing adenocarcinomas (Cardinale *et al.*, 2012a; Nakagawa, 2018; Vijgen *et al.*, 2017; Siegel *et al.*, 2018; Kendall *et al.*, 2019).

Cholangiolocellular carcinoma (CLC) is a rare malignancy first described by Steiner & Higginson (Steiner & Higginson, 1959), which accounts for 0.6–1.0% of primary liver cancers. (Yamamoto *et al.*, 2018). CLC is considered to originate from cells within the ductules (cholangioles)/canals of Hering of liver (Komuta *et al.*, 2008; Brunt *et* al., 2018). This tumor consists of thin, malignant ductular-like structures characterized by low grade cellular atypia that may appear to radiate from or surround a portal tract in a tubular, cord-like, anastomosing pattern within a dense hyalinized (fibrous) stroma, and which may show trabecular and replacing growth at its interface with the surrounding nontumorous liver (Brunt *et al.*, 2018). CLC often exhibits a similar MF type at the periphery of the liver as small duct type of iCCA, and like small duct type MF-iCCA, is mucin negative (Brunt *et a*l., 2018) and associated with cirrhosis or chronic viral hepatitis (Arizumi & Yamamoto, 2015; Yamamoto *et al.*, 2018). Patients with CLC who have undergone curative-intent hepatectomy have been reported to have a significantly better survival rates than those with classic iCCA (Arizumi *et al.*, 2014; Kusano *et al.*, 2020). Integrative genomic analysis revealed CLC to be a distinct biliary-derived entity with chromosomal stability and active transforming growth factor-β (TGF-β) signaling (Moeini *et al.*, 2017). More recent immunochemical features and mutational profiling of CLC (see Molecular Classification subsection below) have further supported classifying CLC as a histological subtype of well differentiated iCCA (Balitzer *et al.*, 2019).

Classic combined hepatocellular-cholangiocarcinoma (cHCC-CCA) is a rare primary MF-type liver cancer, which presents with a more aggressive malignant behavior and a grim prognosis with worse survival outcomes than for HCC and similar to or worse than for iCCA (Gera *et al.*, 2017; Stavraka *et al.*, 2018). Previous reported incidence has ranged between 0.4% and 14.2 % (Stavraka *et al.*, 2018; Wang *et al.*, 2019) and 1.0%−4.7% (Gera *et al.*, 2017) of primary liver tumors, but the true incidence of cHCC-CCA is unclear due to misdiagnosis and a previous lack of consensus on the nomenclature (Stavraka *et al.*, 2018; Wang et al., 2019). Similar to iCCA, the incidence and mortality trends overall for cHCC-CCA have also been reported to have increased in the USA from 2000 to 2014 (Wang *et al.*, 2019).

As the term implies, classic cHCC-CCA contain areas of both unequivocally differentiated HCC and typical iCCA (Gera *et al.*, 2017; Brunt *et al.*, 2018). The HCC component usually is in the form of thickened trabeculae composed of polygonal cells with abundant granular eosinophilic cytoplasm and scant stroma, whereas the iCCA component distinctly presents as an adenocarcinoma with malignant glands comprised of small bile duct-like

cells supported by a conspicuous desmoplastic stroma (Gera *et al.*, 2017; Brunt *et al.*, 2018). The HCC and iCCA components may be intermixed or occupy separate regions of a tumor, but focal areas of intermingling are often observed (Brunt *et al.*, 2018). Mucin production is most often negative. Both MF-iCCA and the cholangiocarcinoma component of cHCC-iCCA in cirrhotic liver were further shown to exhibit higher density of arteries and microvessels than classical iCCA in non-cirrhotic liver, which usually presents with a hypovascular stroma (Xu *et al.*, 2012).

cHCC-iCCA with “stem/progenitor” cell features/phenotypes and intermediate cell carcinoma comprised of tumor cells intermediate between hepatocytes and cholangiocytes are considered non-classical subtypes of HCC or iCCA (Gera *et al.*, 2017; Brunt *et al.*, 2018). CLC, which may be entirely distinct as a primary liver cancer can also be observed variably as a component in either HCC, iCCA, or cHCC-iCCA (Brunt *et al.*, 2018). Other unconventional subtypes of iCCA include rare variants such as adenosquamous type, squamous type, intestinal type, signet ring type, clear cell type, lymphoepithelial type, and others (Vijgen *et al.*, 2017; Kendall *et al.*, 2019). The intestinal-type of iCCA is a particularly interesting variant exemplifying the cellular plasticity of biliary cells in cholangiocarcinogenesis. Reported cases of this rare subtype of large duct iCCA showed absorptive columnar cells, Paneth cell and goblet cell metaplasia, and occasional neuroendocrine cells within carcinomatous epithelium of the tumor (Kozuka *et al.*,1984; Bae *et al.*, 2002;). A similar pattern of intestinal-type differentiation has also been demonstrated in mucin producing iCCA induced by furan in rat livers (Elmore & Sirica, 1993; Elmore & Sirica, Ren *et al.*, 2000).

Lymphoepithelioma-like iCCA is also a distinctive rare sub-type. This primary hepatic tumor is composed of undifferentiated epithelial cells with a dense lymphoplasmacytic infiltration and is characterized by lower rates of recurrence and better overall survival after surgical resection than classic iCCA (Aosasa *et al.*, 2015; Kendall *et al.*, 2019). A recently identified “inflamed” iCCA subtype that presented with a massive T-lymphocyte infiltration and activation of inflammatory and immune checkpoint pathways was also shown to be associated with enhanced patient survival (Job *et al.*, 2019).

2.2 Precursor Lesions of iCCA Subtypes and Cell Origins

Precursor lesions—Presently, no precursor lesions of the MF type of iCCA are known (Kendall *et al.*, 2019), although bile duct adenoma-like and atypical bile duct lesions involving the small bile ducts have been suggested as possibly related to the development of the MF type of iCCA (Nakanuma *et al.*, 2014). However, three preinvasive lesions: (1) biliary epithelial neoplasia (BillN), (2) intraductal papillary neoplasms of the bile duct (IPNBs), and (3) intraductal tubulopapillary neoplasms of the bile duct (ITPNs), which are most frequently observed in high risk conditions affecting the large intrahepatic bile ducts, are now considered as morphologically established precursor lesions for iCCA (Vijgen *et al.*, 2017; Kendall, *et al.*, 2019; Nakanuma *et al.*, 2019; Zaccari *et al.*, 2019). Pancreatic counterparts to these premalignant intraepithelial neoplasms of the biliary tract are also well recognized (Nakanuma *et al.*, 2019; Zaccari *et al.*, 2019).

BillN is a microscopically identifiable dysplastic lesion exhibiting a flat, micropapillary, or pseudopapillary pattern of growth (Nakanuma *et al.* 2019; Zaccari *et al.*, 2019), which is graded as BillN1 (low grade dysplasia), Billn2 (intermediate dysplasia), and BillN3 (carcinoma *in situ*). BillNs are frequently observed in areas of mucosa proximal to classic nodular/sclerosing iCCA and in the context of preneoplastic cholangiopathies, such as PSC, hepatolithiasis, liver fluke infection, and Caroli's disease (Kendall *et al.*, 2019; Zaccari *et al.*, 2019). BillN has also been documented in patients with non-biliary chronic diseases, such as HCV (Vijgen *et al.*, 2017; Kendall *et al.*, 2019), and may also be seen in peribiliary glands (Vijgen *et al.*, 2017; Zaccari *et al.*, 2019).

IPNB can be considered as the biliary counterpart to pancreatic intraductal papillary mucinous neoplasms, while ITNB appears to be the biliary counterpart to intraductal tubular neoplasm of the pancreas (Vijgen *et al*,, 2017; Nakanuma *et al.*, 2019; Kendall *et al.*, 2019). IPNBs precede the growth of IG type and papillary type iCCAs (Nakanuma *et al., 2016*) and are more commonly encountered in the Far East in patients with hepatolithiasis and liver fluke infection, and less frequently in the West (Wan *et al.*, 2013; Kendall *et al.*, 2019). Macroscopically, IPNBs grow as single or multiple yellow, friable papillary mass within large size intrahepatic and extrahepatic bile ducts, which may show unilocular or multilocular cystic dilation (Nakanuma *et al.*, 2019; Kendall *et al.*, 2019; Hucl, 2019). Histologically, IPNBs are classified into four subtypes based on the lining epithelial cells and architecture, including fine fibrovascular stalks: pancreaticobiliary, intestinal, gastric, and oncocytic. The pancreaticobiliary and intestinal subtypes are the most common, with IPNBs often graded as high grade (Kendall *et al.*, 2019; Nakanuma *et al.*, 2019). Invasive iCCA can be found in association with each of the IPNB subtypes, although it is more often associated with the pancreaticobiliary type (Kendall *et al.*, 2019). The invasive parts of IPNBs usually show tubular adenocarcinoma with desmoplastic reaction and only occasionally show oncocytic or colloid (mucinous) carcinoma (Nakanuma *et al.*, 2019). However, invasive iCCAs associated with the intestinal subtype of IPNB are often mucinous adenocarcinomas (Kendall et al., 2019). With respect to mucin types, MUC1, which is associated with iCCA aggressiveness and poor prognosis, is commonly expressed in the pancreaticobiliary subtype, whereas MUC2 is a goblet cell mucin characteristic of the intestinal phenotype of IPNB and of mucinous adenocarcinoma. The gastric and oncocytic subtypes are positive for MUC6, while MUC5AC can be detected in either of the four subtypes (Ishikawa *et al.*, 2004; Rocha *et al.*, 2012; Kasprzak & Adamek, 2019).

ITPN is also a polypoid lesion with a predominant tubular growth pattern. Key histological features of ITPNs are tubular glands densely packed back to back with abortive papillary elements, uniform high grade dysplasia throughout the tumor, no cytoplasmic mucin, and focal necrosis (Vijgen *et al.*, 2017; Kendall *et al.*, 2019; Nakanuma *et al.*, 2019) Although ITPNs have a high risk of malignancy, they generally have a more favorable prognosis when compared with IPNBs (Kendall *et al.*, 2019). 2019).

Cell origins—Histopathological observations of precancerous and borderline lesions that precede the development of iCCA livers of patients with fibroinflammatory cholangiopathies (Cannito *et al.*, 2018; see below) have led to the recognition that iCCA develops through a multistage carcinogenic process, which like other epithelial cancers, may proceed through

a hyperplasia-dysplasia-carcinoma *in situ* sequence (Shimonishi *et al.*, 2000; Kendall *et al.*, 2019). Hyperplasia and dysplasia of the lining biliary epithelial cells and peribiliary glands are characteristically observed in patients with hepatolithiasis and those with liver fluke infection, and also in biliary lining epithelium of patients with PSC and Caroli's disease (Ludwig *et al.*, 1992; Shimonishi *et al.*, 2000; Jang *et al.*, 2014; Zimmermann, 2016; Nakagawa *et al.*, 2018; Carpino *et al.*, 2019). It has also become apparent that the epidemiological, morphological and molecular heterogeneity of iCCA subtypes is linked to potentially diverse cellular origins (Cardinale *et al.*, 2012; Sia *et al.*, 2017; Overi *et a*l., 2018; Bragazzi *et al.*, 2018).

Much of our current understanding of the cell origins of iCCA comes from studies performed using genetic mouse models (Vicent *et al.*, 2019; Erice *et al.*, 2019; Zhu & Kwong, 2020); cell lineage tracing (Fan *et al.*, 2012; Guest *et al.*, 2013; Wang *et al.*, 2018), and immunophenotyping of "stem/progenitor" cells (Sia *et al.*, 2017; Nakagawa *et al.*, 2018; Overi *et al.*, 2018; Vicent *et al.*, 2019). As exemplified in Table 1, and as proposed by Cardinale *et al.* (2012b), iCCA can potentially develop from multiple cells of origin, including immature or more mature cholangiocytes, hepatic stem/progenitor cells localized to the canals of Hering and bile ductules, biliary tree stem/progenitor cells (BTSCs) found in the peribiliary glands, and from transdifferentiation of mature hepatocytes.

While the experimental findings favor a diverse cellular origin of iCCA, it remains unclear as to the extent to which human iCCAs are developed from cholangiocytes *versus* those being derived from hepatic stem/progenitor cells or transdifferentiated hepatocytes. As previously noted, the majority of human iCCAs develop sporadically in livers in the absence of discernable chronic inflammatory liver injury. Also, as has been mentioned, a multistep carcinogenic process indicative of a hyperplasia-dysplasia-cancer *in situ* sequence is well recognized in the pathogenesis of iCCA arising from cholangiocytes within different levels of the hepatic biliary tree. However, the concept of a hepatic stem/progenitor cell origin is increasingly recognized as being relevant to the development of human MF-iCCA arising in a background of chronic viral hepatitis and cirrhosis, and is particularly apparent in the development of cHCC-CCA with stem cell features (Terada, 2013; Brunt *et al.*, 2018; Stavraka *et al.*, 2019; Cai *et al.* 2020). In a limited number of molecular studies involving tissue microarray, gene set enrichment analysis, and integrative genomics, expression of hepatic stem/progenitor traits have been demonstrated in cohorts of surgically resected cHCC-CCA (Woo *et al.*, 2010; Coulouarn *et al.*, 2012). Coulouarn *et al.* (2012) have further shown that cHCC-CCA exhibit down-regulation of the hepatocyte differentiation program and a commitment to the biliary lineage, as well as an activation of the Wnt/β-catenin and TGF-β signaling pathways. Both of these pathways are involved in biliary differentiation. More recently, Xue *et al.* (2019) performing a large-scale integrative analysis of 133 cases of human cHCC-CCA, which included comprehensive genomic and transcriptomic profiling, showed that tumors with clearly defined areas of HCC and iCCA (denoted as combined-type of cHCC-CCA) and those with intimately mixed components of HCC and iCCA in the same tumor without clear boundaries (defined as mixed-type HCC-CCA) both showed stem-like features, were monoclonal in origin, and exhibited a high level of expression of Nestin, a liver stem cell/progenitor cell protein (Gleiberman *et al.*, 2005). Interestingly, the HCC components of the combined-type cHCC-CCA were not clustered into classic HCC

subgroups nor were the iCCA components clustered into classic iCCA subgroups, but rather clustered together, suggesting that the combined-type of cHCC-CCA is not identical to traditional HCC and iCCA, but derived from a common progenitor cell. In this context, is of interest that loss of *p53* has been shown facilitate dedifferentiation of mature hepatocytes into nestin-positive liver progenitor-like cells, which then may be poised to potentially develop into HCC, iCCA, or cHCC-CCA (Tschaharganeh *et al.*, 2014).

Experimental studies have also supported the possibility of adult hepatocytes transdifferentiating into cholangiocytes under conditions of severe chronic hepatic injury and cholestasis (Michalopoulos *et al.*, 2005; Michalopoulos & Khan, 2015; Sadri *et al.*, 2016; Ko *et al.*, 2020). Morphological and phenotypic evidence for putative transdifferentiation of rare ductular hepatocytes from bile duct epithelium within interlobular bile ducts has also been described in human liver in various severe hepatic disease states, including chronic viral hepatitis and end stage cirrhosis (Nomoto *et al.*, 1992), as well as in hyperplastic bile ductular structures formed in rat liver in associated with extreme hepatotoxic injury induced by furan (Sirica *et al.*,1994). Of particular interest are the findings of Seehawer *et al.* (2018) who showed that the hepatic microenvironment epigenetically shapes lineage commitment in mosaic mouse models of liver tumorigenesis. Specifically, these investigators observed that a necroptosis-associated hepatic cytokine microenvironment promotes oncogenetically-transformed hepatocytes to give rise to iCCAs. In contrast, when hepatocytes containing the same oncogenic drivers were surrounded by an apoptotic microenvironment, they formed HCCs. Notably, the transcription factors *Prdm5* and *Tbx3* were singled out as being major microenvironment-dependent and epigenetically regulated lineage commitment factors for ICCA and HCC, respectively (i.e., *Prdm5* overexpression and *Thx3* knockdown resulted in HNF4α-negative and cytokeratin 19 (CK-19)-positive iCCA, whereas *Tbx3* overexpression and *Prdm5* knockdown yielded HNF4α-positive, CK19-negative HCC). Hepatocyte associated transcription factors (HNF4α and HNF6) have been demonstrated in cholangiocytes in chronic HCV infected liver with end stage cirrhosis (Limaye *et al.*, 2008). However, while the apparent transdifferentiation of hepatocytes to cholangiocytes or of rare cholangiocytes to hepatocytes have been observed to occur in various relevant human liver diseases, it remains to be determined if this facultative progenitor cell mechanism plays a plausible role in human iCCA or cHCC-CCA development, since it is more likely to be predominantly manifested in end stage liver diseases where the normal regenerative capacity of liver is severely impaired.

Peribiliary glands are tubuloaveolar glands with mucinous and serous acini that are located in the wall of the large intrahepatic bile ducts and extrahepatic bile ducts, being largely distributed at the branching points of the biliary tree. They are connected to the bile ducts through small canals/tubes and are considered to modulate bile composition by secreting serous and mucinous components, although their pathophysiological role has not been definitively established (Cardinale *et al.*, 2012b; Nakagawa *et al.*, 2018). Peribiliary glands have been implicated in the origin of human large duct mucin-producing CCA and IPNB (Nakanuma & Sato, 2012; Cardinale *et al.*, 2012a; Sato *et al.*, 2014; Carpino *et al.*, 2019).

BTSCs have been localized to the bottom of human peribiliary glands near the fibromuscular layer and have been demonstrated to express specific stem/progenitor cell markers, including

Sox9, Sox17, Pdx1, Foxa2, CD133, CD44, CXCR4, EpCAM, NCAM, Sall4, and Lgr5 (Cardinale *et al.*, 2011; Overi *et al.*, 2018; Nakagawa *et al.*, 2018), but lacked markers of mature cells, such as secretin receptor-SR, albumin, and insulin (Overi *et al.*, 2018). A subpopulation of BTSCs were also found to express markers used to characterize pluripotent stem cells (i.e., Oct4, Nanog, Sox2) (Overi *et al.*, 2018). That the peribiliary gland is a niche for multipotent BTSMs is further highlighted by the demonstration that BTSCs present in peribiliary glands of human extrahepatic biliary tree can give rise to hepatocytes, cholangiocytes, and beta-islet cells in culture and *in vivo* (Cardinale *et al.*, 2011). $Sox17^+$ and/or $Pdx1^+$ cells of the peribiliary glands and peribiliary network of mice were also shown to proliferate in response to viral-induced biliary injury and to biliary obstructive cholestasis caused by bile duct ligation (DiPaola *et al.*, 2013). A recently established novel mouse model of biliary injury-related extrahepatic CCA from peribiliary glands developed by ductal cell-specific activation of KRAS, and deletion of TGF-β receptor 2 and of E-cadherin, together with IL-33 treatment further adds to the likelihood of large duct CCAs arising in humans with underlying fibroinflammatory risk conditions as originating from peribiliary glands harboring BTSCs (Nakagawa *et al.*, 2017; Nakagawa *et al.*, 2018; Nakagawa *et al.*, 2019). Additional support for this possibility also comes from the recent findings of Carpino *et al.* (2019) who demonstrated marked proliferation of BTSCs, expansion of peribiliary glands and dysplasia, and high expression of BTSC markers in mucin-producing CCAs, which emerged in the PSC patients. However, while these findings are compelling, definitive evidence establishing a direct cell lineage relationship between BTSCs and cholangiocarcinoma cells arising within large duct CCAs has yet not been conclusively demonstrated.

2.3 Molecular Classification of iCCA Subtypes

Molecular profiling of human iCCA subtypes has revealed a complex mutational landscape with broad inter- and intra-tumor heterogeneity that are attributed in part to diverse multifactorial etiologies, histological tumor differences and cell origins, and evolving malignant progression (Sirica *et al.*, 2019). However, small duct type and large duct type of iCCAs can be grouped into two distinctive morpho-molecular groups based on unique differences in their gene mutation profiles (Kendall *et al.*, 2019). Isocitrate dehydrogenase 1/2 (*IDH1/2*) mutations and Fibroblast Growth Factor Receptor 2 (*FGFR2*) fusions are almost exclusively detected in small duct type of iCCAs when compared with large type iCCAs (Liau *et al.*, 2014; Akita *et al.*, *2019,* Goeppert *et al.*, 2019; Kendall *et al.*, 2019; Wang *et al.*, 2019; Ma *et al.*, 2020). In contrast to small duct type iCCAs, large duct type iCCAs, as well as extrahepatic CCAs (perihilar and distal) typically show higher mutation frequencies for oncogenes (i.e., *KRAS*) and tumor suppressor genes (i.e., *TP53*) (Liau *et al.*, 2014; Jusakul *et al.*, 2017; Kendall *et al.*, 2019). Loss of *BAP1* was also reported to be restricted to the small bile duct type of iCCA, whereas in the same study, loss of *SMAD4*, as well as *MDM2* amplification were more frequently detected in the large bile duct type (Akita *et al.*, 2019). Genomic profiling of iCCA in cirrhosis (n=10) demonstrated alterations similar to those of iCCA in non-cirrhotic liver, including *IDH1/2* mutations (30%), *FGFR2* fusions (20%), as well as *BAP1* (10%) and *ARID1A* (10%) mutations (Joseph *et al.*, 2019). The genomic profile (*IDH1/2* mutations, *FGFR2* fusions, chromatin-remodeling gene mutations, such as *ARID1A* and *PBRM1* and copy number alterations) were also found

to be similar in a small cohort of analyzed cases of human CLC (n=5), iCCA (n=7), and mixed CLC-iCCA (n=5), with mutations typical of small duct type of iCCA, including *IDH1/2* mutations and *FGFR2* fusions shown to be present in 90% of the cases with a CLC component (Balitzer *et al.*, 2019). Unlike small duct type iCCAs, a limited number of studies have revealed human cHCC-CCA to harbour highly recurrent *TP53* and *TERT* promoter mutations (Sasaki *et al*, 2017; Liu *et al.*, 2018; Joseph *et al.*, 2019), with *TP53* mutations frequently present in both the HCC and CCA components of the tumor (Joseph *et al.*, 2019). None of the cases of cHCC-CCA (n=20) analyzed by Joseph *et al.* (2019) demonstrated *IDH1/2*, *FGFR2*, or *BAP1* mutations. On the other hand, the mutational landscape of cHCC-CCA appears to be more similar to that of HCC and distinctly different from that of iCCA, even in cirrhosis (Liu *et al.*, 2018; Joseph *et al.*, 2019). Mutations in *TERT* promoter, *ARID1A*, *KRAS* and *TP53* were further shown to correlate with different clinical phenotypes of cHCC-iCCA. *TERT* promoter mutations correlated with HBV, an intermediate subtype-predominant histology, higher clinical stage, and higher N-factor (lymph node involvement). *ARID1A* mutations correlated with alcoholic liver disease, smaller tumor size, a lower grade of coexistent HCC, and α-fetoprotein positivity, and were associated with a CLC subtype predominance. *KRAS* mutations correlated with greater histological diversity and a higher M-factor (distant metastasis), while *TP53* mutations correlated with α-fetoprotein positivity (Sasaki *et al.*, 2017).

Integrative molecular analysis was also used to identify two distinct molecular subclasses of human iCCA, respectively designated as an inflammation subclass and a proliferation subclass (Sia *et al.*, 2013; Sia *et al.*, 2017). The inflammation subclass iCCAs was characterized by activation of inflammatory signaling pathways, overexpression of cytokines, including interleukin (IL)-6, IL-10, and IL-17, and STAT3 constitutive activation, whereas the proliferation subclass was based on activation of oncogenic signaling pathways (i.e., KRAS/RAF/ERK, IGF1R, EGFR, MET, NOTCH, ErbB2,, PI3K/AKT/mTOR), oncogenic mutations (i.e., *KRAS, BRAF, EGFR*, *TP53*), DNA hypermethylation, and focal aberrations, including DNA amplifications at Chr 11q13.2 and deletions at Chr 14q22. The proliferation class also included a subtype of iCCA with stem cell-like features, chromosome instability and IDH1/2 mutations. This study also revealed a resemblance between the proliferation class of iCCA and HCC subtypes with poor prognosis and stem cell features (Sia *et al.*, 2017).

The molecular classification profiles described above have significant relevance for the prognosis and treatment of patients with iCCA subtypes. For example, the proliferation subclass of iCCA was demonstrated to exhibit a more aggressive clinical behavior, where as the inflammation subclass defined a class of iCCA with a more favorable prognosis (Sia *et al.*, 2013, Sia *et al.*, 2017). In a separate study, iCCA with CLC differentiation whose transcriptomic profile resembled that of an inflammation-related subtype exhibited less aggressive histopathological features and more favorable survival outcomes and time to recurrence than classic iCCA resembling the proliferation subtype (Rhee *et al.*, 2018). Integrative clustering of genetic and epigenetic data from an analysis of 40 small duct type iCCAs and 12 large duct type iCCAs identified four subgroups with prognostic relevance. These were designated as IDH, high (H), medium (M), and low (L) genetic and epigenetic alteration groups. The IDH group consisted of all samples with *IDH1* or *IDH2* mutations

and showed together with the H group a highly disruptive genome. DNA methylation was highest in both the IDH and H subgroup, intermediate in the M subgroup, and low in the L subgroup, which also had few mutations and a lack of copy number alterations. Patient three-year survival rates were 91% for the L subgroup, 65% for the IDH group, 50% for the H group, and 36 % for the M group (Goeppert *et al.*, 2019). An impressive integrated whole-genome analysis and epigenetic analysis of 489 CCAs from 10 countries carried out by the International Cancer Genome Consortium further demonstrated that an anatomic subset of tumors composed of almost entirely intrahepatic iCCAs, which were mostly liver-fluke negative and characterized by *IDH1/2* and *BAP1* mutations and *FGFR* alterations, had significantly better overall survival relative to other subsets comprised mostly of liver fluke positive tumors (large duct type) and which were enriched in *TP53* mutations and *ERBB2* amplifications (Jusakul *et al.*, 2017). The clinical significance of morpho-molecular subclassification of iCCAs is further highlighted by encouraging phase III clinical trial data supporting the use of IDH1 inhibitors after progression to chemotherapy for the treatment of iCCA with *IDH1* mutations and phase II trial data demonstrating FGFR inhibitors to show therapeutic activity against iCCAs harboring *FGFR2* fusions (Lamarca *et al.*, 2020).

3. Fibroinflammatory Cholangiopathies, Mechanisms, and Cholangiocarcinogenesis

Cholangiopathies are a class of liver diseases of various etiologies that specifically affect the biliary tree (Cannito *et al.*, 2018; Fabris *et al.*, 2019b). Chronic biliary inflammation, bile duct hyperplasia, fibrosis, and cholestasis are common characteristics of cholangiopathies that have been identified as high-risk conditions for cholangiocarcinoma (Cannito *et al.*, 2018; Roy *et al.*, 2019; Guicciardi *et al.*, 2020). As already noted, PSC and liver fluke infestations are two of the most important examples of definitive high risk cholangiopathies for which progressive biliary inflammation and fibrosis have been linked to cholangiocarcinoma development. The main features of PSC and liver fluke infestations are described below in the context of their relationship to cholangiocarcinogenesis.

3.1 Primary Sclerosing Cholangitis

PSC is a rare chronic biliary disease typically diagnosed in the third and fourth decade of life (Lindor *et al.*, 2015; Lazaridis & LaRusso, 2016). PSC is characterized by strong peribiliary fibrosis and inflammation, which can affect both the small and large bile ducts, leading to large bile duct biliary strictures, impaired bile flow, and ultimately to cirrhosis complicated by portal hypertension. Histopathological features of PSC include fibroinflammatory destruction of the interlobular bile ducts with thick concentric fibrosis (“onion skinning”) associated with progressive bile ductopenia, bile duct hyperplasia, and varying degrees of cholestasis (Cannito *et al.*, 2018; Guicciardi *et al.*, 2020). Based on experimental data and anatomic location, it has recently been proposed that in PSC, senescent cholangiocytes and portal fibroblasts participate mainly in the development of persistent peribiliary fibrosis, whereas ductular reactive cholangiocytes and hepatic stellate cells would be involved in the formation of liver parenchymal fibrosis (Guicciardi *et al.*, 2020).

Patients with PSC have been reported to have a 400-to-1500-fold increased lifetime risk of developing cholangiocarcinoma than those without PSC (Roy *et al.*, 2019; Fung *et al.*, 2019), with 20–25% of PSC cases resulting in cholangiocarcinoma (Aron *et al.*, 2009; Karlsen *et al.*, 2010; Karlsen *et al.*, 2013; Williamson & Chapman, 2015, Fung *et al.*, 2019; Buckholz & Brown, 2020). The etiology and pathogenesis of PSC remains unclear (Karlsen *et al.*, 2017, Vesterhus & Karlsen, 2020), but several studies have implicated chronic biliary inflammation as a critical component sustaining the pathogenesis of this disease (Yan *et al.*, 2020). The known association of PSC with inflammatory bowel diseases, the changes in the bile and gut microbiotas, together with genome wide association study data demonstrating the presence of genetic variants related to immune pathways (i.e., IL2, IL2RA, CARD9, and REL) all suggest the involvement of innate immune mechanisms in PSC's pathogenesis. (Melum *et al.*, 2011; Janse *et al.*, 2011, Liu *et al.*, 2013; Mells *et al.*, 2013).

The role of changes in the microbiota is still debated (Hov & Karlsen, 2017). Bacteria and bacterial components, also known as pathogen associated molecular patterns (PAMPs), could be transported to the liver through the enterohepatic circulation, stimulating sustained innate immune responses targeting cholangiocytes (Karlsen *et al.*, 2010; Trauner *et al.*, 2014; Strazzabosco *et al.*, 2018). Katt *et al.* (2013) have further shown that IL-17a is upregulated in livers from patients with PSC and that Th17 cells are increased in peripheral blood from patients with PSC following challenges with candida extracts or toll-like receptor 5 agonists (i.e., flagellin). More recently Tedesco *et al.* (2018) showed in the *Mdr2*$^{-/-}$ mouse model of PSC that gut dysbiosis leads to the translocation of *Lactobacillus gasseri* to the liver and to the stimulation of IL-17 secretion by γδ TCR^{+} cells. Notably, this response was blocked by inhibition of the γδ TCR^{+} cell receptor, resulting in attenuation of peribiliary fibrosis. In addition, Nakamoto *et al.* (2019) reported that the liver of PSC patients is colonized *by Klebsiella pneumonia, Proteus mirabilis, and Enterococcus gallinarum*, stimulating Th17 immune responses and suggesting a possible role of IL-17 and the biliary epithelial cell response to this cytokine in the pathogenesis of PSC.

The persistence of the inflammatory insult in PSC lead to the secretion of numerous fibroinflammatory mediators, in particular of IL-6, interferon (IFN)γ, and tumor necrosis factor (TNF)-α, which, in turn, are able to stimulate inducible nitric oxide synthase (iNOS) expression and activity (Jaiswal *et al.*, 2000; Jaiswal *et al.*, 2001; Spirlì *et al.*, 2003). Increased nitric oxide (NO) synthesized by iNOS stimulates inflammatory cells present in the portal areas to secrete a wide range of cyto- and chemokines, including chemokine (C-C motif) ligand (CCL) 1, 2, and 3, chemokine (C-X-C motif) ligand 2 (CXCL2), IFNβ, IL-6, IL-8, TGFβ, and TNF-α (Cadamuro *et al.*, 2020). Proliferating cholangiocytes also display increased secretions of proinflammatory cytokines, such as TNF-α, and IL-6, as well as chemokines (monocyte chemoattractant protein 1) and growth factors [i.e., platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF] (Guicciardi *et al.*, 2020).The local accumulation of NO_2^- generate reactive oxidative species (ROS), such as peroxynitrites ($ONOO^-$), by interacting with superoxidedismutase-derived O_2^- (Jaiswal *et al.*, 2000; Fang *et al.*, 2004). ROS and NO are involved in the neoplastic transformation inducing lipid peroxidation of the cell membrane, nitrosylation of several proteins, single or double-strand breaks in DNA, and inactivating enzymes involved in DNA proofreading and repair, such as 8-oxo-deoxiguanine DNA glycosylase 1 (Jaiswal *et*

al., 2000). These mechanisms may favour the accumulation of genetic mutations in biliary epithelial cells, prodromal to neoplastic transformation. Moreover, NO and ROS could generate two mutagenic compounds, 8-oxo-7,8-dihydro-20-deoxyguanosine (8-oxodG) and 8-nitroguanine (Jaiswal *et al.*, 2000; Jaiswal *et al.*, 2001; Correia da Costa *et al.*, 2014). The nitrosylation of cysteine residues of the active site of caspase 3, 8, and 9 could inhibit their activity, making tumor cells resistant to apoptosis. NO accumulation also activates the p38 MAPK/JNK pathway, stimulating cyclooxygenase (COX) 2 to generate prostaglandin E_2, which can directly stimulate the proliferation of cholangiocarcinoma cells through interaction with its receptor EP1, or by transactivating the epidermal growth factor receptor (EGFR) through Src protein (Zhang *et al.*, 2007). The resultant activation of the PI3K/AKT encourages increased cell proliferation and chemoresistance. Carpino *et al.* (2019) have further shown that neoplastic ducts of PSC patients are surrounded by an expanded peribiliary plexus and secrete higher amounts of IL-6, IL-8, TGFβ, and VEGF compared to non-neoplastic ducts.

Cholangiocarcinoma developed on a background of PSC is associated with *KRAS* and *TP53* mutations (Ahrendt *et al.*, 2000; Boberg *et al.*, 2000), mutations of the $p16^{INK4a}$ promoter (Taniai *et al.*, 2002), and *FGFR2* fusion IDH 1 and 2 mutations (Wang et al., 2013). Another important mechanism in cholangiocarcinogenesis is the deregulation of specific miRNAs involved in cell proliferation, stemness, and migration. IL-6 is, in fact, able to depress the expression of the tumor suppressor NF2, by upregulating miR-let-7a, and of $p16^{INK4a}$ by increasing miR-148a and miR-152 (Braconi *et al.*, 2010), whilst stimulating p38 MAPK phosphorylation by reducing miR-370 (Meng *et al.*, 2008).

3.2 Fluke Infestations

Liver fluke infestations of *Opisthorchis viverrini* and *Clonochis sinensis* are endemic in several Asian countries (Thailand, Cambodia, Vietnam, Korea, and China), whereas *Opisthorchis felineus* is prevalent in Eastern Europe [Russia, Siberia, Ukraine, Belarus, and Kazakhstan] (Qian *et al.*, 2016; Fedorova *et al.*, 2017; Sripa *et al.*, 2018). The infestation of the biliary network of human patients with *O. viverrini* is due to the ingestion of fluke metacercariae in undercooked or raw fish. Parasites proliferate in bile ducts, releasing eggs that are expelled with stools that contaminate water ways. Here, the eggs are eaten by water snails, the main food source for the fish (Sripa *et al.*, 2007; Qian *et al.*, 2016).

The association between fluke infestation and cholangiocarcinoma is well documented and the International Agency for Research on Cancer (IARC) has included *O. viverrini* and *C. sinensis* as Group I agents/biological carcinogens (van Tong *et al.*, 2017). Within the biliary tree, liver flukes generate a strong immune response and induce an exacerbated chronic inflammatory response that is determined by duration of the infection, by number of infesting flukes, and probably by their modulation of the gut and liver microbiome (Plieskatt *et al.*, 2013; Saltykova *et al.*, 2018). This chronic fibroinflammatory cholangiopathy, as exemplified by *O. viverrini* infestation, is characterized by, cholangitis, eosinophilia, and progressive peribiliary fibrosis (Sripa *et al.*, 2018). Reactive biliary epithelial changes in response to *O. viverrini* include cholangiocyte hyperplasia, goblet cell metaplasia, adenomatous hyperplasia, and dysplasia. Extracellular vesicles from *O. viverrini* have been

also shown to be actively internalized by human cholangiocytes *in vitro*, where they promote cell proliferation and stimulate secretion of the proinflammatory cytokine IL-6 (Chaiyadet *et al.*, 2015a & b).

The *O. viverrini* excretory/secretory products (OvESP), similarly to PAMPs, stimulate the signal cascade initiated by TLR4 activation that induce the NF-kB-mediated hypersecretion of several cytokines, including IL-6 and IL-8. This mechanism was developed as a host defence to microbial or parasitic infection, because it stimulates the action of COX2 and iNOS, leading to the local generation free oxygen and nitric radicals with antimicrobial activity, but which could also promote the malignant transformation of the cholangiocytes (Prueksapanich *et a*l., 2018). *C. sinensis* infestation could also stimulate liver fibrogenesis by activating both TLR2 and 4, leading to the secretion of a wide range of ILs (1β, 4, 6, and 10), TNF- α, and IFNγ. These fibroinflammatory mediators contribute to the recruitment of inflammatory cells and to the proliferation and activation of resident fibroblasts (Sripa *et al.*, 2007; Prueksapanich *et al.*, 2018) leading to chronic inflammation and CCA development. However, it should also be noted that *O. viverrini* infection alone rarely shows cholangiocarcinoma development (Sripa *et al.*, 2018). In this context, fluke-induced carcinogenesis has some peculiar characteristics. Specifically, opisthorchiasis is characterised by the accumulation of hormone-like molecules, such as oxysterol, a compound derived from the oxidation of conjugated bile acids by cytochrome P450. Its metabolites mediate pro-tumorigenic responses in bile ducts generating DNA adducts, increasing nitrosylative and oxidative damages due the hyperexpression and activation of iNOS and COX2, and deregulating the proliferation/death ratio of cholangiocytes (Vale *et al.*, 2020). Similar effects were also induced by catechol estrogen quinone-like hormones of fluke origin, which were able to inhibit the DNA editing action of 8-oxodG and 8-nitroguanine (Correia da Costa *et a*l., 2014). In addition, opisthorchiasis may enhance colonization of the biliary tree by species of *Helicobacter*, including H. pylori, a known GI cancer risk factor (Sripa *et al.*, 2018).

Lastly, using a whole genome sequencing approach, Chan-on *et al.* (2013) depicted the mutational patterns of iCCA on a background of *O. viverrini* infection relative to iCCA without fluke infestation. In a cohort of 108 patients, these authors reported that non-*O. viverrini* related iCCAs showed a higher incidence of mutation in *BAP1*, *IDH1* and *IDH2* genes, while *O. viverrini*-related iCCAs were characterized by a low frequency of *IDH1/2* mutations and a significantly higher frequency of *TP53* and *SMAD4* mutations. Consistent with the findings of Chan-on *et al.*, and as previously noted, Jusakul *et al.*, (2017) had also identified a cluster of liver fluke-associated iCCAs uniquely enriched in *TP53* mutations (and ERBB2 amplifications) together with a low frequency of expression of *BAP1* and *IDH1/2* mutations. Conversely, these investigators also found fluke-negative cholangiocarcinomas to be characterized by increased *BAP1* and *IDH1/2* mutations, together with *FGFR2* gene rearrangements. Collectively, these molecular genomic findings indicate that non-*O. viverrini* cholangiocarcinomas and *O. viverrini*-positive tumors have distinct mutational profiles, strongly implying that different etiologies may induce distinct mutational landscapes even within the same tumor type.

4. The Desmoplastic Reaction and iCCA Progression

Irrespective of etiology (sporadic *versus* known risk factors), iCCAs are most often characterized by a prominent desmoplastic reaction typified by the formation of a hard, stiff, and hypovascularized scaffold comprised of a dense fibro-collagenous enriched matrix along with other ECM proteins, and containing an abundance of activated CAFs positive for α-SMA (a biomarker of myofibroblast differentiation), together with variable numbers inflammatory and vascular cell types (Figure 1, Sirica & Gores, 2014; Brivio *et al.*, 2017; Cadamuro *et al.*, 2018; Banales *et al.*, 2020). In recent years, the desmoplastic stroma of iCCA, also known as tumor reactive stroma or TRS (Brivio *et al.*, 2017), has become the focus of a growing research interest due to its prognostic potential and possibilities for therapeutic targeting. Moreover, there is now a growing body of evidence to convincingly implicate the tumor reactive microenvironment in iCCA as playing an active and crucial role in promoting and or modulating the major hallmarks of cancer (Hanahan & Weinberg, 2011). The stromal cellular and ECM components of iCCA, which frequently comprises the bulk of the tumor, provide critical molecular and biomechanical signals for perpetuating cholangiocarcinoma cell proliferation, promoting iCCA progression, facilitating iCCA invasion and metastasis, resisting apoptotic cell death, regulating iCCA angiogenesis and lymphangiogenesis, mediating epigenome and metabolic reprogramming, enabling resistance to chemo- and targeted agent therapies, and avoiding immune destruction (Sirica, 2012; Sirica & Gores, 2014; Affo *et al.*, 2017; Brivio *et al.*, 2017; Cadamuro *et al.*, 2018; Mancinelli, *et al.*, 2019; Cadamuro *et al.*, 2019; Fabris *et al.*, 2019; O'Rourke *et al.*, 2019; Pant *et al.*, 2020; Banales *et al.*, 2020).

A critical aspect of the relationship between the desmoplastic reaction in iCCA and increased malignant behavior is the deleterious interplay between cholangiocarcinoma cells and α-SMA+CAFs, which are considered the most important host-derived stromal cells within the tumor microenvironment. Activated CAFs in iCCA are a major source of secreted growth factors [e.g., hepatocyte growth factor (HGF), PDGFs (-BB & -D), heparin-binding epidermal growth factor (HB-EGF)], chemokines /cytokines [e.g., IL-6, TGF-β, stromal derived factor-1 (SDF-1), also known as CXCL12,] ECM proteins [e.g., fibrillar collagens, fibronectin], matricellular proteins [e.g., periostin (Postn), tenascin-C, and osteopontin], proteinases [e.g., metalloproteinases (MMPs)-2 and −9], and angiogenic modifiers [e.g., VEGFs A & C, thrombospondin-1], which collectively function to promote or modulate cholangiocarcinoma cell behavior and progression. (Sirica *et al.*, 2009; Fingas *et al.*, 2011; Sirica, 2012; Clapéron *et al.*, 2013; Cadamuro *et al.*, 2013; Sirica & Gores, 2014; Affo *et al.*, 2017; Brivio *et al.*, 2017; Cadamuro *et al.*, 2018; Gentilini *et al.*, 2018; Cadamuro *et al.*, 2019; Nissen *et al.*, 2019; Labib *et al.*, 2019; Banales *et al.*, 2020; Vaquero *et al.*, 2020). Exosome crosstalk (Goulet *et al.*, 2018; Yang *et al.*, 2019), exchange of metabolites, such as pyruvate and lactate (Fiori *et al.*, 2019; Yoshida *et al.*, 2019), activation of mechanosensitive signaling cascades associated with activation of Yes-associated protein (YAP)/transcriptional coactivator with PDZ-binding motif (TAZ), desmoplastic ECM stiffness, and hypoxia (Sahai *et al.*, 2020, Banales *et al.*, 2020), and aberrant integrin, sonic hedgehog, and Wnt/β-catenin signaling (Utispan *et al.*, 2012; Gascard & Tisty, 2016; Labib *et al.*, 2019; Banales *et al.*,

2020) are also important to the interactive relationships that exist between cancer cells and CAFs, as well as other cell types of the iCCA tumor reactive microenvironment.

4.1 CAF Origins and Heterogeneity in iCCA

CAFs in iCCA are phenotypically heterogenous and are likely derived from various cell lineages (Sirica *et. al.*, 2011; Sirica & Gores, 2014; Affo *et al.*, 2017; Vaquero *et al.*, 2020), although the actual cellular origins of these tumor stromal cells remains unclear due largely to a lack of *in vivo* cell fate tracing data. Moreover, CAFs are not normal fibroblasts, and their differentiation and phenotypic biomarker profiles can be influenced by the tumor microenvironment and molecular cross talk with cancer cells. However, based on what is known of fibrogenic cell responses in cirrhosis and fibroinflammatory/cholestatic liver diseases like PSC, it is generally believed that the most likely major source of CAFs in iCCA are likely originated from resident hepatic nonparenchymal cells, notably hepatic stellate cells and activated portal fibroblasts (Okabe *et al.*, 2016; Manzanares *et al.*, 2017; Affo *et al.*, 2017; Vaquero *et al.*, 2020).

Taking into account the limitations of using potential phenotypic biomarkers to identify the cell origins of CAFs in iCCA, such analyses can still be of value in suggesting possible cell lineage relationships, as well as potential prognostic relevance. Okabe *et al.* (2009) first reported that hepatic stellate cell markers (i.e., desmin, glial fibrillary acidic protein) were expressed in α-SMA+CAFs in human iCCA, suggesting a hepatic stellate cell origin. In contrast, Itou *et al.* (2019) more recently showed that α-SMA+CAFs in the stroma of primary human iCCAs expressed portal fibroblast markers [i.e., fibulin-2, thymus cell antigen-1 (Thy-1], and except for a small subset, were largely negative for fascin, a hepatic stellate cell biomarker. It was also reported by these investigators that α-SMA+CAFs in metastatic lymph nodes from iCCA patients, while positive for Thy-1 and negative for fascin, were also negative for fibulin-2. On the other hand, hepatic stellate cells in human cirrhotic liver were found to be negative for both Thy-1 and fibulin-2. In addition, Itou *et al.* also detected a small number of α-SMA+CAFs in the primary human iCCAs that were immunoreactive for bone-marrow derived fibrocyte biomarkers (CD34, CD45), suggesting that bone marrow derived cells may also contribute to some population of iCCA α-SMA+CAFs. Overall the findings by Itoh *et al.* imply that CAFs at the primary site of iCCA are similar to activated portal fibroblasts, but different from hepatic stellate cells and from CAFs in metastatic lymph nodes. Further, small subsets of CAFs may have also been derived from bone marrow derived fibrocytes, and in the case of the metastases, from resident cells of lymph nodes.

Manzanares *et al.* (2017) further showed by transcriptomic analysis and by immunophenotyping that α-SMA+CAFs purified from a rat orthotopic desmoplastic iCCA prominently overexpressed portal fibroblast biomarkers (e.g., gremlin 1, fibulin-2, Thy-1, cofilin-1, NTPDase2, elastin), but were negative for desmin, and lacked biomarkers for bone marrow derived fibrocytes or hematopoietic stem cells. Moreover, in this study, only a small number of desmin-positive fibroblastic cells were detected in the desmoplastic stroma of iCCA from which the α-SMA+CAFs were isolated from, as well as in human iCCA stroma.. Moreover, biomarker and karyotypic analyses supported a resident liver fibroblastic origin

of the orthotopic iCCA-derived α-SMA+CAFs and argued against epithelial-mesenchymal transitional (EMT) as a source of these myofibroblastic cells. Lineage cell tracing has also presented evidence against the possibility of epithelial mesenchymal transition of neoplastic cholangiocytes as being a likely contributing source for α-SMA+CAFs accumulating within desmoplastic stroma of iCCA (Cadamuro *et al.*, 2013).

It is also not known to what extent other cells, such as endothelial cells, may give rise to iCCAs. Recently, Zhang *et al.* (2020) using a droplet-based single-cell RNA sequencing platform to profile single cells from human iCCA identified six distinct fibroblast clusters, five of which were mainly enriched in the iCCA tissues and one primarily present in the adjacent tissues. All six subclusters expressed high levels of *ACTA2* that encodes for α-SMA, in addition to other canonical CAF markers. Interestingly, one of these subclusters (vCAFs), which accounted for 57.6% of the iCCA fibroblast population, was characterized by microvascular signature genes, including CD146 (an endothelial cell marker), as well as high levels of IL-6 expression. CD146 expression has also been demonstrated in human liver endothelial cells and pericytes surrounding the portal vasculature (Strauss *et al.*, 2017), as well as in mouse liver Thy-1-positive periportal fibroblasts and hepatic stellate cells, although at more variable levels in the hepatic stellate cells (Katsumata *et al.*, 2017). Without cell fate tracing, however, it is not possible to conclude that vCAFs were derived from resident portal fibroblasts, hepatic stellate cells or subsets of hepatic endothelial cells and vascular associated pericytes.

In addition to α-SMA, other biomarkers used to delineate CAFs in iCCA include fibroblast specific protein 1 [FSP-1, also known as S100A4], fibroblast activating factor [FAP] and platelet-derived growth factor receptor-β [PDGFR-β] (Zhang *et al.*, 2017; Sha *et al.*, 2018; Itou *et al.*, 2019; Vaquero *et al.*, 2020). In a number of independent studies, iCCA patients whose tumor stroma was enriched in α-SMA+CAFs were demonstrated to have significantly shorter overall survival and worse recurrence-free survival rates following surgical resection than those whose iCCAs expressed low or negative levels of stromal α-SMA (Okabe *et al.*, 2009; Chuaysri *et al.*, 2009; Sha *et al.*, 2018). High expression of FAP in iCCA has also been reported to be predictive of poor prognosis in iCCA patients (Yang *et al.*, 2016). Zhang *et al.* (2017) have further shown histological categorization of CAFs in human iCCA to be a potentially useful predictor for prognosis, with surgically resected iCCA patients whose tumors showed a more normal fibroblastic-like morphology (termed mature CAFs) having a significantly better overall survival than those that were characterized by a myofibroblast morphology (termed immature CAFs). In this study, high stromal α-SMA expression was found to be associated with poor iCCA differentiation, but surprisingly, not with improved overall survival. This discrepancy may be due to a relatively small sample size and use of a different immunohistochemistry scoring system than those used by others.

Survival rates of iCCA resected patients with stromal CAFs immunoreactive for the transmembrane glycoprotein podoplanin (D2–40-positive myofibroblasts) were found to be significantly lower than those of patients without podoplanin expressing CAFs (Aishima *et al.*, 2008). Podoplanin has also been shown to be a potentially useful stromal prognostic marker for perihilar cholangiocarcinoma (Obulkasim *et al.*, 2018). Furthermore, CD10 (neprilysine), a membrane bound metalloprotease expressed in CAFs, may be more involved

with the progression of perihilar and extrahepatic CCAs than of iCCAs (Nishihara *et al.*, 2009). High tissue and serum Postn, which in both human and rat is produced solely by α-SMA+CAFs in iCCA tissue (Utispan *et al.*, 2010; Dumur *et al.*, 2010) have also been demonstrated to be a particularly promising predictor of poor prognosis in human iCCA patients (Utispan *et al.*, 2010); Thuwajit *et al.*, 2017) and increased malignant tumor grade and progression in a well established syngeneic rat model of desmoplastic iCCA (Manzanares *et al.*, 2018). In this same rat model, high accumulation of α-SMA+CAFs in the tumor stroma was also found to be associated with increased iCCA tumor grade and enhanced malignant aggressiveness (Sirica *et al.*, 2011).

4.2 PDGF-D and TGF-β and the Desmoplastic Reaction in iCCA

PDGF-D and TGF-β play key roles in driving the recruitment and expansion of CAFs in iCCA. Cadamuro *et al.*, (2013) have presented compelling data supporting a model of CAF recruitment into iCCA based on a PDGF-D-mediated paracrine fibroblastic cell recruitment by cholangiocarcinoma cells. In this model, PDGF-D, which is up-regulated in cholangiocarcinoma cells by a hypoxia-mediated mechanism, is secreted into the tumor cell microenvironment, where it in its active ligand form binds to and activates its cognate receptor PDGFRβ expressed by resident liver fibroblastic cells/myofibroblasts and CAFs. This interaction, in turn, promotes proliferation and elicits a strong migratory response in these cells via activation of Rho GTPases (i.e., Rac1, Cdc42) and JNK, thereby providing a mechanism for CAF recruitment in iCCA as illustrated in Sirica *et al.* (2019).

It has also been shown that PDGF-D, and also PDGFBB, each of which are abundantly produced by cholangiocarcinoma cells, can sensitize activated CAFs to apoptotic cell death triggered by BH3 mimetics, such as navitoclax and ABT-199 (Rizvi *et al.*, 2014). Similar apoptotic sensitization was demonstrated in co-cultures of myofibroblasts and cholangiocarcinoma cells. Furthermore, this study also showed that PDGF-linked apoptotic priming of CAFs occurs via Puma-mediated Bak activation, which can then be converted to full-blown apoptosis induced by navitoclax or ABT-199. Notably, navitoclax or ABT199 have been demonstrated to significantly reduce iCCA tumor burden in mouse (Rizvi *et al.*, 2014) and rat cholangiocarcinoma models (Mertens *et al.*, 2014; Cadamuro *et al.*, 2019) by depleting CAFs from the tumor stroma. Navitoclax treatment was also demonstrated to be associated with decreased lymphatic vascularization and lymph node metastasis in the rat cholangiocarcinoma model (Cadamuro *et al.*, 2019).

TGF-β plays a key role in the acquisition of the CAF phenotype and is well known inducer of myofibroblast differentiation (i.e. α-SMA induction) and fibrosis (Giscard, P. & Tlsty, 2016; Caja *et al.*, 2018). Exosomal TGF-β has also been identified as a molecular mechanism involved in CAF activation (Goulet *et al.*, 2018). In relation to iCCA, TGF-β, expressed in both cholangiocarcinoma cells and in α-SMA+CAFs, was demonstrated to be essential for provoking a dramatic desmoplastic-like reaction *in vitro* (prominent overproduction of dense fibrocollagenous matrix concomitant with significantly enhanced accumulation and proliferation of α-SMA+CAFs) within the matrix of a three-dimensional organotypic cholangiocarcinoma culture model that closely resembled the desmoplastic features of the *in situ* tumor. This model was established by co-culturing of

cholangiocarcinoma cells derived from a syngeneic rat iCCA with α-SMA+CAFs exhibiting portal fibroblast biomarkers, obtained from the same tumor type, within a dilute collagen type I hydrogel (Manzanares *et al.*, 2017). Three-dimensional co-culturing of the activated CAFs with the cholangiocarcinoma cells was further found to markedly increase mature TGF-β production within the gel cultures over that elaborated by CAFs alone, to cause marked gel contraction (shrinkage), an indicator of increased substratum stiffness, and to promote significantly increased cholangiocarcinoma cell growth and elevated expression of phenotypic markers of malignant progression (i.e., Muc1). Cholangiocarcinoma spheroid ductal-like structures which formed in the gel cultures were further observed to become increasingly more anaplastic, as well as invasive when co-cultured in the presence of α-SMA+CAFs (Campbell *et al.*, 2012; Manzanares *et al*; 2017; Manzanares et al., 2018; Sirica *et al.*, 2019). TGF-α, an epidermal growth factor receptor (EGFR) ligand expressed in iCCA, was further determined to be a key factor for further enhancing cholangiocarcinoma anaplasia, hyperproliferation, and higher malignant grading in this 3-D culture model (Manzanares *et al*; 2017).

The interplay between activated CAFs and cholangiocarcinoma cells through TGF-β expression and EGFR activation in relation to cholangiocarcinoma progression is further emphasized by the interesting findings of Clapéron *et al.* (2013), who showed that cholangiocarcinoma cells produced TGF-β1, which, in turn, induced HB-EGF expression in CAFs leading to paracrine activation of EGFR on the cancer cells, promoting cholangiocarcinoma cell invasion. Of further interest, TGF-β1 expression in cholangiocarcinoma cells was also found to be enhanced by HB-EGF stimulation. These results support a model as first proposed by Clapéron *et al.* depicting a reciprocal paracrine loop between cholangiocarcinoma cells and CAFs through the HD-EGF/EGFR axis that contributes to cholangiocarcinoma progression and also triggers TGF-β1 production in cholangiocarcinoma cells, which stimulates CAF activation and functions. TGF-β1 has also been demonstrated to promote iCCA tumor growth and metastasis in a rat cholangiocarcinoma model (Huang *et al.*, 2016).

Mesothelin (Msln) is a glycosylphosphatidylinositol-anchored cell membrane glycoprotein that is expressed in liver portal myofibroblasts (Fausther *et al.*, 2017; Koyama *et al.*, 2017), as well as shown to be highly overexpressed in human and rat cholangiocarcinoma cells, but less prominently expressed or not detected in CAFs in desmoplastic human or rat iCCA (Yu *et al.*, 2010; Tang *et al.*, 2013; Manzanares *et al.*, 2017; Manzanares *et al.*, 2018). Msln, however, has been detected by Western blotting in α-SMA+CAFs expressing portal myofibroblast biomarkers, including Thy-1, which were derived from a syngeneic rat iCCA, but expressed at a lower level than Msln protein amounts detected in cell lysates from intrahepatic and extrahepatic metastatic cholangiocarcinoma cells obtained from the same iCCA model (Manzanares *et al.*, 2018).

Msln overexpression has been suggested to be a prognostic factor for patients with intrahepatic cholangiocarcinoma (Nomura *et al.*, 2013) and more recently demonstrated to predict malignant progression in the rat iCCA model described above (Manzanares *et al.*, 2018). In relation to cholestatic liver fibrosis associated with early activation of portal fibroblasts, Koyama *et al.* (2017) have shown Msln to regulate TGF-β1-inducible

activation of portal fibroblasts by disrupting the formation of an inhibitory Thy-1-TGFβR1 complex, as well as to facilitate fibroblast growth factor-mediated proliferation of these cells. However, while it may be assumed, it remains to be determined if Msln effects Thy-1 and TGFβR1 in portal fibroblast-derived CAFs in cholangiocarcinoma in a similar manner. Of added interest is the identification of two distinct molecular weight forms of Msln being expressed in rat cholangiocarcinoma cells *in vivo* and in 3-dimensional culture, notably a 40 kDa form (mature form), which was associated with a cytoplasmic and diffuse cell membrane immunostaining pattern, and whose increased expression predicted a more aggressive iCCA phenotype compared with a more heavily glycosylated 50 kDa form that was expressed predominantly at the apical luminal surface of well differentiated cholangiocarcinoma ducts and which predicted a less aggressive iCCA phenotype. Complementary to these findings, it could be further shown in 3-dimensional culture that co-culturing α-SMA +CAFs with cholangiocarcinoma cells contributed to a significant increase in the production of the 40 kDa form of Msln, which correlated with an increase in cholangiocarcinoma cell anaplasia and disruption of cholangiocarcinoma "ductal-like" polarity (Manzanares *et al.*, 2018). In comparison, highly tumorigenic rat cholangiocytes harboring mutationally activated rat *neu* oncogene (homolog of *erbB2*) were found to primarily express the 40 kDa form of Msln, which was associated with enhanced malignant cell features (e.g., higher malignant grade, disruption of polarized morphogenesis and underexpression of cell polarity regulating proteins, oncogenic *neu* activation with corresponding overexpression of phosphorylated Erk1/2 and AKT, and invasiveness) even when cultured in the absence of CAFs (Mazanares *et al.*, 2018; Wei *et al.*, 2018).The relationship between Msln isoforms, CAFs, and cholangiocarcinoma progression is illustrated in Figure 2. Further studies are needed now to extend these preclinical findings to human iCCA.

TGF-β is also a known inducer of α-SMA+CAF-derived Postn, which is highly germane to supporting and sustaining iCCA's desmoplastic microenvironment and promoting ICCA invasiveness (Utispan *et al.*, 2012; Sirica *et al.*, 2014) Moreover, Postn along with other matricellular proteins, such as tenascin-C and Msln, may be playing a pivotal role in conditioning metastatic niches and the formation of metastases (Ma *et al.*, 2012; González & Alonso, 2018; Lowy & Oskarsson, 2015; Coelho *et al.*, 2020). Postn, like TGF-β, has also been shown to stimulate liver fibrogenesis by activating lysyl oxidase in hepatic stellate cells, inducing the expression of collagen type I and fibronectin, and stimulating the phosphorylation of SMAD2/3, as well as of focal adhesion kinase and AKT in hepatic stellate cells (Kumar *et al.*, 2018). A novel TGF-β induced long noncoding RNA has further been demonstrated to promote an inflammatory microenvironment in human intrahepatic cholangiocarcinoma (Merdrignac *et al.*, 2018). In addition, preclinical targeting of TGF-β in rat cholangiocarcinoma models has been demonstrated attenuate the desmoplastic reaction generated by co-culturing α-SMA+CAFs with cholangiocarcinoma cells in 3-dimensional culture (Manzanares *et al.*, 2017) and to reverse preexisting fibrosis induced by thioacetamide *in vivo* (Ling *et al.*, 2013), leading in both cases to a significant reduction in cholangiocarcinoma.

4.3 The Matrisome, Tumor Stiffness, and Hypovascularity in iCCA

In a recent (albeit limited) analysis by Carpino *et al.*, (2019b) to characterize the matrisome or ECM proteome of human iCCA, the proteomic profile revealed a unique signature of ECM proteins in iCCA when compared with that of paired samples of non-cancerous liver tissue (NCT). As expected, the iCCA ECM exhibited high levels of fibrillar collagen type I components (COL1A1 and COL1A2) and of the matricellular protein Postn, which is known to interact with and regulate collagen fibrillogenesis (Norris *et al.*, 2012; Sirica, A.E., *et al.*, 2014), as well as bind to other ECM proteins (i.e, tenascin-C) promoting iCCA malignant behavior (Sirica *et al.*, 2014). Col3A1, a component of fibrillar collagen type III was also observed to be overexpressed in 6 of 9 analyzed samples of iCCA ECM when compared to NCT and further shown to be a component of tumor-associated aligned collagen fibers. Also, in contrast to the NCT, the iCCA ECM was characterized by an almost total absence of elastic fibers and a low expression of proteoglycans and downregulation of laminins, type IV collagen, and basement membrane-specific heparan sulphate proteoglycan. The angiogenesis factors angiopoietin-related protein 6 and sushi repeat protein X-linked 2 were also seen to be dramatically downregulated in the iCCA samples. Taken together, these findings are compatible with an increased tumor stiffness, a breakdown of basement membrane leading to disruption of cholangiocarcinoma cell polarity, and a hypovascularized iCCA stroma.

Increased tumor stiffness, as exemplified by desmoplastic epithelial cancers like iCCA not only affects malignant cell progression by triggering cell survival, proliferation and motility signaling cascades within the cancer cells (Noguchi *et al.*, 2018; Kalli & Stylianopoulos, 2018; Sahai *et al.*, 2020), but also impacts on surrounding stromal cells, whereby tumor stiffness mechanoactivates YAP/TAZ in fibroblastic cells, leading them to acquire a CAF phenotype (Noguchi *et al.*, 2018; Yoshida *et al.*, 2019). Moreover, YAP activation is enhanced by ECM matrix stiffness, thus establishing a feed-forward self-reinforcing loop to maintain the CAF phenotype. Increased mechanical stress associated with tumor stiffness produced by desmoplasia can also lead to collapse of micro-blood vessels within the tumor, leading to hypovascularization, hypoxia, and reduced bioavailability for therapeutic drugs (Sahai *et al.*, 2020). Enhanced expression of thrombospondin-1, immunohistochemically localized to both CAFs cholangiocarcinoma cells in iCCA, has also been reported to be related to diminished microvessels in human iCCA (Kawahara *et a*l., 1998; Aishima *et al.*, 2002; Tang *et al.*, 2006).It is also relevant here that thrombospondin-1 is an activator of latent TGF-β (Murphy-Ullrich & Suto, 2018).

5. Immune Milieu in iCCA.

5.1 The Innate Immune System in iCCA

The iCCA tumor microenvironment is populated to various degrees by cells of the innate immune response, including macrophages (in particular M2 or alternatively activated macrophages), neutrophils, and natural killer (NK) cells. In the TRS of iCCA, two populations of macrophages are present, those derived from Kupffer cells localized to the perisinusoidal space of Disse, and TAMs.

TAMs are CD86 $^{+}$/CD206 $^{+}$ M2 macrophages, mainly derived from CD14^{+}/CD16^{+} circulating monocytes, which play a major role in suppressing T lymphocyte activity and proliferation, thereby having a pronounced anti-inflammatory action (Rőszer *et al.*, 2015). M2 TAMs promote angiogenesis, induce tissue remodeling, and stimulate the apoptosis of M1 macrophages (Duluc *et al.*, 2007; Rőszer *et al.*, 2015). Monocytes accumulated within TRS are promoted by tumor cells through the local secretion of a variety of cytokines, including IL-1β, IL-4, IL-8, IL-10, IL-13, colony-stimulating factor 1, and growth factors (i.e., VEGF-A) and through the secretion of CCL2 by Kupffer cells (Li *et al.,* 2018).

In iCCA, TAMs mainly localize to the tumor invasive front, with high counts of M2-TAMs in iCCA patients shown to correlate with poor disease-free survival (Hasita *et al.*, 2010) and poor overall survival in surgically resected iCCA patients (Sun *et al.*, 2020). M2 TAMs could, in fact, sustain tumor invasive ICCA growth and promote metastasis by various mechanisms, including (1) via the release of proangiogenic growth factors (e.g., angiopoietin 1, IL-8, VEGF-A) (Leyva-Illades *et al.*, 2012; Corliss *et al.*, 2016), (2) secretion of soluble factors that stimulate the migration of neoplastic cells (e.g., VEGF, fibroblast growth factor (FGF)-1/−2, PDGF, and TGF-β) (Roy *et al.*, 2019), and (3) through the action of MMP-9, a key metalloproteinase involved in tumor spread (Raggi *et al.*, 2017). Furthermore, TAMs promote cholangiocarcinoma cell proliferation by secreting IL-6 and VEGF into the tumor microenvironment, which, in turn, can stimulate the activation of the p42/p44 MAPK and AKT/mTOR signaling pathways, (Banales *et al.*, 2016; Roy *et al.*, 2019).TAMs may drive iCCA progression by their increased expression of WNT ligands, such as WNT3a and WNT7b (Loilome *et al.*, 2014; Boulter *et al.*, 2015; Saito *et al.*, 2018) and enzymes, such as COX2 and iNOS (Chariyalertsak *et al.*, 2001; Wójcik *et al.*, 2012). ECM modification in iCCA may also modulate the accumulation of M2 TAMs within the tumor microenvironment. Notably, Postn has been shown to stimulate the migration of TAMs *in vitro* and to spatially correlate with M2 TAM density in primary human iCCA tissue. (Zeng *et al.*, 2018).

Tumor associated neutrophils (TANs) represent another cell population belonging to the tumor reactive microenvironment that is of increasing importance in cancer (Jaillon *et al.*, 2020). CD66b^{+} neutrophils are recruited through the local secretion of CXCL5 (Zhou *et al.*, 2014), but very little is known about the pathogenic mechanisms of these cells in iCCA. Current data show that accumulation of TANs within the TRS of iCCA is predictor of poor prognosis (Mao *et al.*, 2015), and that a high neutrophil-to-lymphocyte ratio is an independent predictor of poor survival outcome and insensitivity to chemotherapy and radioembolization (Tan *et al.*, 2016; Lin *et al.*, 2016; Wang *et al.*, 2018; Filippi *et al.*, 2020). One may speculate that accumulation of TANs contributes to the generation of a microenvironment prone to immunosuppression that favors neoplastic cell growth through the local release of CCL2 and CCL17 and recruitment of M2 macrophages.

NK cells, immunophenotypically recognized as CD3^{-}CD56^{+} cells, represent between 30 and 40% of the total normal human liver lymphocyte population (Björkstrom *et al.*, 2016, Martín-Sierra *et al.*, 2019). Their biological function is to protect against bacterial invasion via the release of cytotoxic granules, which also produce lytic death of tumor cells. In iCCA, NK are recruited in the tumor area in response to the secretion by cholangiocarcinoma

cells of CXCL9, a INFγ-dependent chemokine. Notably, the ablation of CXCL9 in a mouse model of iCCA was observed to lead to a reduced accumulation of NK cells in peritumoral area, resulting in an increased volume of the tumor (Fukuda *et al.*, 2020). In a xenotransplant model of human cholangiocarcinoma, the infusion of mice with NK cells was also shown to significantly reduce tumor growth as a result of the lytic and cytotoxic actions of the infused cells (Jung *et al.*, 2018). The mechanisms regulating the antitumor effects of NK cells in iCCA are not well understood, but may involve the secretion of several immunomodulatory mediators, such as adenosine, PGE_2, and TGF-β (Melaiu *et al.*, 2020).

Dendritic cells (DCs) represent a bridge between innate and adaptive immune response due to their ability to function as antigen presenting cells to prime $CD4^+$ and $CD8^+$ T-lymphocytes. $CD83^+$ DCs accumulated at the invasive front of iCCA have been shown to significantly correlate with the number of $CD4^+$ and $CD8^+$ T effector cells in the cancerous region, leading to a significantly lower incidence of lymph node metastasis and a better survival outcome for the $CD83^+$ patients than that of CD83-negative patients. (Takagi *et al.*, 2004). Suppression of the expression of receptors for TGF-β and IL10 (immunosuppressive cytokines) on DCs was further demonstrated to result in an increased activation of the adaptive immune response, consequently leading to an enhanced secretion of IFNγ and increased cytolytic action of T-lymphocytes against cholangiocarcinoma cells (Thepmalee *et al.*, 2018; Thepmalee *et al.*, 2020). Furthermore, a limited number of studies on small cohorts of cholangiocarcinoma patients have yielded promising results when coupling chemotherapy (i.e., with gemcitabine) with immunotherapy using protein or RNA-pulsed DCs (Higuchi *et al.*, 2006; Kobayashi *et al.*, 2013; Junking *et al.*, 2017; Sawasdee *et al.*, 2020).

5.2 Adaptive Immune System and Tumor-infiltrating Lymphocytes in iCCA

The adaptive immune response, which involves tumor infiltrating lymphocytes (TILs) and their modulating effects on iCCA growth and spread, is becoming more apparent. This is particularly highlighted by the fact that reduced recruitment of TILs or their inactivation has been shown to correlate with worse patient survival outcomes and increased tumor growth and malignant aggressiveness (Zheng *et al.*, 2020). In an analysis of human iCCA biopsies, TILs accounted for about 8.0% of the inflammatory cells present in the tumor sample (Martìn-Sierra *et al.*, 2019). $CD4^+$ T cells have been shown to be mainly localized at the tumor invasive front of iCCA, whilst $CD8^+$ T cells were usually, but not exclusively, recruited into the tumor mass (Kasper *et al.*, 2009). Patients with iCCA whose tumors exhibited an increased presence of $CD3^+$ and $CD8^+$ infiltrating T cells were also found to exhibit higher overall and disease free survival rates, as well as a lower tumor recurrence risk after curative-intent tumor resection (Vigano *et al.*, 2019; Tian *et al.*, 2019; Tian *et al.*, 2020). In contrast, patients whose iCCAs were characterized by increased infiltration of $Foxp3^+$ regulatory T cells (Tregs) exhibited worse overall survival rates (Vigano *et al.*, 2019), likely due to the ability of Tregs to secrete IL-10 and TGF-β, cytokines able repress the cytotoxic activity of $CD8^+$ T and of NK cells in the tumor microenvironment (Tu *et al.*, 2014). $CD20^+$ B lymphocytes are unfrequently detected in iCCA (Kasper *et al.*, 2009), but their presence also seems to be predictive of a better overall survival when compared with iCCA patients in which B cells were absent (Goeppert *et al.*, 2013).

One of the salient mechanisms used by the cancer cells to survive is to escape immune surveillance of immune cells by modulating the expression of several immune checkpoints, such as programmed death-1 (PD-1) and its ligand PD-L1, or cytotoxic T-lymphocyte antigen-4 (CTLA-4). PD-L1 is variably expressed by cholangiocarcinoma cells, (Sabbatino *et al.*, 2016; Kitano *et al.*, 2020). The rational for the use of a PD-1 based immunotherapy in iCCA is that cholangiocarcinoma cells could promote their survival by inducing depletion of PD-1-expressiong T cells through the PD-L1/PD-1 route. Moreover, CTLA-4 is expressed by Tregs that are known to depress the activity of DCs by binding to its specific surface receptor CD80, thus blocking the cytotoxic action of DCs (Goeppert *et al.*, 2013). In addition, it was shown that targeting of PD-1 and CTLA-4 expressed by iCCA-derived TILs could induce, *ex vivo*, an increased activation of tumor-infiltrating T cells (Zhou *et al.*, 2019). Notwithstanding this, data regarding the importance of PD-L1 expression in cholangiocarcinoma cells are conflicting. In conflict with studies implicating the expression of this ligand as an independent prognostic factor for predicting poor overall survival of iCCA patients (Sabbatino *et al.*, 2016; Kitano *et al.*, 2020), a recent metanalysis of 1066 cholangiocarcinoma patients subdivided in 11 studies showed that the association among PD-L1 expression and overall or disease free survival was not significant (Xu *et al.*, 2019).

To date, various clinical trials for iCCA based on PD-1/PD-L1 modulation are now in the recruiting phase (e.g., NCT04157985, NCT04440943, NCT02834013, NCT04295317, NCT03111732), but no data are yet available. As alluded to in a previous section of this review, Job *et al.* (2019) reclassified ICCA on the basis of the different inflammatory infiltrates into 4 subtypes that included immune desert (non-inflammatory), lymphoid, myeloid, and mesenchymal types. The lymphoid (immunogenic) subtype, which represented 11% of the total iCCAs analyzed, was characterized by a massive T-lymphocyte infiltration, an activation of inflammatory and immune checkpoint pathways, and was associated with the best patient survival outcome when compared with the other subtypes. Job *et al.* (2019) further suggested that this distinct inflamed iCCA subtype could be amenable for treatment with checkpoint blocking immunotherapy due to the unique composition of the inflammatory infiltrate.

6. Challenges and Clinical Implications

Despite improvements in the standard of care and multidisciplinary management of iCCA, the overall global 5-year survival of patients with iCCA has not changed appreciably over the past 20–30 years, currently reported as ≤ 10% (Dhanasekaran *et al.*, 2013; Mody *et al.*, 2018; Simile *et al.*, 2019). As already noted in the Introduction, the majority of patients with iCCA (70–80%) present with advanced disease where therapeutic options are limited to palliative treatments. Gemcitabine plus cisplatin remains the standard of care as a first line systemic treatment for non-resectable cholangiocarcinoma. This palliative treatment, however, provides only a modest survival benefit (Plentz & Malek, 2016; Buettner *et al.*, 2017; Vienot & Neuzillet, 2019). Furthermore, iCCAs, which most often develop sporadically in the absence of known risk factors, are asymptomatic in their early stage. Even in patients with known risk factors, early diagnosis is problematic, and further, most patients that are diagnosed with late disease (locally advanced or metastatic iCCA) usually present with ambiguous or nonspecific symptoms.

Currently there are no curative medical treatments for iCCA and the only hope for a potentially curative therapy is complete surgical resection. However, only a minority of iCCA patients (-i.e., 10%-to ~25%) are eligible for curative-intent surgical resection. (Buettner *et al.*, 2017; Ma *et al.*, 2020; Banales *et al.*, 2020). Moreover, even with curative-intent resection, an estimated median disease free survival in various patient series has been reported to range from 12 to 36 months (Rizvi *et al.*, 2018), with overall 5-year survival rates after resection ranging between 20% and 35% in some series (Mavros *et al.*, 2014; Chun & Javle, 2017; Waisberg *et al.*, 2018; Ma *et al.*, 2020).

The BILCAP phase 3 randomized trial (NCT00363584) aimed at evaluating capecitabine as an adjuvant treatment for biliary tract cancers of all types (including iCCA) following curative-intent surgical resection did not meet its end point of improving overall survival, but demonstrated a median overall survival of 51.1 months in the capecitabine group *versus* 36.4 months in the observational group not receiving capecitabine (Primrose *et al.*, 2019). The PRODIGE12-ACCORD 18-UNICANCER GI phase 3 trial (NCT01313377), which evaluated adjuvant gemcitabine and oxiplatin in resected biliary tract cancers of all types, failed to show any demonstrable benefit (Edeline *et al.*, 2019).

Molecular profiling of iCCA has revealed a complex and evolving genomic and epigenomic landscape characterized by extensive inter-tumor and intra-tumor heterogeneity that has been attributed in part to diverse multifactorial etiologies, histological tumor differences, adverse stromal microenvironments, and evolving tumor progression (Jusakul *et al.*, 2017; Pellino *et al.*, 2018; Rhee *et al.*, 2018; Sirica *et al.*, 2019; Ma *et al.*, 2019; Goeppert *et al.*, 2019; Banales *et al.*, 2020). This inter-tumor and intra-tumor heterogeneity serves as a major obstacle to the development of molecular targeted and immune-based therapies for iCCA. It has also hindered the development of non-invasive serological tests for iCCA early diagnosis, as well as for monitoring iCCA treatment responses and prognosis. Research aimed at developing noninvasive serological tests based on multiplexing of discreet cholangiocarcinoma cell and tumor stromal markers that may include tumor exosomes, mRNAs, and distinct protein biomarkers linked to malignant progression, such as Muc1, Postn and Msln, could be highly useful towards advancing this clinical need.

The results from earlier clinical trials aimed molecular therapeutic targeting of altered signaling pathways in iCCA, including EGFR, ERBB2, MET, PDGFR, VEGF, and VEGFR have, to date, been disappointing (Mertens *et al.*, 2018; Pellino *et al*, 2018; Labib *et al.*, 2018; Simile *et al.*, 2019). Direct therapeutic inhibition of activated *KRAS* has also proved elusive (Mertens *et al.*, 2018). However, with the advent of next-generation sequencing technology to identify oncogenic driver mutations, there is now a powerful tool to identify novel molecular targets and to allow personalized targeted approaches to iCCA therapy. (Mertens *et al.*, 2018; Pellino *et al.* 2018, Dabney *et al.*, 2019).

The value of the personalized approach to iCCA targeted therapy is exemplified by the rapid translation of FGFR2 fusion/translocation and IDH1/2 mutations, respectively, into promising therapeutic targets for particular subsets of iCCA patients (Pellino *et al.*, 2018; Dabney *et al.*, 2019; Krook *et al.*, 2020). Mutated FGFR2 has been described in approximately 10%-to-23% of patients with iCCAs (Nakamura *et al.*, 2015; Pellino *et al.*,

2018; Dabney *et al.*, 2019; Simile *et al.*, 2019; Krook *et al.*, 2020). Sia *et al.* (2015), using RNA- and exome-sequencing analysis, reported a higher rate of FGFR2 fusions (45%) in a cohort of 107 resected iCCA patients, which represented the most recurrent targetable alteration. IDH1/2 mutations also commonly occur in patients with iCCA at reported rates of 13% up to 30% (Pellino *et al.*, 2018; Mertens *et al.*, 2018; Goeppert *et al.*, 2019; Lowery *et al.* 2019; Abou-Alfa *et al.*, 2020a).

The efficacy of pemigatinib, a selective oral FGFR 1,2, and 3 inhibitor, was recently investigated in FIGHT-202 (NCT02924376), a multicenter open-label single arm phase II study that included a cohort of 107 cholangiocarcinoma patients with locally advanced or metastatic cholangiocarcinoma harboring FGFR2 gene rearrangements/fusions and who had received at least one systemic cancer therapy that did not include selective FGFR inhibitors. Among these 107 patients, permigatinib elicited an overall response rate of ~36% (38 of 107 patients), with 3 patients having a complete response. Kaplan-Meier estimates of overall survival further demonstrated a 12-month survival rate of 68% in the permigatinib-treated cholangiocarcinoma patients with FGFR2 fusions or rearrangements compared to 23% for treated cholangiocarcinoma patients with other FGF/FGFR alterations and 13% for those with no FGF/FGFR alterations (Abou-Alfa *et al.* 2020b). Based on these results, the FDA fast tracked the approval of pemigatinib for the treatment of adult patients with previously treated, unresectable, locally advanced or metastatic cholangiocarcinoma with FGFR2 fusions or rearrangements, making it the first targeted agent FDA approved for cholangiocarcinoma therapy. Of further note, in a limited study involving six patients with advanced FGFR2 fusion-positive iCCA, treatment with TAS-120 (an irreversible FGFR inhibitor) was shown to overcome acquired resistance that had developed in these patients to previous treatments with the ATP-competitive FGFR inhibitors BGJ39 or Debio (Goyal *et al.*, 2019).

ClarlDHy, an international multicenter, randomized, double-blind, placebo-controlled, phase III study, evaluated the therapeutic efficacy and safety of ivosidenib (AG-120), a small molecule targeted inhibitor of mutated IDH1, when administered to patients with chemotherapy-refractory advanced IDH1 mutant iCCA. (Abou-Alfa *et al.* 2020a). This clinical trial demonstrated encouraging therapeutic benefits for the IDH-1 mutant iCCA patients over those treated with matched placebo. Specifically, in the ivosidenib-treated group, progression free survival was reported to be 32% at 6 months and 22% at 12 months, *versus* no patients in the placebo group being free from progression at 6 months or more.

The complex microenvironment of desmoplastic iCCA and the deleterious interplay between activated CAFs, inflammatory and immune cells, ECM, and vascular components clearly provides a rationale for devising novel therapeutic strategies aimed at targeting stromal components of iCCA. Activated CAFs, by virtue of being the most prominent cell component and driver of the desmoplastic reaction, are emerging as an appealing target for iCCA therapy. As previously cited, preclinical results with TGF-β inhibitors and with BH3 mimetics offer proof of concept that depleting CAFs and ablating the desmoplastic stroma can potentially have significant therapeutic efficacy for iCCA. In further support of this concept, photothermal depletion of CAFs preferentially loaded with hybrid iron oxide-gold nanoparticles was recently shown to normalize tumor stiffness and produce tumor

regression in desmoplastic human EGI-1 cholangiocarcinoma formed in a mouse xenograft model (Nicolás- Boluda *et al.*, 2020). The FDA-approved antifibrotic drug nintedanib has also been recently demonstrated to reduce xenografted iCCA growth and activated CAFs expressing α-SMA, and further, combinational treatment with nintedanib and gemcitabine against CAFs and iCCA cells was shown to exhibit the strongest inhibition of tumor growth compared with control and single-treatment groups (Yamanaka *et al.*, 2020).

Another potential strategy suggested for targeting CAFs for clinical benefit relates to developing treatments that would cause activated CAFs to revert to a phenotype comparable to that of normal fibroblasts, as exemplified by the targeting of the vitamin D receptor in pancreatic cancer (Sahai *et al.*, 2020), or by the normalizing effect of dasatinib, a FDA approved inhibitor of PDGFR, on CAFs from primary lung cancer (Haubeiss *et al.*, 2010). Targeting specific tumor promoting factors produced by CAFs, such as Postn, SDF-1, or FAP may also provide a potentially useful strategy for iCCA therapy (Sirica *et al.*, 2014; Liepelt & Tacke, 2016; Lin *et al.*, 2019*).*

Activated CAFs have also emerged as central players in immune regulation that shapes the immune microenvironment, including that of iCCA (Monteran & Erez, 2019; Fabris *et al.*, 2019). FAP^{+}CAFs have been shown to be a major source of CCL2, which promotes recruitment of myeloid-derived suppressor cells (MDSC) and immunosuppression via a STAT3-CCL2 signaling. FAP, phospho-STAT3 and CCL2 are expressed in the desmoplastic stroma of iCCA (Yang *et al.*, 2016). Furthermore, TGF-β and VEGF, each of which are hypersecreted into the iCCA microenvironment, are both potent suppressors of the tumor immune response (Thepmalee *et al.*, 2018; Batlle & Massagué, 2019; Fukumura *et al.*, 2018; Ma *et al.*, 2019).

As alluded to earlier in this Review, a majority of desmoplastic iCCAs have been found to exhibit a sub-optimal response rate of less than 10% to immune check point blockade monotherapy (Loeuillard *et al.*, 2020). This is consistent with data already cited that most iCCAs are characterized by an immune desert phenotype (Job *et al*, 2019), with a non-T cell infiltrated tumor immune microenvironment. Loeuillard *et al.* (2020) have recently shown that TAMs are the major source of PD-L1 in murine and human cholangiocarcinomas and dual inhibition of TAMs and granulocytic-MDSC potentiated PD-1 blockade in a murine cholangiocarcinoma model.

With respect to other gastrointestinal cancer types, but relevant to iCCA, combined targeting of TGF-β and PD-L1 receptors has been shown to promote T-cell mediated tumor regression and improved survival in a genetic mouse model (KPC) of advanced pancreatic ductal adenocarcinoma (Principe *et al.*, 2019). When tested against the highly immunogenic mouse MC38 colon adenocarcinoma model, combinational inhibition of TGF-β signaling and PD-L1 blockade was also found to be significantly more effective than corresponding single agent treatments in improving survival, which was associated with an influx of $CD8^{+}$ T-cells in the tumor microenvironment. (Sow *et al.*, 2019). In addition, anti-PD-L1 therapy was demonstrated to sensitize RT2-PNET murine pancreatic neuroendocrine tumors to antiangiogenic therapy, and conversely to improve anti-PD-L1 therapy specifically though the generation of high endothelial venules in the tumor, which in turn, promoted cytotoxic

T-cell infiltration, activity, and tumor destruction (Allen *et al.*, 2017). Of added interest, inhibition of the ROS-producing enzyme NOX4, which is upregulated in activated CAFs, has most recently been shown in murine colorectal (as well as lung and breast tumor models), to normalize CAF myofibroblastic cells to a quiescent state. This effect, in turn, was found to re-sensitize the CAF-rich tumors to PD-1 check point inhibition by overcoming activated CAF-mediated $CD8^+$ T-cell exclusion (Ford *et al.*, 2020).

As implied by the findings described above, therapeutic strategies that incorporate targeting of the tumor stroma and novel immunotherapies in conjunction with standardized chemotherapy or targeted agents against cholangiocarcinoma cells offer the possibility of increased survival outcomes and possibly even cures, particularly when considered as a second line treatment for distinct subsets of iCCA patients with recurrent disease. Mou *et al.* (2018) ostensibly was the first to describe in a case report the complete remission of a postoperatively recurred metastatic PD-L1-positive iCCA with a high tumor mutational burden following combination treatment with the anti-PD-1 antibody pembrolizumab together with chemotherapy with oxiplatin, and tegafur. A significant response to anti-PD-1 based immunotherapy with nivolumab administered in combination with the multi-kinase tyrosine kinase inhibitor lenvatinib was also demonstrated against recurrent iCCA with bone metastasis (Chen *et al.*, 2019). The patient's initial primary iCCA, which had a high tumor mutation burden and actionable mutations in *KIT*, *NRAS*, *TP53*, *MET*, and *PDGFR*, recurred at 5 months after surgery and adjuvant chemotherapy. Following combinational treatment with nivolumab plus lenvatinib, the recurred liver tumor completely resolved and the bone metastases were stable after 9 months of therapy.

Bintrafusp alpha is a bifunctional fusion protein composed of a monoclonal antibody against PD-L1 fused to a TGF-β "trap" (Lind *et al.*, 2020). In various preclinical mouse carcinoma models, bintrafusp alpha, previously designated M7824), demonstrated superior anti-tumor activity over that produced by treatments with anti-PD-L1 antibody alone or TGF-β trap alone by a mechanism involving simultaneous blockade of immunosuppressive PD-L1, as well as of TGF-β in the tumor microenvironment (Lan *et al.*, 2018). In this study, treatment with bintrafusp alpha was also shown to reduce α-SMA expression and stromal collagen fiber density in syngeneic EMT-6 mouse breast tumors. In addition, combinations of bintrafusp alpha with chemotherapy (oxaliplatin/5-fluorouracil) or radiotherapy was demonstrated to be effective at improving antitumor efficacy and enhancing $CD8^+$ T-cell activation in various preclinical mouse carcinoma models (Lan *et al.*, 2018; Lind *et al.* 2020).

An open-label Phase I clinical trial of bintrafusp (MSB0011359C) has been completed, showing early signs of efficacy in patients with heavily pretreated advanced solid cancers without prior immune checkpoint inhibitor treatment (Strauss *et al*, 2018). More recently, Yoo *et al.* (*2020*) described results from an expansion cohort of a Phase I open-label trial of bintrafusp in 30 Asian patients with metastatic or locally advanced biliary tract cancers. Included in this trial were 10 patients with iCCA. The objective response rate for the iCCA patients treated with bintrafusp was reported to be 30%, with one patient showing a complete response. While these findings are encouraging, they are limited by the fact that this study lacked of a comparator group, had a small patient enrollment, and did

not include non-Asian patients as part of the cohort. Multiple clinical trials are currently ongoing involving the use of bistrafusp alpha as a monotherapy or in combination with other immunotherapeutics, chemotherapy, or radiation therapy in patients with a variety of solid cancer types, including breast, prostate, biliary, and pancreas (Lind *et al.*, 2020). The results of these trials should hopefully provide new insights concerning the effectiveness of targeting the tumor microenvironment as a strategy for enhancing immunotherapies and other treatment modalities against desmoplastic cancers, such as iCCA.

A variety of combination regimens with PD-1/PD-L1 immune checkpoint inhibitors (ICI) are also now being tested in ongoing key clinical trials against various advanced gastrointestinal malignancies, including biliary cancers (Wang *et al.*, 2019). In this context, there is increasing preclinical and clinical trial evidence to support combined antiangiogenic therapy and immunotherapy with ICIs as a promising strategy for advanced solid cancer treatment (Fukumura *et al.*, 2018; Song *et al.* 2020). VEGF, which is abundantly expressed in iCCA, impairs dendritic cell maturation, resulting in reduced T-cell priming, as well as stimulates expansion of Tregs, M2-TAMs and MDSCs. These actions, in turn, lead to an immunosuppressive tumor microenvironment and weakened anti-tumor immune response, together with dysregulating tumor neovascularization associated with impaired T cell infiltration and cell death (Fukamura *et al.*, 2018; Kudo, 2020). Anti-VEGF agents have been shown to reprogram the tumor milieu from an immunosuppressive to an immune permissive microenvironment by normalizing the tumor vasculature, decreasing the activity of MDSCs, Tregs, and TAMs, and by suppressing dendritic cell maturation (Fukamuro *et al.*, 2018; Kudo et al., 2020; Song *et al.*, 2020). Immune checkpoint blockade has also been shown to facilitate anti-angiogenesis by downregulating VEGF and alleviating hypoxia (Song *et al.*, 2020). These findings, in turn, have served as the basis for multiple clinical trials, many of which have demonstrated improved anti-cancer efficacy and prolonged survival when anti-angiogenic agents were combined with ICIs in the treatment of variety of advanced carcinoma types (Fukamura *et al.*, 2018; Song *et al.*, 2020).

The therapeutic benefit of combining anti-angiogenic therapy with ICIs is exemplified by the results of the phase III IMbrave 150 study-NCT03434379 (Cheng *et al.*, 2019; Kudo, 2020; Nakano *et al.*, 2020). This study evaluated combinational therapy with the anti-PD-L1 antibody atezolizumab + the anti-VEGF antibody bevacizumab for unresectable HCC without prior systemic therapy *versus* sorafenib monotherapy (the standard of care for advanced HCC). The results revealed that atezolizumab + bevacizumab-treated group had a significantly improved overall response rate and progression free survival compared to the sorafenib-treated group. Moreover, six percent of the HCC patients treated with the atezolizumab + bevacizumab combination were found to be in complete remission. The patients in the combination group also reported a considerably better quality of life than those in the sorafenib group.

In July 2019, the FDA granted a Breakthrough Therapy designation for lenvatinib, a multikinase inhibitor targeting VEGFR 1–3, FGFR 1–4, PDGFR-α, RET, and KIT) in combination with the PD-1inhibitor pembrolizumab for the first-line treatment of patients with unresectable HCC, based on the results from the phase 1b KEYNOTE-524/Study 116 trial-NCT03006926 (Nakano *et al.*, 2020; Finn *et al.*, 2020). An ongoing double-blind

randomized controlled phase III study of lenvatinib plus pembrolizumab as a first-line treatment for HCC *versus* lenvatinib plus placebo (NCT03713593) to further assess the efficacy and safety of this combination in patients with HCC is now ongoing (Finn et al., 2020), the results of which are also likely to have potential implications for cHCC-CCA and iCCA, notably those formed in association with viral hepatitis and cirrhosis.

Other suggested considerations for targeting of tumor stroma and immune checkpoints in iCCA include the use of BH3 mimetics such as navitoclax to deplete activated CAFs, together with PD-L1 inhibition to reconstruct T-cell response and anti-tumor immunity (Mertens *et al.*, 2018). A number of phase I/II clinical trials (e.g., NCT02341625, NCT03644550, NCT03371381) assessing targeted therapies for Msln expressing cancers based on antibody-based drugs (8BMS-98614; LMB-100) or vaccines (live attenuated *Lysteria monocytogenes*) in combination with immune checkpoint inhibitors (nivolumab; pembrolizumab) are currently recruiting or ongoing. In addition, a limited number of clinical trials (e.g., NCT03615313; NCT03030001) for targeted therapy of Msln positive advanced solid tumors with PD-1 antibody expressing mesothelin specific chimeric antigen receptor T (CAR-T) cells have also been reported to be in development (Lv & Li, 2019). In support of this strategy, Lal *et al* (2019) have recently demonstrated that CAR-T cells targeting Msln and secreting PD-L1 antibodies enhanced antitumor efficacy when compared with Msln-targeted CAR-T cells in xenograft mouse models of mesothelioma, pancreatic, and ovarian cancers. The results of these trials are awaited with anticipation, since they may have important implications for the treatment Msln-expressing iCCA.

Last, a better understanding of cellular heterogeneity and functions of the desmoplastic reaction in iCCA is needed inorder to successfully exploit its potential as a therapeutic target while avoiding any possibility of accelerating malignant aggressiveness. Multiple CAF subtypes demonstrating inter- and intra-tumoral heterogeneity have been demonstrated in human desmoplastic pancreatic desmoplastic adenocarcinoma, which may account for inconsistencies in preclinical results and the failure of some stromal targeted agents (Neuzillett *et al.*, 2019). Two separate classes of CAFs, one expressing myofibroblastic features (α-SMA) and the other expressing inflammatory fibroblast features (i.e., IL-6 secretion) have also been shown to coexist in the desmoplastic stroma of pancreatic cancer and to show distinct transcriptional profiles (Öhlund *et al.*, 2019). Furthermore, as previously alluded to, the reactive microenvironment in primary iCCA is distinct from that of metastatic iCCA. In this context, careful attention needs to be given to advancing the development of *in vivo* models closely reproducing the morpho-molecular pathology and TRS of early, advanced, and metastatic human iCCA in an effort to precisely identify and test primary and adjuvant combinational therapeutic strategies against advanced disease that can predictively translate into more novel systemic targeted agent and immunotherapeutic treatments for iCCA patients, as well as for those with other more common desmoplastic cancers.

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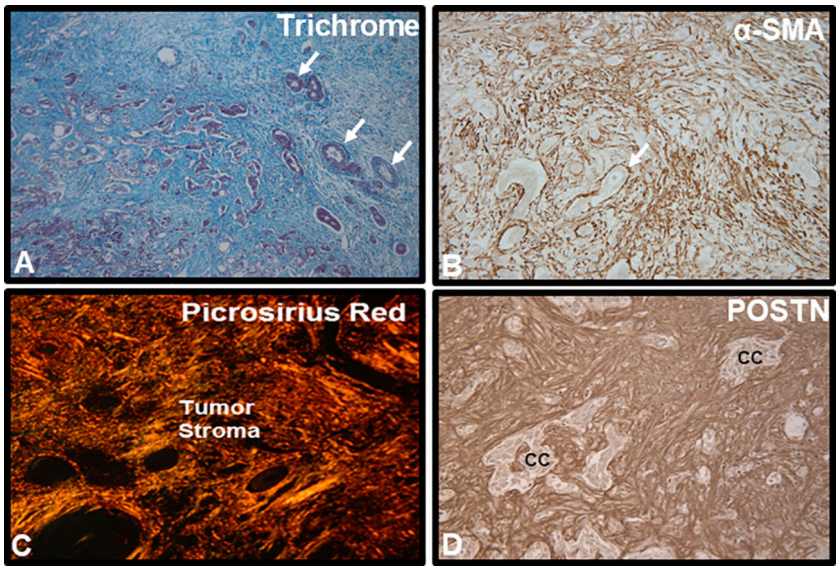

Figure 1.

Representative histological images illustrating characteristic features of the desmoplastic microenvironment in human iCCA. **A.** Masson trichrome staining of a moderately to poorly-differentiated mass-forming iCCA demonstrating the tumor to be largely comprised of a prominent desmoplastic stroma strongly stained for collagen (blue staining). Arrows point to representative small clusters of cholangiocarcinoma. **B.** CAFs comprising the vast majority of cell types populating the desmoplastic stroma of iCCA are seen to be strongly immunoreactive for α- smooth muscle actin (α- SMA), a biomarker of myofibroblast differentiation, whereas cholangiocarcinoma cells (arrow) are negatively stained for α- SMA. **C.** Picrosirius red staining for collagen (orange-staining under polarized light) typically reveals the extracellular matrix of desmoplastic CCA to be comprised of thick collagen fiber bundles that are largely comprised of collagen type I. **D.** Immunostaining for matricellular periostin (Postn), produced by α-SMA+CAFs and which has a binding site for collagen, is exclusively localized to the desmoplastic stroma of iCCA. **cc**, cholangiocarcinoma. Increased numbers of α-SMA+CAFs within the iCCA microenvironment together with corresponding strong stromal immunoreactivity for Postn have been shown to be predictors of poor survival outcomes for iCCA patients following curative-intent surgical resection.

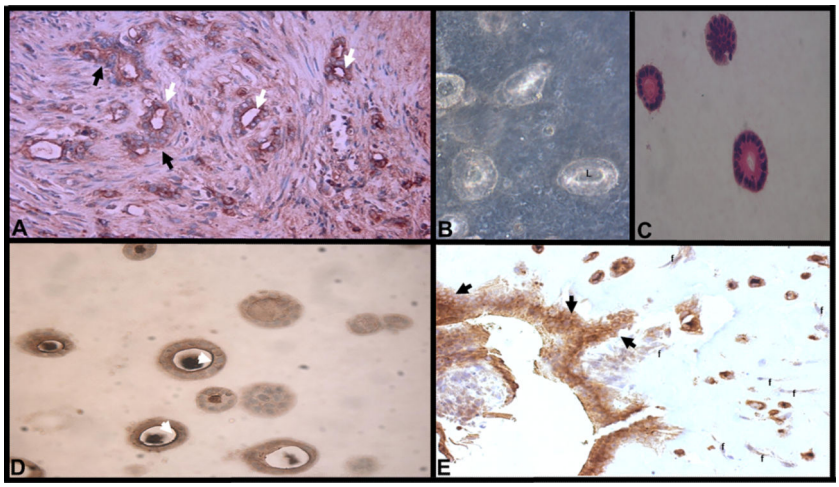

Figure 2.

Relationship between mesothelin (Msln) immunostaining pattern, disruption of polarized morphogenesis, and invasiveness in a syngeneic orthotopic rat tumor model and corresponding 3-dimensional (3-D) organotypic culture model of cholangiocarcinoma progression. **A.** Immunohistochemical demonstration of luminal Msln immunoreactivity expressed in differentiated cholangiocarcinoma ductal structures (white arrows) contrasted with diffuse cell membrane/cytoplasmic Msln immunoreactivity (black arrows) expressed in invasive cholangiocarcinoma emanating from the ductal structures in an orthotopic rat iCCA (TDE_{CC} iCCA). **B.** Phase contrast image of viable spheroids and well-differentiated ductal-like structures with distinct lumens (L) formed in 3-D organotypic culture of a cholangiocarcinoma cell strain (TDE_{CC} cells) derived from rat TDE_{CC} iCCA. **C.** Hematoxylin & eosin stained section demonstrating polarized ductal-like structure formed from rat TDE_{CC} cholangiocarcinoma cells in 3-D culture. **D.** In the absence of CAFs, polarized ductal-like structures formed from TDE_{CC} cholangiocarcinoma cells in 3-D culture exhibit strong luminal surface immunoreactivity for Msln (white arrow heads). **E.** In sharp contrast, polarized morphogenesis is dramatically disrupted when TDE_{CC} cholangiocarcinoma cells are maintained in 3-D co-cultured with α- SMA+CAFs, also derived from a rat TDE_{CC} iCCA. Under these co-culture conditions, the cholangiocarcinoma cells can organized into hyperproliferative, invasive structures exhibiting diffuse cell membrane/cytoplasmic immunoreactivity for Msln (black arrows). In both the orthotopic tumor and organotypic culture models, a more heavily glycosylated 50-kDa form of Msln was demonstrated to be associated with the apical surface pattern of expression of Msln, whereas a 40-kDA Msln form was shown to be associated with the diffuse cell membrane/cytoplasmic immunostaining pattern. Data based on results presented in Manzanares *et al.*, 2018. Images in 2A and 2E are slightly modified versions of those published as Figure 2B and Figure 7B, respectively, in Manzanares *et al*, 2018.

Table 1.

Representative Genetic Mouse Models Supporting Different Cells of Origin of Intrahepatic Cholangiocarcinoma

Model	Method	Tumor	Suggested Cell Origin[2]	Reference
AhCreER^{T};Kras$^{V12/+}$;Pten$^{f/f}$ (*PTEN* loss; *KRAS* activation)	GEMM[1]	Non-invasive papillary neoplasms of intrahepatic biliary tract, including major interlobular bile ducts and small bile duct radicles	BEC/HPC	Marsh *et al.*, (2013)
Alb-Cre^{+};LSL-Kras$^{G12D/+}$;Pten$^{f/f}$	GEMM	Well differentiated, desmoplastic intrahepatic cholangiocarcinoma	BEC/HPC	Ikenoue *et al.*, (2016); Lin *et al.*, (2018);
Sox9-Cre^{ERT2};LSL-Kras$^{G12D/+}$;Pten$^{f/f}$	GEMM	Intrahepatic and extrahepatic cholangiocarcinomas[3]	BEC	Lin *et al.*, (2018)
Alb-Cre,Smad4$^{f/f}$; Pten$^{f/f}$ (*Smad4* and *PTEN* ablation)	GEMM	Bile duct dysplasia, carcinoma *in situ,* well differentiated, desmoplastic intrahepatic cholangiocarcinoma	BEC/HPC	Xu *et al.*, (2006)
Ck19-CreER^{T}eYFP^{R26}p53$^{f/f}$ + thioacetamide (*p53* deletion + chronic liver injury)	GEMM	Intrahepatic cholangiocarcinoma	BEC[4]	Guest *et al.,* (2013)
Sox9-Cre^{ERT2};Kras$^{LSL-G12D}$;Tp53$^{f/f}$ (*KRAS* activation; *Tp53 deletion*)	GEMM	BilIN lesions[5]; desmoplastic intrahepatic cholangiocarcinoma	BEC	Hill *et al.*, (2018)
AAV8-TBG-Cre;Kras$^{LSL-G12D}$;Tp53$^{f/f}$ plus 2 week dietary treatment with DCC[6] (KRAS activation; Tp53 deletion; dietary-induced liver injury)	GEMM	Intrahepatic cholangiocarcinoma; hepatocellular carcinoma; mixed hepatocellular carcinoma-cholangiocarcinoma	HEP[4]	Hill *et al.*, (2018)
Alb-Cre;LSL-IDH2R172K; Kras^{G12D} (mutant *IDH* expression; *KRAS* activation)	GEMM	Intrahepatic cholangiocarcinoma	HPC	Saha *et al.*, (2014)
Alb-Cre;NotchIC (Notch overexpression)	GEMM	Desmoplastic intrahepatic cholangiocarcinoma	HPC	Zender *et al.*, (2013)
AAV8-TBG-Cre;Pten$^{f/f}$;TBR2$^{f/f}$ (*PTEN* and *TGFβR2* ablation)	GEMM	Desmoplastic intrahepatic cholangiocarcinoma	HEP[4]	Mu *et al.*, (2016)
Prom1-CreERT2;Pten$^{f/f}$;TBR2$^{f/f}$ or *K19-CreERT; Pten$^{f/f}$;TBR2$^{f/f}$*	GEMM	Desmoplastic intrahepatic cholangiocarcinoma	BEC[4]	Mu *et al.*, (2016)
Alb-Cre;Nf2$^{lox/lox}$ (N*eurofibromatosis type 2 gene deletion*)	GEMM	Oval cell proliferation; hepatocellular carcinoma; intrahepatic cholangiocarcinoma	HPC	Benhamouche *et al.*, (2010)
K19-Cre^{ERT};LSL-Kras^{GD12D}; Tgfbr2$^{f/f}$ + IL-33 (*KRAS* activation, *TGFβR2* loss, IL-33 mediated biliary epithelial injury response	GEMM	Extrahepatic cholangiocarcinoma	PBG[4]	Nakagawa *et al.*, (2017)
AAV8-Tte-Cre; Hydrodynamic tail vein injection NICD/AKT plasmids (activated Notch and AKT)	SB[7] transposon-transfection	Intrahepatic cholangiocarcinoma lacking desmoplastic stroma	HEP[4]	Fan *et al.*, (2012)
Hydrodynamic tail vein injection of HA-tagged pT3-EF5α-PIK3CAH1047R and Flag-tagged pT3-EF5αYapS127A (Co-activation of PI3CA and Yap)	SB transposon transfection	Hepatocellular carcinoma; intrahepatic cholangiocarcinoma; mixed hepatocellular carcinoma-intrahepatic cholangiocarcinoma	HPC/HEP	Li *et al.*, (2015)
Intrabiliary injection of murine myr-AKT and human YAP127A combined with systemic IL-33 (or IL-6) injection and lobar bile duct ligation (Transduction of constituitively activated AKT	SB transposon transfection	Desmoplastic intrahepatic cholangiocarcinoma	BEC	Yamada *et al.*, (2015)

Model	Method	Tumor	Suggested Cell Origin[2]	Reference
and Yap facilitated by a IL-6-mediated process and lobar bile duct obstruction)				

[1] GEMM, genetically engineered mouse model

[2] BEC, biliary epithelial cell; HPC, hepatic progenitor cell; HEP, hepatocyte; PBG, peribiliary gland cell

[3] Pancreatic ductal adenocarcinomas also developed in this model

[4] Lineage tracing employed

[5] BilIN, biliary intraepithelial neoplasia, which only preceded development of intrahepatic cholangiocarcinoma from BEC and not hepatocytes in this model

[6] DDC, 3,5-diethoxycarbonyl-1,4 dihydrocollidine

[7] SB, Sleeping Beauty