CHOLANGIOCARCINOMA: NEW PERSPECTIVES FOR NEW HORIZONS

ABSTRACT

INTRODUCTION: Biliary tract cancer represents a heterogeneous group of malignancies characterized by dismal prognosis and scarce therapeutic options.

AREA COVERED: In the last years a growing interest in BTCs pathology has emerged, thus highlighting a significant heterogeneity of the pathways underlying the carcinogenesis process, from both a molecular and genomic point of view. A better understanding of these differences is mandatory in order to deepen the behavior of this complex disease, as well as to identify new targetable target mutations, with the aim to improve the survival outcomes. We decided to provide a comprehensive overview of the recent highlights on BTCs, with a special focus on the genetic, epigenetic and molecular alterations which may have an interesting clinical application in the next future.

EXPERT OPINION: In the last years, the efforts resulted from international collaborations have led to the identification of new promising targets for precision medicine approaches in the BTC setting. Further investigations and prospective trials are needed, but the hope is that these new knowledge in cooperation with the new technologies and procedures including bio-molecular and genomic analysis as well radiomic studies, will enrich the therapeutic armamentarium thus improving the survival outcomes in a such lethal and complex disease.

KEYWORDS: cholangiocarcinoma, precision medicine, immunotherapy, genomic analysis

ARTICLE HIGHLIGHTS:

- Biliary tract cancer (BTC) remains nowadays one of the more lethal malignancies and the identification of new therapeutic strategies is urgent.

- In the last years, huge efforts have been made with the goal to identify the molecular and genomic pathways which underly the carcinogenesis in BTC, thus highlighting several promising targets.
- The incidence of genomic aberrations, including FGFR2 fusions and IDH1 mutations, have been recently investigated in BTC, and some molecules have showed promising results in targeting the subpopulations of patients carrying these aberrations, in terms of both efficacy and survival outcomes. Other molecular and genomic alterations are currently on investigation in this setting, including NTRK fusions and BRCA mutations, as well as epigenetic phenomena, like DNA methylation.
- Despite a number of promising preclinical evidences, therapies targeting the neo-angiogenesis pathways have not showed the desired results. A deeper insight into the underlying molecular pathways is needed.
- The role of immunotherapy in BTC is undefined. Many studies are investigating the role of immune-checkpoint inhibitors in both monotherapy and combination in this setting, but, nowadays, no specified indications exist. Further results are awaited.

1. INTRODUCTION

Biliary tract cancer (BTCs) represents a heterogeneous group of malignancies accounting the 10-15% of primary liver cancers, and cholangiocarcinoma (CCA) is the second most common primary liver tumor worldwide after hepatocarcinoma (HCC) (1). Nowadays, surgery and/or chemotherapy with or without radiotherapy constitute the standard treatment for the early and locally advanced stages, whereas systemic platin-based chemotherapy remains the backbone treatment of advanced and metastatic disease. Of note, only about one in five patients with CCA is eligible for surgery at the time of presentation, with a significative recurrence rate after liver resection. The ABC-02 trial leaded to the approval of the doublet cisplatin-gemcitabine as first line standard treatment in advanced CCA, whereas no second line treatments are currently approved for this disease (2). The results obtained with the standard treatment in advanced stages are unsatisfactory, with a dismal prognosis and a five-year survival rate of about 2% for stage IV (3). For this reason, a better understanding of the biological pathways underlying the carcinogenesis in CCA and an individual characterization of these tumors at the genomic, epigenetic and molecular levels has turned to be

an urgent need, and many efforts have been made in the last years to identify targets for new therapeutic approaches. In the present review we provide a comprehensive overview of the most recent and attractive insights on CCA, with a special focus on the genetic, epigenetic and molecular alterations which may have an interesting clinical application in the next future.

2. RISK FACTORS AND ONCOGENESIS'S PATHWAYS

A first big challenge in the CCA field involves the difficulties existing in the definition of the population at risk which could be potentially eligible for an intense screening protocol. The majority of CCA diagnosis are attributable to sporadic events (4); nevertheless, several risk factors, including genetic predisposition and environmental factors, have been recognized, with several geographical differences. The incidence of BTC is manifold higher in Eastern word compared to Western word, with differences between countries and regions too, which reflect the presence of different risk factors as well as genetic determinants. Generally, several factors have been recognized as involved in the process of CCA carcinogenesis. A predisposition to CCA has been highlighted in patients with several biliary pathologies, including primary sclerosing cholangitis (5), choledocholithiasis (6), bile duct cysts (7), inflammatory bowel disease, pancreatitis, parasitic infections (8, 9), metabolic disorders (diabetes, non-alcoholic fatty liver disease) and liver disease (10-13). Moreover, the alcohol consumption and tobacco use have been put in correlation with the development of CCA (13), as well as the presence of genetic polymorphism (14). If in the East regions of the world the parasitic infestation with liver flukes, which occur by ingestion of raw, undercooked, or pickled fish constitute the most important risk factor for CCA, as well as infection by HBV, in the Western part of the world the strongest association has been reported with primary sclerosing cholangitis, with or without bowel disease, and with altered metabolism conditions (14).

All the overmentioned risk factors convert in damage to the biliary epithelium which is sustained by both cholestasis and chronic inflammation. Chronic inflammation induces activation of cytokines and growth factors, leading to the increase of the influx of immune cells and the development of aberrant vascular network, which all characterized an oncogenic microenvironment (15, 16).

Unfortunately, several mechanisms involved in the oncogenesis of CCA are still unknow, and further investigations are needed in order to clarify which patients could be eligible for an intense screening procedure due to the high risk to develop cancer.

Recently, researchers have focused on epigenetic alterations involved in biliary tract carcinogenesis, especially DNA methylation. DNA methylation is a stable epigenetic mark consisting in the addition of a methyl group to the fifth carbon atom of a cytosine mainly occurring in the context of cytosine-guanine dinucleotides (CpG sites). These epigenetic modifications frequently occur in the regions of gene promoters , thus playing a crucial role in the regulation of gene expression by influencing the accessibility of DNA to transcription factors (17).

DNA methylation alterations are early events during tumorigenesis and may be detected even in preneoplastic lesions in many types of tumors (18-25), including CCA (26-28) and even several years prior to tumor diagnosis (29, 30), thus making this process likely to be a good tool to predict cancer development and improve early diagnosis.

A growing body of evidence suggest that DNA methylation alterations play a crucial role in the onset and progression of CCA. The mechanisms responsible for the altered methylation patterns observed in CCA have been explored, and include an increased expression of the methyltransferase DNMT1 by eventual action of interleukin-6 (31,32) and the IDH1 gene mutations (33, 34)Of interest, although DNA methylation predominantly affects polycomb repressive complex 2(PRC2) targets genes such as homebox genes and genes involved in transcription regulation, the altered genomic regions can be different among tumors from different etiology. In fact, a comprehensive integrative clustering of CCA revealed two hypermethylated clusters. The first one was enriched in fluke positive CCAs, characterized by hypermethylation of promoter CGIs, downregulation of TET1 and upregulation of EZH2. The other cluster was enriched in fluke negative tumors with IDH or BAP1 mutations and mainly shows hypermethylation of promoter shores. These findings suggest that, in the first case, CCA development can be driven by pathogenic agents that can be responsible for early epigenetic alterations and, in the second case, by genetic mutations in driver genes such as IDH that can consequently induce DNA hypermethylation (35). All these findings suggest that these epimutations might represent potential biomarkers for cancer early detection, but a role as important prognostic and predictive markers to improve therapeutic interventions has been recognized too. Moreover, since the screening of epigenetic alterations can also be carried out in circulating tumor DNA (ctDNA) and in DNA isolated from different biological matrices other than tissues, the detection of these biomarkers through less invasive procedures makes their identification of great value.

Several CCA methylation-based biomarkers with good specificity and sensitivity in tissues have been proposed, including *OPCML* (specificity and sensitivity respectively of 100% and 89%) and *SFRP1* (specificity and sensitivity respectively of 100% and 84%) (36), *SHOX2-SEPT9* biomarker panel (specificity of 100% and sensitivity of 75%) (37), a four biomarker panel (including *CDO1*, *SFRP1*, *ZSCAN18* and *DCLK1*, with specificity of 100% and sensitivity of 87%) (38). In a series of 15 CCAs, *SEMA3B* hypermethylation showed the power to distinguish tumor tissues from their normal matched with 100% specificity and sensitivity (39). *CDO1* was also evaluated by Nakamoto et al, reaching a sensitivity of 76%, and specificity of 92% (40). However, many of these studies focused on biomarkers that are frequently hypermethylated also in other types of cancers.

Other putative CCA methylation biomarkers have been suggested by the analysis of case series available online, such as the panel composed by *F2*, *AHSG*, *ALDH8A1*, *SERPIND1* and *AGXT*, showing higher DNA methylation levels of promoters in CCA samples compared with normal liver tissues (41). Mishra and colleagues identified 17 differentially methylated promoter CpGs, two of which, together with differentially expressed genes and miRNAs are likely associated with patient survival (42). Zhang and colleagues identified a hub of nine altered genes (*AURKB*, *PLK1*, *CCNA2*, *ASPM*, *RRM2*, *TOP2A*, *BIRC5*, *F2*, and *AHSG*), where more than 40% of CCAs had at least one hub gene alteration, with *ASPM* (29%) as the most frequently altered (43).

Methylation alterations at *DLEC1* gene have been reported as characterizing biliary tumors of different localizations (44), underlining their importance as topographic biomarkers, already known in different types of cancer (45-47). Other methylation changes correlated with overall patient survival, as in the study conducted by Nanok and collaborators, showing that CCA patients with high methylation level of *HTATIP2* and low methylation level of *UCHL1* were associated with longer overall survival (48).

As said above, an important advantage for clinical implementation is that methylation alterations can also be detected in cell free DNA from different matrices such as blood, urine and stool (49-53). Several studies have demonstrated that selected methylation alterations can also be detected in bile (54, 55), biliary brush cytology specimens (56-58), plasma (46) and serum samples (59) from patients with BTC. For instance, a four-biomarker panel (*CDO1*, *CNRIP1*, *SEPT9*, and *VIM*) achieved 85% of sensitivity and 98% specificity in distinguishing CCA from PSC by analyzing biliary brush samples (38). In the same biological matrix, *HOXA1* and *NEUROG1* showed sensitivity of 89% and 100% respectively, and sensitivity of 100%, although in a rather small number of samples (60, 61). DNA methylation of

CDKN2A (p16) and *CDKN2A* (p14) in bile showed specificity of 94% and 97%, and sensitivity of 52% and 48% (54); a two-biomarker panel (*HOXD9* and *OPCML*) showed a 100% specificity and 63% sensitivity in blood samples(59).

The possibility of detecting DNA methylation alterations in these minimally- and non-invasive matrices rather than tissue samples would allow a useful implementation in the clinical setting, as less invasive procedures are needed to obtain the samples. DNA methylation alterations are therefore very promising biomarkers for early detection of BTC.

3. NEW INSIGHTS IN THE DIAGNOSTIC WORK FLOW OF CCA

Another important concern in CCA is diagnosis. CCA is often diagnosed following presentation with non-specific symptoms such as weight loss or abdominal pain, with 20-25% of cases being an incidental finding whereas later stage's presentation frequently includes biliary obstruction and jaundice, mainly in case of iCCA (62, 63). The diagnostic algorithm in case of suspected CCA includes a first-level investigation with ultrasound, and then contrast-enhanced computer tomography (CT) and/or magnetic resonance imaging (MRI). Moreover, CT and MRI with magnetic resonance cholangiopancreatography have an essential role in the diagnostic and staging process of pCCA and dCCA (64). Due to the frequent difficulties in discriminating between CCA and hepatocarcinoma (HCC), and with the aim to better plan the therapeutic protocol, a cytological and/or anatomopathological diagnosis is needed to establish a correct diagnosis. Endoscopic ultrasonography (EUS) provides the possibility of sampling tumor tissue at the price of risk of infections, bleeding and tumor seeding. Endoscopic retrograde cholangiopancreatography permits a good evaluation of the biliary structures, and the placement of stent to solve the biliary obstruction, as well as allows brushing sampling for cytological analysis. Percutaneous transhepatic cholangiography (PTC) is frequently performed with a both diagnostic and therapeutic role too, and recently the choledochoscopy is playing an increasing role in the diagnosis of eCCA achieving a specificity of 90% when implemented with new digital fiberoptic techniques (65). The difficult accessibility of CCA, especially when located in the perihilar region, as well as the high desmoplastic reaction which frequently characterized this disease, makes the cytology sensitivity as low as 20-

40% (66). This date is even more unsatisfactory if we consider the importance of molecular and genetic analysis, which will be increasingly important in this type of pathology. All the work flow in the diagnosis, staging and treatment planning of CCA has to involved a number of specialists, including oncologists, surgeons, radiologists with hepatic expertise; for this reason, nowadays, the tendence is to centralize patients in high volume institutions. Nevertheless, over half of the patients are currently diagnosed at advanced stages, when treatments with curative intent are not suitable options and prognosis is very poor (67-69). In this setting, novel tools able to improve diagnosis and managing of CCA patients are needed. Recent advances in the medical fields have opened new horizons in several oncologic settings, including CCA: liquid biopsy and radiomics appear of particular interest, as both consist in no-invasive procedures which could anticipate the diagnostic work flow. The term liquid biopsy includes a group of methodologies centering on detection of tumoral biomarkers released in organic fluids, such as plasma, urine and bile. The samples obtained could be analyzed through a range of technologies, with next generation sequencing (NGS) providing a high level of sensitivity on a small amount of tumor-derived genetic material (70). In the CCA setting, liquid biopsy has the fascinating advantage to increase the changes of achieving a diagnosis at early stages with less invasiveness, as well as to be serially repeated in order to perform a dynamic study of the disease's biology during treatment with chemotherapy or target therapies. In fact, the genomic assess on fluid samples obtained by liquid biopsy could offer the possibility to evaluate the heterogeneity of CCA and to monitor changes in tumor biology by the detection of cell free DNA (cfDNA) and circulating tumor DNA (ctDNA) and the use of genomic platforms (70). The larger experience involving the use of liquid biopsy in biliary tract cancer setting setting was conducted by Mody and collaborators, who performed a large profiling ctDNA series on 138 patients with biliary tract cancer. They found at least one genomic alteration in 89% of the cohort, including TP53, FGFR2, IDHA1 and BRAF mutations, ERBB2 amplifications and FGFR fusions (71). Unfortunately, to date, the concordance between mutations observed in ctDNA/cfDNA with those detected in tissue samples remains uncertain and based on small case series, and the role of liquid biopsy in clinical practice of patients with CCA is still marginal: further investigations are needed.

Another important diagnostic tool under investigation in many oncologic settings, including CCA, is radiomics.

The advancements in machine learning and data mining of the last years opened the door to a new comprehensive method of imaging analysis called Radiomics. The term Radiomics refers to the high-throughput extraction of quantitative features from digital medical images by a multistep

process which includes data selection, volume segmentation, features extraction, exploratory analysis and modeling (72). Through this complex process, the texture analysis based on medical imaging allows the extraction of a huge amount of information which are invisible to the human eye, thus potentially constituting a new important tool in the decision making.

In the field of primary liver malignancies, an increasing number of evidences emerged about the prognostic potential of texture analysis, mainly in the hepatocarcinoma settings (73-75). To date, only few studies focused on biliary tract cancer (BTC), but results are promising, and suggest a potential utility of texture analysis in different phases of the diagnostic-therapeutic flow-chart of this disease, including diagnosis, staging, molecular characterization and treatment choice.

Several evidences reported an important role of radiomics in predicting the lymph-nodal involvement before surgery. In particular, in a large retrospective study conducted on 247 biliary tract cancer patients treated with curative-intent surgery and lymph-node dissection, Ji et al. elaborated and validated a radiomics-based model for noninvasive individualized prediction of lymph-node metastasis, which resulted to be more sensible than the simple macroscopic appearance of lymph-nodes on images (76).These results were in line with a previous work, where it was ideated a nomogram including texture features extracted from MRI-images and clinical parameters, thus providing an individualized Lymph-nodes evaluation and therefore a useful clinical tool to guide surgical decisions (77). In terms of prediction of tumor biological behavior and genetics, the evidences about the role of radiomics in BTC are still not conclusive. The first evidence appeared in 2011, when Kim and colleagues highlighted the correlation between the improved arterial enhancement pattern and better DFS after surgical resection (78). These findings were later confirmed in a cohort of 47 ICC patients, where it was observed that those patients with hypovascular cancers showed to have more instances of lymphatic invasion, perineural invasion and, in conclusion, poorer DFS when compared to those patients with hypervascular cancers (79). More recently, Aherne and collaborators highlighted the correlation between three radiomic features extracted from CT images (necrosis, satellite nodules and vascular encasement) and poorer clinical outcomes. In the same work, no association was identifying between imaging features and genetic pathways (80). Very few works have focused on the correlation between imaging and tumor genotype in the BTC setting. Sadot et al. highlighted that qualitative and quantitative imaging features correlated to hypoxia specific marker's expression, including hypoxia inducible factor-1 alpha (HIF-1α), vascular endothelial growth factor (VEGF), and epidermal growth factor receptor (EGFR) (81), as previously noted from Segal and collaborators (82). More recently, Peng and

collaborators performed a high-throughput radiomics analysis based on US medicine images of 128 cholangiocarcinoma patients, and proposed a radiomics signature as a prediction tool of biological profile, including the tumor differentiation, perineural and microvascular invasion, and few immunohistochemistry patterns (Ki67, CK7 and VEGF expression) (83). The information extracted through the radiomics analysis could guide treatment choices. In the retrospective study conducted by Mosconi et all on 55 pre-TARE CT scans of cholangiocarcinoma patients, texture analysis resulted to quantify vascularization and homogeneity of the cancer architecture, thus providing information useful in identifying ideal TARE candidates (84). In two previous studies it emerged a potential utility of radiomics in differential diagnosis (85), and in defining patients likely to benefit from surgical treatment with radical intent (86).

The reported data are still preliminary and more investigations are needed to explore all the potentialities of texture analysis.

In the era of the precision medicine, the hope is that radiomics analysis could be integrated as a new noninvasive tool in the diagnostic therapeutic flow chart of cholangiocarcinoma, with the aim to improve the survival outcomes of this complex disease.

4. THE PATHOLOGIC HETEROGENEITY OF CHOLANGIOCARCINOMA

BTCs are a heterogeneous group made of benign, premalignant and malignant lesions, mainly classified anatomically in intrahepatic and extrahepatic according the WHO 2019 recommendation. TNM staging system subdivides these lesions in 3 groups: intrahepatic CCA (iCCA) is located proximal to the second order bile ducts; the extrahepatic cancer is perihilar adenocarcinoma of the bile ducts that develop in the right and left hepatic duct or at the confluence between the two and the last is the distal bile duct cancer that involves the common bile duct.

The intrahepatic group includes benign lesions such as biliary hamartomas, bile duct adenomas and biliary adenofibroma (this last one was not included in WHO 2010) which is very rare and considered a premalignant neoplasm (4) (figure 1A).

The malignant intrahepatic lesions and mainly represented by iCCA which is an adenocarcinoma with variable morphology. In particular the new WHO subdivides between small duct type" and "large duct

type" iCCA (WHO 2019). The "small duct type" is made of small tubules and ductulus, generally constituting a mass forming lesion that does not produce mucus (figure 1B). The "large duct type" more typically presents as a highly desmoplastic lesion with poorly defined margins, typically with periductal infiltrating pattern of growth, behaving like an extrahepatic bile duct adenocarcinoma 87). The small duct type iCCA is commonly an "occupying space" mass forming lesion with irregular but defined margins, that can have different morphological aspects and don't produce mucus. The "large duct type" iCCA produces typically mucus as the extrahepatic bile duct cancer and, because of the location, is commonly aggressive to the perihilar structures and extrahepatic hilar connective tissue with local (other than systemic) aggressiveness (figure 1C).

The intrahepatic mass can also be a combined hepatocellular-cholangiocarcinoma (cHCC-CCA) (figure 1D), a category that now is made simpler in the last WHO and that still is under investigation because it is a rare disease and because the diagnosis can be challenging too. According the new WHO recommendation, the diagnosis is to be made based on morphology and confirmed by immunohistochemistry. Some reports found out that it behaves more as HCC 88), but actually their treatment is still without defined guidelines 89). The so called "cholangiolocarcinoma" can be a part of the cHCC-CCA, but is more commonly found of part of an iCCA, then considered a subtype of iCCA.

The extrahepatic bile duct cancer is commonly an adenocarcinoma, although various morphologies and subtypes exist (WHO 2019), morphologically similar to a ductal adenocarcinoma of the pancreas and typically has local aggressiveness manifesting with periductal connective tissue invasion and neurotropism (figure 1E).

Concerning premalignant non-invasive lesions of both intrahepatic and extrahepatic bile ducts, they are the microscopic biliary intraepithelial neoplasm (BilIN) (figure 1F) and grossly visible, radiologically identifiable, intraductal papillary neoplasms of the bile duct (IPNB) (figure 1G), both of them subdivided between low grade and high grade. They have the pancreatic counterpart in pancreatic intraepithelial neoplasms (PanIN) and intraductal mucinous papillary neoplasms (IPMN), although there are some differences (90). The IPNB are subdivided between type I and type II. Type I is more architecturally regular with gastric/intestinal phenotype, whilst the type II has a complex architecture and can have intestinal phenotype but more frequently pancreatico-biliary, showing greater aggressiveness (WHO 2019).

Moreover, mucinous cystic neoplasm of the liver is a cystic lesion that can have variable amount of mucinous epithelium and ovarian-like stroma (figure 1H) (91), making the diagnosis sometimes

challenging and can be a precursor of cholangiocarcinoma. These neoplasms are rare and represent around 10% of resected hepatic cysts. They develop exclusively in females, usually are large and intrahepatic, regarding mainly the left lobe. Rarely they are invasive, but when it happens, they behave as ICC. Incomplete excision, as it happens in fenestration, conditions a relapse.

Carcinosarcomas and undifferentiated carcinomas are very rare entities. From the anatomopathological point of view, carcinosarcomas show both the components, with the sarcomatous component being characterized by the loss of epithelial differentiation (figure 1l); the undifferentiated carcinomas show basically the only epithelial differentiation.

5. NEW OPTIONS IN THE SYSTEMIC TREATMENT OF LOCALLY ADVANCED AND METASTATIC CCA

The new insights described in recent years have opened the way of the precision medicine in the CCA setting, with some promising results from randomized controlled trials. To date, the only curative option for patients with CCA was surgery, as performed by high volume institutions with high expertise. Unfortunately, a small percentage of patients are candidate to surgical option at the diagnosis, since the majority are initially diagnosed with locally advance or metastatic disease. In the advanced and metastatic CCA setting, the gold standard has been constituted by systemic chemotherapy with a combination of gemcitabine/cisplatin, but no standardized second line therapy have been approved (92). The new insights on CCA biology have recently changed the ideal paradigm of treatment in this setting, since the advent of larger scale genomic profiling permitted to identify new pathways which could constitute a target for new molecules. Moreover, the recent knowledges have highlighted several pathways which are of particular interest in the CCA carcinogenesis, including neo-angiogenesis, DNA methylation, and checkpoint inhibitors pathways. Here we report the most promising results from randomized controlled trials designed on the basis of the most promising and recent evidences about CCA biology.

5.1 NEW HIGHLIGHTS IN CCA'S BIOLOGY

Starting from 2014 (93), especially in intrahepatic cholangiocarcinoma, targetable alterations have been reported (Table 1), mainly regarding the fibroblast growth factor receptor 2 (FGFR2) and Isocitrate dehydrogenase 1 (IDH1).

FGFR2 chromosomal rearrangements are found in 10 to 20% of intrahepatic cholangiocarcinoma, seems to be altered mainly in young adults and possibly confers a better prognosis (93).

FGFR2 aberrations are represented mostly by fusions (93), in a small percentage by mutations, but studies have demonstrated the importance of fusions over other genetic alterations for therapy (94). Fusions induce the constitutive activation of FGFR2 (95) and happen between the N-terminus containing exons 1-19 of FGFR2 with a functional tyrosine kinase domain and the C-terminus that contains the partner dimerization domain. This partner gene can be represented by different genes, the most common are Periphilin 1 (PPHLN1) and Bicaudal family RNA binding protein 1 (BICC1) (96). Recently it has been stated that the breakpoint in FGFR2 in almost always within intron 17 or exon 18 but several other partners for fusions have been reported (97). Recently also a deletion-in-frame alteration has been described, that seems to be responding to FGFR2 inhibition too (98). Starting from these premises, a number of clinical trials have been conducted to assess the efficacy of agents targeting FGFR, including pemigatinib,infigratinib, futinatinib and derazatinib, in patients affected by CCA with FGFR2 fusions, and the FDA has recently approved the use of pemigatinib in refractory iCCA. Further studies investigating the role of FGFR inhibitors in chemo-naive patients are currently ongoing, and head-to-head trials trials will eventually be needed in order to better understand the benefits of each agent on the basis of their reversible or irreversible FGFR2 inhibition (99, 100). IDH1 mutations are missense mutations that generally involve a single residue in the active site of the enzyme. They have been described to be more commonly associated to" small duct type" ICC than "large bile duct type" and with poor grade of differentiation in CCA (101). The involved residue is more commonly R132C (70%) and less frequently R132L (15%) or R132G (12%) (102), which are mutant alleles distinct from those found in glioma and acute myeloid leukemia (enriched for IDH1R132H and IDH2R140Q) (103). IDH1 harbors missense mutations confined predominantly to a single residue (e.g., R132) in the active site of the enzyme. The mutation of IDH1/2 has been found to be correlated with poor differentiation (102, 103). Recently Lee K et al found association between the small duct mass-forming iCCA , which do not produce mucus, and better prognosis (101); nevertheless, further evaluations on larger series are needed to confirm this prognostic correlation.. From the clinical point of view, the phase III ClarIDHy trial has recently showed a survival benefit in previously treated patients with advanced IDH1-mutated CCA treated with Ivosidenib, the first-in-class oral small molecule inhibitor of mutant IDH1 (104). Due to the promising results obtained by IDH1 inhibitors in other oncologic setting, and the limited therapeutic approaches for metastatic BTC patients, IDH1 pathways have become an attractive therapeutic target, and are currently under

investigation four IDH1 inhibitors (e.g., pan-IDH1mut AG-120 and BAY1436032; specific-IDH1mut FT-2102 and IDH305) in six clinical trials. The data from these trials are awaited in order to clarify the role of this class of molecules in the subset of IDH1-mutant patients.

NTRK fusions are really rare in BTC, tested around 0,75% in a large series (105-107) but they are of great interest because they represent a direct target for therapy (108).

BRCA mutations are found in around 3,6% of CCA, without correlation with location (109), providing rationale for targeted therapies (110). BRCA mutant BTCs are more frequently associated with MSI but not with PD-L1 expression and show high mutational burden (111). Nevertheless, the clinical significance of BRCA status in BTC needs further investigation.

Concerning MSI, only a small percentage (around 5%) of ICC have microsatellite instability and are at least partially associated in the literature with Lynch syndrome (112,113).

It is important to detect such cases, although very rare, since microsatellite instability has been proven to be a predictive marker of response to immune checkpoint inhibitors (114) and until now the immune-expression of PD-L1 in cholangiocarcinoma has not been proven to be relevant for defining response or not to immunotherapy and it is usually found in macrophages and not in cancer cells (115) but more studied are needed to investigate this important aim.

Another rare mutation regards BRAFV600E, accounting for only 1% of all cases (116) can be screened with immunohistochemical staining and/or directly investigated with DNA-based methods (117).

Rarely HER2 can be found rarely overexpressed by immunohistochemistry in CCA, especially in the extrahepatic adenocarcinoma (118). Guidelines for scoring the immunohistochemical expression are lacking and actually relies on the scoring in gastric cancer (119) so FISH is recommended for confirmation of amplification.

In the previous paragraph we reported the importance of DNA methylation as potential biomarker able to early detect CCA and to predict prognosis. Due to the reversibility, DNA methylation changes have been proposed as potential therapeutic target too. Epigenetic drugs, such as 5'-azacitidine and decitabine, are routinely used as a cancer treatment for acute myeloid leukemia. Their potential use has been also explored for CCA. Decitabine and zebularine has showed anti-tumor properties *in vitro* (120). Given the cooperation of DNA and histone modifications in cancerogenesis, inhibition of both DNA and histone methyltransferases represents a clever solution for CCA treatment. Recently, Colyn

and colleagues, demonstrated that the concomitant inhibition of these two epigenetic regulators reduced CCA proliferation, inhibited CCA tumoroids and xenograft growth (121).

5.2 NEO-ANGIOGENESIS IN CCA

Neo-angiogenesis is the biological process that led to the origin of dysregulated and dysfunctional vessels from pre-existing vascular vessels through different pathological pathways and genic regulations, and it has been demonstrated to be closely linked to cholangiocytes inflammation and CCA development and progression (122-124).

Nowadays, the role of antiangiogenic drugs in CCA is debated.

Starting from promising results obtained in preclinical setting (125-135), the activity of many anti-angiogenic treatments with antibodies (Bevacizumab and Ramucirumab), trap (Aflibercept) and TKIs (Sorafenib, Vandetanib, Sunitinib and Regorafenib) have been explored alone or in association to chemotherapy and/or anti-EGFR drugs in several phase I and II CCA trials.

In a phase II non-comparative study, Bevacizumab in association with gemcitabine and oxaliplatin performed a mPFS of 7.0 months (95% CI 5.3-10.3), without reaching the target rate in six-month PFS (67% Vs 70%) (136). Patients wild-type EGFR and wild-type RAS tumors demonstrated to be more likely to respond to Bevacizumab in combination with chemotherapy and erlotinib (an anti-EGFR TKI) (137). As second line therapy, Bevacizumb achieved encouraging results in combination with both FOLFIRI (138), and capecitabine, gemcitabine and irinotecan (139).

In the randomized phase II placebo-controlled ABC-03 study the association of cisplatin/gemcitabine and Cediranib, and oral VEGFR1-3, platelet-derived growth factor (PDGF) receptor and c-Kit inhibitor, conferred improved response rate (44% vs 19%, p=0.0036) and improved 6-months PFS (70.5& vs 61.3%; p>0.05) in patients with advanced BTCs. Unfortunately, the study did not meet its primary endpoint, and showed an unfavorable toxicity profile of Cediranib (140).

Apatinib, a selective suppressor of VEGFR2, showed to reverse the VEGF signal by the inhibition of PI3K/Akt and VEGFR2/RAF/MEK/ERK pathways, which are normally upregulated during cell proliferation and differentiation (141).

Recently, the co-administration of pazopanib and trametinib appeared to prevent the cancer neo-angiogenesis through inhibition of RAF/MEK/ERK cascade, thus being a promising treatment strategy in refractory CCA (142).

The survival benefit provided from Regorafenib, an oral multikinase inhibitor targeting VEGFR1-3, PDGF receptor and several oncogenic kinases (KIT, RET, RAF), has been showed in two single-arm, phase II studies enrolling pretreated advanced CCA patients. In the first one Regorafenib showed a mPFS of 15.6 weeks and a mOS od 31.8 weeks, with Grade 3-4 toxicities experienced in 40% of patients (143). In the second one, the 51% of patients treated with regorafenib showed an OS > 6 months, thus reaching the primary endpoint (144).

Disappointing results have been achieved from Sorafenib in monotherapy and in combination with chemotherapy (gemcitabine or gemcitabine plus cisplatin) or target therapy (erlotinib) in untreated CCA patients (145-149).

The strong VEGFR-2 inhibitor Vandetanib was evaluated in two large phase II trials: Vandetanib in addition to gemcitabine failed to confer a survival benefit in comparison to gemcitabine in monotherapy in advanced CCA setting (150, 151). Finally, Sunitinib showed both to confer a marginal advantage in untreated and pretreated, respectively, advanced CCA patients, at the expense of relevant toxicities (152).

Hopefully, these studies will give us some answer about the efficacy and safety of the combo anti-angiogenetic drugs and checkpoint inhibitors in CCA (Table 2).

In conclusion, despite the strong biomolecular rationale, the available clinical evidences showed unsatisfactory benefit from antiangiogenic therapies in CCA patients. A deeper understanding of the biomolecular pathways involved in CCAs neo-angiogenesis will guide the development of new therapeutic approach and will permit the identification of biomarkers to select patients which are likely to respond to these therapies.

5.3 IMMUNOTHERAPY IN BILIARY TRACT CANCER

Nowadays, immunotherapy has radically changed the therapeutic algorithm of several cancers. In particular, immune-checkpoint inhibitors (ICIs) have become the mainstream treatments which enhance adaptative immunity against cancer increasing OS and response rate in several malignancies. With this in mind, it needs to be said that the role of ICIs and their combinations with other therapies in advanced CCA is still undefined. Several preclinical studies have tried to demonstrate a therapeutic role of immunotherapy in BTC (153, 154). Since CD47 is expressed in several cancer cells and it displays a protective signal for phagocytic elimination, a CCA tumor specimens as well reveal elevated levels of CD47, with a high activity of immune escape (119). Moreover, in a CCA mouse model, anti-CD47 mAb infusion increased innate immunity and inhibit the cancer proliferation (153). Zhou et al. demonstrated that programmed cell death protein 1 (PD1) and Cytotoxic T-Lymphocyte Antigen 4 (CTLA4), the mainstream checkpoint in immune escape, were more expressed in tumor-infiltrating T cells when compared with T cells in non-tumoral tissue and blood. Ex vivo, targeting of PD1 or CTLA4 enhanced effector proteins production and T cell proliferation in TILs derivative from CCA (154).

Currently, CTLA4, PD-1, and its ligand (PD-L1) are at this moment the main target for immune-checkpoint inhibitors (ICIs). It is interesting to note that in CCA, PD-1 and PD-L1 were assessed. In CCA tumor specimens, PD-L1 overexpression correlated with survival rates of patients (155, 156). In particular, PD-L1 overexpression (≥5%) was associated with superior OS (P=0.012) and DFS (P=0.018) (155).

5.3.1 Monotherapy with Immune checkpoint inhibitors

Several studies evaluated the activity of ICIs monotherapy in BTCs with conflicting results in patients without stratification for MSI, PD-1, and PD-L1 expression (157) (Table 3).

First data in CCA derived from the combined analysis of KEYNOTE-028 and KEYNOTE-158 trials (158). These studies, a phase Ib and II, respectively, presented the efficacy and safety of an anti-PD-1 namely pembrolizumab in BTC patients who progressed after standard chemotherapeutic regimen(s) (158, 159). Both these studies showed promising data in terms of OS (5.7 and 7.4 months in KEYNOTE-028 and KEYNOTE-158, respectively) and PFS (1.8 and 2.0 months in KEYNOTE-028 and KEYNOTE-158, respectively).

Nivolumab was evaluated in a phase I trial that enrolled 30 Asiatic BTC patients (160), and displayed a manageable safety profile with median OS and PFS of 5.2 months (90% CI, 4.5-8.7) and 1.4 months (90% CI, 1.4-1.4), respectively (160). A phase II multicenter trial investigated Nivolumab

in in 54 pretreated BTC patients (161) according to the following schedule: 240 mg on day 1 every 2 weeks for 16 weeks followed by 480 mg every 4 weeks. Median PFS and OS were 3.6(95% CI, 2.30-5.69) and 14.2 (95% CI, 5.86- not reached) months; ORR and DCR were 22% and50%, respectively (127). Moreover, PD-L1-positive BTC-patients obtained a significantly higher PFS compared to PD-L1 negative group (10.4 and 2.3 months, respectively; HR 0.23; 95% CI, 0.10-0.51; $P < 0.001$).

A Phase 1 study evaluated Durvalumab (an anti-PD-L1) and Tremelimumab (an anti-CTLA-4) in Asian cancer patients. This study demonstrated that no dose-limiting toxicities were observed for the use of Durvalumab alone or in combination with Tremelimumab. The trial was subsequently expanded to larger cohorts of patients with advanced cancers including BTC. Median duration of response for the durvalumab combination cohorts were 9.7 and 8.5 months, respectively, with a mOS of 8.1 (95% CI, 5.6-10.1) and 10.1 (95% CI, 6.2-11.4) months, respectively (162).

Bintrafusp-alfa (M7824), an innovative first-in-class bifunctional fusion protein composed of a human IgG1 monoclonal antibody, has been assessed against PD-L1 fused with 2 extracellular domains of TGF-β receptor in a phase I study in advanced BTC (163). Thirty Asian patients were treated with M7824 achieving an ORR of 20% (95% CI 8-39), even if 2 deaths were reported, one due to severe interstitial lung disease and the second one due to septic shock consequent to bacteremia (163). Recently the long-term follow-up data have been reported, showing a manageable safety profile and long-lasting response after a median follow up of 28 months (164).

Today, several clinical trials assessing ICIs as single agent are currently ongoing: nivolumab (NCT02829918), pembrolizumab (NCT03110328), atezolizumab (NCT03201458,).

At the moment, even if there are no indications on single agent immunotherapy in BTC, FDA recommends pembrolizumab for the treatment of any dMMR or MSI-H (165) and TMB high malignancies (more than 10 mutations per megabase) (166).

5.3.2 Combination therapy with Immune checkpoint inhibitors

There is growing evidence that cytotoxic drugs can also strengthen the immune system by increasing the ratio of cytotoxic lymphocytes to regulatory T cells and the number of antigen-presenting cells (167) (Table 4, Table 5). In vitro, cholangiocarcinoma (CCA) cells treated with gemcitabine can induce the mRNA expression of PD-L1, and thus combined with immunocheckpoint inhibitors (ICI) may enhance antitumor immunity (168, 169). In fact, the use of an ICI could increase the efficacy of

gemcitabine, which in turn increases the antigenicity of tumor cells and partially reduces the immunosuppressive effect of chemotherapy, which may be associated with the role of tumor associated macrophages (169). A phase II trial (NCT03311789) studied the clinical response to nivolumab in combination with gemcitabine and cisplatin in 27 response evaluable patients with biliary tract cancer (BTC), and found an ORR of 55.6%, including 5 CRs and 10 PRs. Of the 6 patients who were resistant to chemotherapy, one CR and one PR were achieved. This result indicates that, as a PD-1 inhibitor, nivolumab is able to re-sensitize BTC to gemcitabine and cisplatin chemotherapy (169). Another phase II trial (NCT04413734) is now recruiting patients to evaluate safety and efficacy of an anti PD-1 antibody, triprilumab, in combination with doublet chemotherapy of gemcitabine plus cisplatin in patients with unresectable intrahepatic cholangiocarcinoma (iCCA) (170).

In recent years, research into the efficacy of combinations of multiple immunotherapy drugs (171) has begun, and their respective strengths are emerging. A clinical trial, NCT02923934, explored the efficacy and safety of a combination of two immunotherapy drugs, nivolumab and ipilimumab, for the treatment of CCA. This phase II clinical trial reported an ORR of 23%, DCR of 44%, mPFS of 2.9 months and OS of 5.7 months. Notably, all patients who responded had received prior chemotherapy, and none of them had a microsatellite unstable tumor. Although the combination of immunotherapy drugs proved clinically effective in the study, dual drug immunization was not superior to single drug immunotherapy, with the exception of its effects on ORR (172).

Preclinical evidence indicates a close correlation between angiogenesis and the suppression of anti-tumor response. Vascular endothelial growth factor (VEGF) increases T cell exhaustion by enhancing the expression of inhibitory checkpoints on T cells, while simultaneous blocking VEGF receptor (VEGFR) and PD-1/PD-L1 can induces cumulative antitumor effects (173). These effects would be obtained by supporting vascular changes, such as vessel normalization and high endothelial venule formation, that facilitate enhanced cytotoxic T-cell infiltration, activity and tumor cell destruction (173). A non-randomized, open label, phase I trial of ramucirumab and pembrolizumab was the first to combine antiangiogenic therapy with an ICI to treat advanced BTC. The ORR was 3.8%, with mPFS and OS times of 1.64 and 6.44 months, respectively (174). Furthermore, two phase II trials are now recruiting patients to investigate the safety and efficacy of this combination. In particular, the NCT03895970 trial investigates the combination of pembrolizumab and lenvatinib (a small molecule tyrosine kinase inhibitor (TKI) that inhibits VEGFR1-3, fibroblast growth factor receptor 1-4, platelet-derived growth factor receptor α, stem cell factor receptor and rearranged during transfection protein) in second line therapy of advanced hepatobiliary malignant tumors. Instead, the

NCT04642664 analyzes the combination of the anti PD-1 camrelizumab and of the VEGFR-2 TKI apatinib in pretreated patients with advanced biliary tract malignant tumors (170).

Furthermore, there are recruiting phase II clinical trials that aim to evaluate the efficacy and safety of the combination of immunotherapy with new small molecules that have very selective biological targets in patients with advanced or metastatic BTC. The small molecules involved in these studies are entinostat that is a synthetic benzamide derivative histone deacetylase inhibitor (NCT03250273), AZD6738 that is a selective ataxia telangiectasia and Rad3 (ATR) kinase inhibitor (NCT04298008), nedisertib that is an inhibitor of DNA-dependent protein kinase (NCT04068194), the Colony Stimulating Factor-1 R inhibitor (NCT04301778) and cobimetinib that is a MEK inhibitor (NCT03201458) (170).

When radiotherapy is administered, the sensitivity of the immune system to the tumors is increased (175). In fact, radiofrequency or cryoablation could induce a peripheral immune response which may enhance the effect of anti-CTLA-4 treatment. A case report showed that radiotherapy can improve the efficacy of immunotherapy in patients with late stage or recurrent iCCA with low TMB, microsatellite stability and negative PD-L1 expression status (176). A multicenter phase 2 randomized controlled trial (NCT04333927) aims to evaluate the efficacy and safety of adjuvant immunotherapy combined with chemoradiation for patients with high-risk resectable extrahepatic cholangiocarcinoma and gallbladder cancer. This study has finished recruiting and results are expected. Moreover, the CORRECT is a recruiting multicenter phase II randomized trial with the purpose to investigate the efficacy and safety of radiotherapy combined with the anti-PD-1 antibody camrelizumab and chemotherapy (gemcitabine plus cisplatin) in unresectable iCCA patients (170).

Finally, Xie and collaborator investigated whether tremelimumab could be safety with microwave ablation in twenty patients with refractory CCA. The combination showed an acceptable safety profile; 12.5% of patients achieved a partial response, whereas 31.3% achieved a stable disease. Of interest, peripheral blood immune cell subset profiling showed an increased activated CD8+Tcells and TCR repertoire expansion induced by tremelimumab, which could contribute to the treatment benefit (177).

5.3.3. EMERGING BIOMARKERS OF RESPONSE TO IMMUNOTHERAPY IN BTC

The randomized trials investigating immunotherapy both as monotherapy and combination therapy

showed controversial results, since responses seem limited to a small percentage of BTC patients. Starting from this premise, many efforts have been made in order to find biomarkers able to identify patients likely to respond to immunotherapy and in order to understand the resistance mechanisms in non-responders. To date, few data are available regarding the role as predictive biomarker of PD-L1 as assessed by immunohistochemistry in BTC patients treated with immune-checkpoint inhibitors. From the subgroup analysis of the KEYNOTE-158, the ORR of patients treated with pembrolizumab was 6.6% and 2.9% in PD-L1-positive patients and in PD-L1-negative patients, respectively (158, 159). In the phase 3 trial investigating the role of nivolumab as second line therapy in advanced BTC patients, Kim and collegues reported a statistically improved PFS in patients PD-L1-positive compared PD-L1-negative patients (10.4 months versus 2.3 months; HR, 0.23; 95% CI, 0.10–0.51; $p < 0.001$). Moreover, a clinically meaningful superior median OS was showed in PD-L1-positive patients, without reaching statistical significance. Overall, the role of PD-L1 in predicting patients likely to respond to immune-checkpoint inhibitors is still unclear, also due to the different PD-L1 assays and scoring system.

Tumor mutational burden (TMB), commonly defined as the overall number of somatic nonsynonymous mutations per megabase, has been associated to response to immunotherapy in several solid tumors (178). In the BTC setting, data are inconsistent and anecdotal. Zang and collegues reported a case series of three BTC patients with TMB-H treated with immune-checkpoint inhibitors: of note, two patients reported partial response and one patient a complete response (179). Unfortunately, these data have not been confirmed by other publications, and further investigations are needed. Moreover, as in the case of PD-L1, TMA assessment is strongly influenced by the methods and cutoffs used.

The evaluation of the Mismatch repair deficiency as a potential biomarker of response to immunotherapy has been suggested in several oncologic setting (180, 181). The proportion of MSI-H status among BTC patients is controversial (182), and few data are available regarding its role as predictor of response to immunotherapy. In the overmentioned phase II trial of nivolumab monotherapy conducted by Kim and collegues, all responders were MSS patients, which is consistent with the report by Zhang and collaborators, where the three patients who achieved PR or CR with immune-checkpoint inhibitors were all MSS (179). Moreover, in the KEYNOTE-158 and KEYNOTE-028 all patients responders to immunotherapy were MSS, thus adding confusion on the putative role of MSI (158, 159). Data are still scarce, but available evidence seems to suggest a modest value of MSI/MSS as biomarker of response to immunotherapy Recent years have witnessed growing attention toward DDR gene aberrations, which seem to constitute a promising predictive biomarker of response to immunotherapy (183). DDR gene alterations impair DNA damage repair mechanisms, thus leading to accumulation of DNA damage and genomic instability. DDR gene mutations in BTC has been reported to occur in approximately 30% of cases (184). Recently, Spizzo and collegues by analyzing tumor samples from 1292 BTC patients using NGS reported an interesting association between

BRCA mutations, MSI/dMMR and TMB-H with DDR gene mutations, thus supporting a razionale of DDR gene mutations as biomarker of response to immunotherapy (185). However, few data are already available on the potential role of DDR gene mutations in BTC; further studies are warranted in this direction.

Finally, tumor microenvironment (TME), on the basis of preclinical studies suggesting its role as modulator of the host immune response against tumors, is currently under investigation as potential predictor of response to immune-check point inhibitors in several solid tumors, including BTC (186). BTCs are desmoplastic tumors with the TME showing immunosuppressive innate tendency. The existence of different subgroups of tumors have been suggested, with immunologically "hot" BTC characterized by higher CD8+ cell density more likely to respond to immunotherapy, compared to the "cold" tumor, which present a prevalence of immunosuppressive cells. Overall, these data remain preliminary (187).

In conclusion, no validated predictors of response to immunotherapy are already available in the BTC setting, and further translational investigations in this direction are needed due to the aggressiveness of this malignancy which presents scarce treatment option.

6. CONCLUSION

Despite its low incidence, BTC remains a worldwide emergency due to its dramatic prognosis and to the lack of effective treatments. In the last years, the efforts resulted from international collaborations between research groups have led to a deeper insight into its biological pathways and genomic profile, which allow a new awareness about the disease's molecular heterogeneity. The multiple failures experienced looking for an effective therapeutic strategy for advanced BTC could be consequence of such important heterogeneity of biology and behavior, as well as response to specific treatments. However, these new insights have permitted the identification of new promising targets for precision medicine approaches. Further investigations and prospective trials are mandatory in order to define this new paradigm of treatment. The hope is that these new knowledge in cooperation with the new technologies and procedures including biomolecular and genomic analysis as well radiomic studies, will enrich the therapeutic armamentarium thus improving the survival outcomes in a such lethal and complex disease.

EXPERT OPINION:

The therapeutic chances for patients by BTC are scarce, and the only standardized treatment for advances stages is cisplatin-based chemotherapy. Nevertheless, the recent advent of new technologies and new techniques of genomic and molecular analysis have permitted to deeper the knowledge about the molecular, genomic and epigenetic alterations which underly the carcinogenesis in such a complex disease. All these new insights have made clear that the BTC could not be considered as a single entity, but, contrarily, includes a number of malignancies s with different genomic profile and, consequently, different behavior and response to specified treatments. The big challenge of the last years was to find the driven mutations to target with new therapeutic approaches. In the precision medicine optic, some molecular aberrations have been reported as of special clinical interest, and clinical trials have been conducted to evaluate the clinical impact on populations previously stratified for such alterations. FGFR2 fusions and IDH1 aberrations were the first targeted genomic alterations of clinical interest in BTC setting by reporting promising results from early clinical trials with small molecules inhibiting FGFR2 and IDH1, respectively.

Beyond the results showed by target therapies against the FGFR2 and IDH1 aberrations, many others potential targets are currently under investigation, including BRCA and BRAF mutations, NTRK fusion and HER2 amplifications. The pending results will give us several important information about the clinical impact of these driven mutations. Other therapeutic strategies, including immunotherapy and antiangiogenic treatments, have been recently investigated in several trials: unfortunately, the results obtained are contradictory, thus making difficult the definition of the role of immune checkpoint inhibitors and antiangiogenic treatments in BTC nowadays. On the other hand, a number of preclinical trials has highlighted a strong rationale in combining immunotherapy and antiangiogenic therapies, thus opening a new chapter of investigations which are currently ongoing.

In the last years another technologic advance has shown to be interesting in many cancer fields, including BTC: the radiomics. Through the texture analysis made on radiological imaging, the radiomics permits the extraction of a huge amount of information, including biomolecular and genomic ones, which could be integrated with the information obtained with the analysis on tissue and/or circular ctDNA. In the BTC field, some promising data have been reported, mainly in genomic profiling and predicting response to specified treatments like antiangiogenic compounds. In the era

of the precision medicine, the hope is that machine learning could be integrated as a new noninvasive tool in the diagnostic and therapeutic flow chart of many malignancies, including BTC. Further investigation on the diagnostic, prognostic and predictive role of radiomics in BTC are deserved.

Through international collaborations, BTC is no longer considered “too rare” for powered clinical trials, and prospective validations of all the recent discovers are an urgent need in this setting. The hope is that all these new finding, including the knowledge about molecular and genomic aberrations, combined with new technologies like radiomics, could led to a deep comprehension of the BTC, thus helping in improve therapeutic strategies and, consequently, the survival outcomes of patients.

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LEGEND

FIGURE

Figure 1A: Biliary adenofibroma (Haematoxylin-eosin staining, 10x)

Figure 1B: iCCA small duct type with mass forming type pattern of growth (Haematoxylin-eosin staining, 20x)

Figure 1C: iCCA large duct type with periductal infiltrating pattern of growth (Haematoxylin-eosin staining, 20x)

Figure 1D: Combined HCC-iCCA (Haematoxylin-eosin staining, 20x)

Figure 1E: Perihilar extrahepatic bile duct cancer with periductal infiltrating pattern and local aggressiveness (Haematoxylin-eosin staining, 20x)

Figure 1F: Biliary intraepithelial neoplasm, high grade (Haematoxylin-eosin staining, 20x)

Figure 1G: Intraductal papillary neoplasm of the bile ducts, high grade (Haematoxylin-eosin staining, 20x)

Figure 1H: Mucinous cystic neoplasm of the liver (Haematoxylin-eosin staining, 20x)

Figure 1I: Carcinosarcoma of the liver (Haematoxylin-eosin staining, 20x)

TABLE

Table 1: immunotherapy studies in BTC.

Table 2: Combination-therapy studies in BTC.