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The immune milieu of cholangiocarcinoma: From molecular pathogenesis to precision medicine

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ABSTRACT

Cholangiocarcinoma (CCA) is a deadly cancer of the biliary epithelium with limited therapeutic options. It is a heterogeneous group of cancer that could develop at any level from the biliary tree and is currently classified into intrahepatic, perihilar and distal based on its anatomical location. With incidence and mortality rates currently increasing, it is now the second most common type of primary liver cancer and represents up to 3% of all gastrointestinal malignancies. High-throughput genomics and epigenomics have greatly increased our understanding of CCA underlying biology, however its pathogenesis remains largely unknown. CCA is characterized by a highly desmoplastic microenvironment containing stromal cells, mainly cancer-associated fibroblasts, infiltrating tumor epithelium. Tumor microenvironment in CCA is a highly dynamic environment that, besides stromal and endothelial cells, encompass also an abundance of immune cells, of both the innate and adaptive immune system (including tumor-associated macrophages, neutrophils, natural killer cells, and T and B lymphocytes) and abundant proliferative factors. It is orchestrated by multiple soluble factors and signals, that eventually define a tumor growth-permissive microenvironment. Through complicate interactions with CCA cells, tumor microenvironment profoundly affects the proliferative and invasive abilities of epithelial cancer cells and plays an important role in accelerating neovascularization and preventing apoptosis of neoplastic cells. In this review, we discuss recent developments regarding the characteristics of the tumor microenvironment, the role of each cellular population, and their multiarticulate interaction with the malignant population. Further we discuss innovative treatment approaches, including immunotherapy, and how identification of CCA secreted factors by both the stromal component and immune cell subsets are leading towards a precision medicine in CCA.

1. Introduction

Cholangiocarcinoma (CCA) is a heterogeneous group of cancer that arises from epithelial cells lining the bile duct. With incidence and mortality rates currently increasing, it is now the second most common type of primary liver cancer and represents up to 3% of all gastrointestinal malignancies [1]. Based on the anatomical location, biliary tree cancers (BTC) are classified into intrahepatic CCA (iCCA), perihilar CCA (pCCA), distal CCA (dCCA), and gallbladder cancer (GBC), the most frequent tumor originating from the extrahepatic biliary tract [2].

CCA is commonly asymptomatic in early stages and no clinical

Abbreviations: Cholangiocarcinoma, (CCA); intrahepatic, (iCCA); perihilar, (pCCA); distal, (dCCA); and gallbladder cancer, (GBC); biliary tree cancers, (BTC); cancer-associated fibroblasts, (CAFs); tumor-associated macrophages, (TAMs); cytotoxic T-lymphocyte antigen-4, (CTLA-4); primary sclerosing cholangitis, (PSC); C-X-C motif chemokine ligand, (CXCL); fibroblast growth factor, (FGF); hepatocyte growth factor, (HGF); granulocyte-macrophage colony-stimulating factor, (GM-CSF); and insulin-like growth factor, (IGF); natural killer cells, (NK cells); cyclooxygenase, (COX); inducible nitric oxide synthase, (iNOS); tumor-associated neutrophils, (TANs); Tumor-infiltrating lymphocytes, (TILs); dendritic cells, (DCs); biliary tree cancers, (BTC); Hazard Ratio, (HR); confidence interval, (CI); Wilms' tumor gene-1 antigen, (WT1); Mucin-1, (MUC-1); adoptive cell therapy, (ACT); programmed death-1, (PD-1); programmed death-1 ligand, (PD-L1); hepatocellular carcinoma, (HCC); tumor mutational burden, (TMB); mucin domain-containing 3, (TIM3); indoleamine 2,3-dioxygenase, (IDO); lymphocyte activation gene 3, (LAG3); combined positive score, (CPS); anti-vascular endothelial growth factor receptor 2, (VEGFR-2); mismatch repair, (MMR)

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molecular markers for early diagnosis are known. Despite improvements in treatment and diagnosis, it is frequently diagnosed in advanced phases when the disease is found disseminated. Late diagnosis highly compromises surgery, the only potentially curative option. CCA recurrence risk is elevated and even among the 10–30% patients eligible for resection at diagnosis, 50% recur within the first 12 months. Further, effective targeted therapies are yet to be approved [3]. Thus, there is an imperative need for better understanding of CCA characteristics and pathogenesis, and eventually for effective therapeutic strategies based on precision medicine.

Accumulating evidence suggests that CCA phenotype is dictated not only by genetic and epigenetic alterations in the cancer cells per se, but also influenced by molecular cross-talk between neoplastic cells and the surrounding microenvironment. Tumor microenvironment is a complex, multicellular functional compartment that may profoundly impact the growth of the tumor mass. The development of a highly reactive microenvironment is a functional hallmark of many epithelial cancers, including CCA.

We herein aim to present a complete review of the current understanding of the immune milieu of CCA and the tumor biology. A thorough understanding of the tumor microenvironment will shed some light on the development of new therapeutic methods and personalized medicine for treating CCA.

2. Genetics and epigenetics of cholangiocarcinoma

Recent omics–based studies (including transcriptomics [4,5], whole-exome sequencing [6,7], and epigenomics [8]) have greatly helped to unravel the genetic landscape of CCA, and to increase our knowledge of its underlying biology. Although our understanding of the genetic mechanisms involved in CCA development remains inadequate, these studies provide promising treatment options for CCA patients (Table 1).

2.1. Genomic heterogeneity of cholangiocarcinoma

Both the anatomical location of the tumor and various risk factors have been related to the genomic heterogeneity of CCA. The majority of CCAs showed a driver gene mutation, although CCA from different anatomical location had different genetic profiles, with RAS mutations being prevalent in the dCCA [9]. The most common genetic variants in CCA involve key networks such as DNA repair (TP53) [7,10] the WNT–CTNNB1 pathway [11], tyrosine kinase signaling (KRAS, BRAF, SMAD4 and FGFR2) [6,7,12], and protein tyrosine phosphatase (PTPN3) [13]. Further, epigenetic factors and pathways are largely involved, and Notch signaling, key component in cholangiocyte differentiation and biliary duct development, is deregulated.

Whole-genome analyses of CCA have provided additional information. Two distinct genomic classes of iCCA have been identified: an inflammatory class with predominant activation of inflammatory pathways, and a proliferation class with predominant activation of oncogenes that correlate with worse patient outcome [14]. In 2015 Nakamura and colleagues performed a comprehensive whole-exome and transcriptome sequencing of 260 CCAs from Japanese patients, including 145 iCCA, 86 extrahepatic CCA (pCCA and dCCAs were grouped together), and 29 cases of GBC [6]. Importantly, 40% of the included cases had genetic aberrations. While FGFR gene fusions were found exclusively in iCCA, PKA gene fusions were more common in extrahepatic CCA, and EGFR or ERBB3 mutations in GBC. Importantly, significant enrichment of hypermutated tumors was associated to a worse prognosis and a characteristic elevation in the expression of immune checkpoint molecules and antiapoptotic signatures, suggesting an important role of the neoplastic immune environment [6].

Recently, a stratification of iCCA patients based on occurrence of the three most frequently mutated genes (IDH, KRAS, TP53) was performed. The study revealed unique oncogenic programs that influence pharmacologic response and, most importantly, highlighted the potential of individual mutations to induce extensive molecular heterogeneity and response to treatment [15]. Taken together, up to 70% of iCCA patients harbor at least one actionable molecular alteration (FGFR2 fusions, IDH1/2, ARAF, KRAS, BRAF and FGF19) that is suitable for therapeutic targeting [10]. While no targeted therapy has been approved to date, FGFR2 inhibitors may be the first to transform iCCA clinical management, displaying prospective efficacy in fusion-positive

Table 1
Genetic variants and current targeted therapy clinical trials in cholangiocarcinoma Modified from Ref. [112].

Molecular target	Prevalence in CCA	Gene significance	Agent	ClinicalTrial.gov reference
FGFR2	10–16% iCCA	cell proliferation, differentiation, and angiogenesis.	Derazantinib	NCT03230318
			TAS 120	NCT02052778
			Debio 1347	NCT01948297
			INCB054828	NCT02924376
			Ponatinib	NCT02265341
			NVP-BGJ398	NCT02150967
			Erdafitinib	NCT02699606
			INCB054828	NCT02924376
ROS1 rearrangements	8–9%	Proto-oncogene encoding a membrane protein with tyrosine kinase activity	Ceritinib	NCT02374489
			Crizotinib	NCT02638909
				NCT02034981
IDH1/IDH2 mutations	25% iCCA	Metabolic enzymes involved in oxidative stress, glutathione metabolism, and citric acid cycle;	Ivosidenib	NCT02989857
			BAY 1436032	NCT02746081
			Olaparib	NCT03212274
EGFR overexpression	16% iCCA	Tyrosine kinase receptor for EGF regulating cell proliferation, cell-cell interactions, and cell morphogenesis	NCT03093870	NCT03093870
HER2 amplification	11–20% pCCA/ dCCA	Tyrosine kinase receptor belonging to the EGF receptor family	Trastuzumab	NCT02836847
TP53 mutation	27% iCCA 40% pCCA/dCCA	Tumor suppressor gene involved in cell cycle arrest, cell apoptosis, cell senescence, and DNA repair		
KRAS mutations	22% iCCA 42% pCCA/dCCA	Proto-oncogene encoding for a protein belonging to the small GTPase family		
BRAF mutation	1–5%	Proto-oncogene with serine/threonine kinase functions, regulating cell proliferation, secretion, and differentiation	Dabrafenib, trametinib	NCT02034110
PIK3CA mutation	4–9%	Catalytic subunit of the phosphoinositide 3-kinase		
PTEN mutation	4%	Tumor suppressor gene preventing cell proliferation		
CDKN2A mutation	47% iCCA	Tumor suppressor gene inhibiting cell cycle and proliferation		
MET amplification	2% iCCA	Proto-oncogene member of the tyrosine kinase receptor family		

patients [16].

2.2. Epigenetics of cholangiocarcinoma

Epigenetic dysregulation, including DNA methylation and histone acetylation, has been implicated in the pathogenesis of a number of tumors, including CCA. The epigenetic landscape of CCA is deregulated both during carcinogenesis and disease progression. Gene alterations in epigenetic (both IDH1 and IDH2) [10,17] and chromatin-remodeling factors (ARID1A, BAP1, PBRM1, SMARCB1) have been reported in one third of CCA cases [9].

Further, epigenetic changes in the genes linked to signaling pathways have also been implicated in CCA. For instance, the promoter of SOCS3, the upstream regulator of JAK/STAT cytokine signaling, is frequently hypermethylated in CCA determining a sustained IL-6/STAT-3 signaling and enhanced Mcl-1 expression in CCA [18]. Similarly, SFRP1, Wnt signaling modulator, has been found to be hypermethylated in CCA in almost 85% of cases [19].

3. Cholangiocarcinoma and inflammation

It is well recognized that CCA often arises in the context of biliary inflammation; indeed, well recognized risk factors for CCA include persistent biliary inflammation from cholestatic injury, such as primary sclerosing cholangitis (PSC), liver flukes, or chronic viral infections and liver cirrhosis. In a chronically inflamed environment, cholangiocytes are constantly subjected to proliferative stimuli that are responsible for initiating and perpetuating the tumorigenesis [14,20,21]. As previously mentioned, molecular analysis of iCCA identified two different biological subtypes of the tumor, an inflammatory class, and a proliferation class [14]. The inflammatory iCCA is characterized by activation of inflammatory pathways and overexpression of different cytokines. Importantly, high levels of IL-6 confer a significant increased risk (above 100-fold) for CCA development in *Opisthorchis viverrini* infected subjects [22]. Further, IL-6, constitutively secreted by neoplastic cholangiocytes, has crucial stimulatory effects on cholangiocyte growth [23].

All things considered, these data suggest a key role of inflammation in the development of CCA. However, our knowledge of the microenvironment and crosstalk between the immune system and neoplastic cholangiocytes is still limited.

4. The tumor microenvironment and immune milieu of cca

CCA is characterized by a rich desmoplastic stroma [24], with an inflammatory and immune cell-controlled microenvironment composed primarily of cancer-associated fibroblasts (CAFs) and a lesser proportion of tumor-associated macrophages (TAMs) and other immune cell subsets. The CCA specific microenvironment has a key role in the regulation of its growth, angiogenesis, invasion, and metastasis through reciprocal interactions with malignant cells. Indeed, immune cells release a wide spectrum of chemokines and growth factors that stimulate cancer growth, invasion, and recruitment of macrophages and T lymphocytes (Fig. 1).

4.1. The emerging role of cancer associated fibroblasts in cholangiocarcinoma

The presence of an abundant stroma is a histological hallmark of CCA and clinically relevant genomic alterations in the stroma of CCA have been identified, supporting the idea that tumor stroma is an important factor for CCA onset and progression [25]. CAFs are activated myofibroblasts that represent a major cellular population of the desmoplastic stroma of CCA. CAFs may potentially derive from quiescent hepatic stellate cells, portal fibroblasts, bone marrow-derived fibroblasts, as well as cholangiocyte derived fibroblasts via epithelial mesenchymal transition; however, the sources of CAFs in CCA remains largely unknown and are probably multiple [26].

A number of soluble mediators have been reported to be responsible for the persistent activation of CAFs in CCA, including MCP-1, C-X-C motif chemokine ligand (CXCL)-12, CXCL-14, PDGF, TGF-β, fibroblast growth factor (FGF)-1/2, hepatocyte growth factor (HGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and insulin-like growth factor (IGF)-1 [26]. Activated CAFs are capable to stimulate tumor overgrowth by secreting several factors. Accordingly, patients with high levels of CAFs in CCA tissue samples have worse prognosis [27].

Finally, through the secretion of a vast number of cytokines, chemokines (MCP-1, SDF-1, CXCL-14), and growth factors (VEGF, FGF), CAFs also contribute to recruit inflammatory cells, and even endothelial cells, thus providing stimuli to maintain the tumor microenvironment, neoangiogenesis and lymphangiogenesis [28].

4.2. The role of the innate immune system in cholangiocarcinoma

The CCA microenvironment is characterized by the presence of innate immune cells, including macrophages, neutrophils and natural killer cells (NK cells), that significantly impact cholangiocarcinogenesis. TAMs are the most relevant infiltrating immune cell population within the tumor microenvironment and high tissue macrophage density correlates with poor prognosis of patients with CCA [29,30].

TAMs are mainly a subtype of M2 macrophages (alternatively activated macrophages) with particular powerful anti-inflammatory action, thus resulting in tumor progression [31]. Indeed, neoplastic cholangiocytes induce macrophage polarization toward the M2 phenotype via the STAT-3 pathway [32]. Importantly, TAMs associated with the cancer stem cell niche display unique features, including expression of both M1 and M2 phenotypic traits, increased adhesive and invasive capabilities, *in vitro*, and enhanced tumor-promoting activities, *in vivo* [33]. This has supported the conviction that different TAM subsets are present within the tumor, reflecting different hints derived from various cell niches.

TAMs, through their crosstalk with CCA cells are able to accelerate tumor progression on multiple levels by releasing a variety of inflammatory, growth, and proliferative factors [11,34,35]. They support neoangiogenesis via the secretion of pro-angiogenic (e.g. VEGF-A, angiopoietin, IL8) and pro-inflammatory mediators, such as cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS), supporting tumor growth [36]. Further, macrophage-derived Wnt ligands, activate canonical Wnt pathway, contributing to CCA cell proliferation [11,35].

Although the role of neutrophils in the CCA microenvironment is still largely unknown, some evidence suggests a key involvement in CCA development and infiltration of tumor-associated neutrophils (TANs) has also been associated with poor prognosis in CCA [37,38]. Moreover, neutrophil gelatinase-associated lipocalin expression in bile has been propose as a valid biomarker to discern malignant from benign biliary strictures [39].

NK cells are innate lymphocytes with natural cytotoxicity, recognizing and lysing malignant and virally infected cells without prior sensitization [40]. This recognition is regulated by a plethora of activating and inhibitory immune receptors expressed on the surface of NK cells [40]. The liver is enriched in NK cells and they represent up to 30–40% of all liver lymphocytes [41]. Nevertheless, little is known regarding NK cells in CCA. *In vitro*, activated NK cells have been shown to enhance the cytotoxic efficacy of cetuximab against human CCA cell lines [42]; *in vivo* Jung and colleagues recently showed that the infusions of ex vivo-expanded human NK cells (SMT01) in HuCCT-1 tumor-bearing nude mice resulted in significant inhibition of CCA growth [43]. Immunotherapy with infusion of activated allogeneic NK cells has been proposed as an innovative immune-therapy against various types of cancer with promising outcomes [44,45]. However, it is still necessary to determine the pathogenetic role of NK cells in CCA development and progression before NK manipulation can be proposed as a

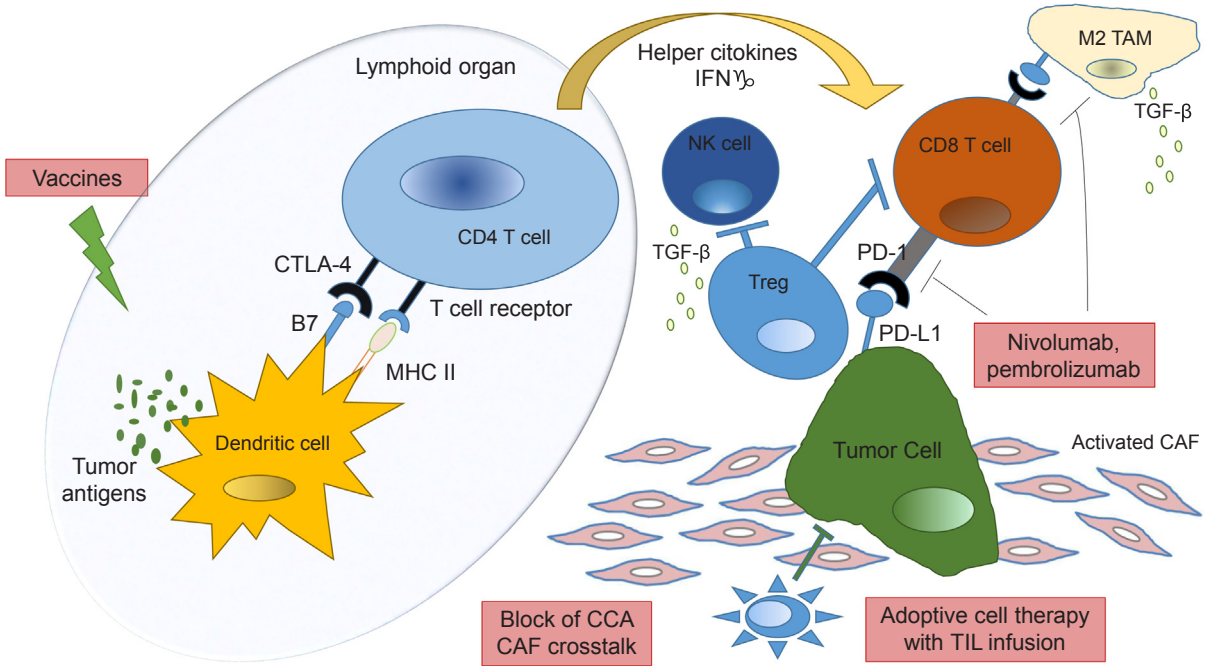

Fig. 1. The mechanisms of action behind current immunotherapy approaches in CCA.

therapeutic option in CCA.

4.3. The role of adaptive immune system in cholangiocarcinoma

Convincing evidence support the ability of the adaptive immune system to identify and target arising tumor cells, and therefore, to act as a primary defense against cancer [46]. Tumor-infiltrating lymphocytes (TILs) are present in many solid tumors and form highly heterogeneous populations [47].

B lymphocytes, $CD8^+$ cytotoxic T lymphocytes, $CD4^+$ T helper lymphocytes, and Tregs have been studied in the CCA microenvironment. Moreover, dendritic cells (DCs) are abundant in the tumor microenvironment, and act as an important linkage between adaptive and innate immune responses shufflering antigen towards the draining lymph node for immune activation [48].

Overall, $CD4^+$ TILs prevail in the peritumoral region [49], while $CD8^+$ TILs are mostly prevalent in the intratumoral CCA tissue [49,50]. Following the release of CCL2 that is produced by neoplastic cells, TAMs, and CAFs cytotoxic T cells acquire CD4/CD25 expression and become Treg [51]. Within tumors, Tregs secrete TGF-β and IL10, which leads to an immunosuppressive environment. Further, Tregs also bind to IL2, making this cytokine unavailable in the tumor microenvironment and therefore preventing the activation of additional immune cells [52].

Multiple studies confirm that high levels of $CD4^+$ and $CD8^+$ within CCA microenvironment are associated with better prognosis [50,53–56], whereas low numbers of $CD8^+$ TILs are associated with poor overall survival [57]. In accordance with these findings, in CCA, the total amount of TILs has been demonstrated to decrease in invasive and metastatic tumors compared with non-invasive precursors [50], suggesting a gradually establishing immune escape of the tumor.

DCs might also influence the number of $CD4^+$ and $CD8^+$ TILs in CCA. In fact, it has been demonstrated that the number of DCs at the invasive margin correlated with the number of $CD4^+$ and $CD8^+$ TILs within the tumor core. Immature $CD1a^+$ DCs reside only in the tumor, while mature $CD83^+$ DCs are found predominantly at the invasive front [56]. Furthermore, patients classified with an advanced tumor stage showed significantly lower numbers of DCs.

Although several studies have investigated T cells in CCA, the role of B lymphocytes is still largely unknown. B cells have been identified in TIL populations in CCA, and high densities of $CD20^+$ cells have been observed in low-grade tumors and associate with a favorable overall survival [49,50]. However, no data regarding their pathogenic role in CCA are available and future studied are needed to clarify their relevance.

4.4. Immune escape in cholangiocarcinoma

FoxP3, a distinctive feature of Tregs, is overexpressed also by CCA cells and correlates with lymphatic metastasis and poor survival [57,58]. Indeed, knockdown of FoxP3 in CCA cells *in vitro* reduced proliferation and invasiveness, inhibited T cell survival, and reduced IL10 and TGF-β signaling in the tumor microenvironment [58]. Furthermore, FoxP3 overexpression is accompanied by cytotoxic T-lymphocyte antigen-4 (CTLA-4) overexpression [59]. CTLA-4 is expressed on the surface of Tregs and binding CD80 on antigen-presenting cells determines inhibitory effects on cytotoxic cells [60]. The expression of CTLA4 in CCA has prognostic value highlighting the concept that immune escape in CCA leads to poor prognosis [59].

In order to escape immune surveillance, cancer cells frequently manipulate immune checkpoints such as programmed death-1 (PD-1) and CTLA-4, that promote peripheral T cell exhaustion once activated by specific ligands (PD-L1 and CD152, respectively). Both PD-L1 and PD-1 are up-regulated in tumor cells [61–63], and their overexpression is associated with increased invasiveness, and poor outcome, especially when accompanied by low $CD3^+$ or $CD8^+$ infiltrate [55,59,61,64]. High expression of PD-L1 was demonstrated in a subset of CCA patients (5.9%) with high mutational load and poor prognosis [6]. On the contrary, low PD-L1 expression was found to be related to favorable prognosis [65]. Importantly, the PD-L1/PD-1 pathway is considered to be responsible, to some extent, for CCA progression and account for an increased malignant potential.

Notch signaling, an important pathway in the liver, is a signaling mechanism associated with iCCA [66,67]. Among divers functions, it can also modulate the immune cell regulation determining activation of Th1 [68] and Tregs [69]. Further, Notch may contribute to M1 macrophages polarization and to their relationships with CAFs. Notch is also involved in T cell induction and stimulation of effector T cell. It is

therefore hypothesized a crucial role of Notch for directing T cell infiltrates in CCA [68,69].

5. Medical treatment for cholangiocarcinoma

5.1. Overview on medical treatment of advanced cholangiocarcinoma

Gemcitabine and platinum agents are among the most active drugs in advanced biliary tree cancers (BTC). Almost 20 years after the first report on the role of palliative chemotherapy in advanced BTC [70], the phase 3 ABC-02 trial demonstrated a benefit from cisplatin plus gemcitabine over gemcitabine alone (11.7 vs. 8.1 months; Hazard Ratio (HR), 0.64; 95% confidence intervals (CI), 0.52–0.80; $P < 0.001$) [71]. Also, these findings were confirmed in a Japanese randomized phase 2 study [72] and in a subsequent meta-analysis involving the two studies [73]. Given these premises, for first-line treatment of BTC, cisplatin added to gemcitabine currently represents the reference regimen, and a benchmark for ongoing first-line trials. However, in the ABC-02 trial, a deeper analysis shows that the 95% CI of the HR for risk of death cross the unit when smaller subgroups, such as pCCA, are considered [71].

In the adjuvant setting, according to the results of the BilCap study still reported in abstract form, capecitabine has demonstrated efficacy after surgical resection for cholangiocarcinoma or gallbladder cancer. In the intent-to-treat population, the median overall survival (OS) was 51 months in the capecitabine arm and 36 months in the observation arm, nonetheless these results were not statistically significant (HR 0.81; 95% CI 0.63–1.04; $P = 0.097$) [74]. Additionally, in a phase 3 French trial, after R0 or R1 resections, adjuvant gemcitabine and oxaliplatin did not significantly improve disease-free survival (HR 0.83; 95% CI 0.58–1.19; $P = 0.31$) when compared with observation alone [75]. As such, the role of adjuvant chemotherapy after surgery in BTC still needs to be fully elucidated.

Of interest, most chemotherapeutic agents, including gemcitabine, have been developed *in vitro* with cancer cell lines harvested in Petri dishes, or *in vivo* by adopting immunodeficient mice models. However, similar approaches, even in clinical studies, have precluded a deeper appraisal of innate as well as treatment-induced immune responses. Although these aspects have been widely overlooked, several lines of evidence suggest that gemcitabine lowers the amount of circulating myeloid-derived suppressor cells [76] and promotes a switch of tumor-associated macrophages towards antitumor phenotypes [77]. Besides these direct immunostimulatory effects, gemcitabine has been reported to upregulate the expression of HLA class I molecules by cancer cells [78], resulting in a more pronounced T-cell activity. Whereas neither oxaliplatin nor cyclophosphamide seem to produce similar effects, it remains to be determined whether tumor-targeting immune responses could be primed in patients undergoing gemcitabine-based chemotherapy.

While robust evidences support current use of cisplatin and gemcitabine as first-line treatment in BTC, no individual agents or combination regimens have been identified as standard of care in the context of second-line treatments.

At the same time, the use of whole genome sequencing has underscored the substantial molecular heterogeneity underlying the broad definition of BTC. As previously mentioned, specific genetic alterations occur in nearly 40% of patients with BTC and the repertoire observed seem to differentiate each CCA subtype [6]. Whereas several targetable driver alterations have been found and several clinical trials are underway [79], this topic is beyond the scope of the present review.

5.2. Cancer vaccines

Cancer-associated antigens produced by cancer cells may elicit the host immune response through antigen-presenting cells. In theory, these are the ideal targets for cancer immunotherapy, since they are not expressed in non-tumoral tissues, thereby allowing a mature T-cell repertoire to recognize them as neo-antigens. However, candidate epitopes detected on their ability to bind to a specific MHC molecule may not always be processed on cancer cells. In fact, derangements in HLA class I and class II antigen expression are among those escape mechanisms observed in cancer to escape host immune surveillance. From a prognostic standpoint, iCCA with downregulation of HLA class I antigen expression present with more advanced disease than tumors with high HLA class I antigen expression [65]. The underlying mechanisms leading to downregulation of HLA class I antigen expression in iCCA are unknown, even if epigenetic events could be involved.

Historically, two such cancer-associated antigens have been targeted for vaccine therapy, namely Wilms' tumor gene-1 antigen (WT1) and Mucin-1 (MUC-1). In a Japanese phase I trial enrolling 16 patients with advanced BTC, the median OS observed with a WT1 vaccine plus gemcitabine was 9.5 months [80]. Although the primary endpoint of this study was the assessment of toxicity, safety, and optimal immunologic dose, the WT1 vaccine plus gemcitabine did not seem to lead to a meaningful survival improvement.

MUC-1 is a glycoprotein expressed in up to 85% of iCCA, and it is associated with a negative prognostic impact [81]. Small-scale studies with MUC1 peptide vaccination have accrued mixed cohorts of patients with BTC and pancreatic cancers, either in the advanced [82] or adjuvant setting [83]. Vaccine consisted of MUC-1 peptides and incomplete Freund's adjuvant [82] or dendritic cells loaded with MUC-1 [83]. Despite a satisfactory safety profile, the survival data do not allow to conclude on the efficacy of such strategies.

In general, the unique set of mutations that characterize every patient's tumor is a major hurdle that hampers the development of a vaccine targeting cancer-associated antigens. This should prompt the development of more personalized approaches based on identification of the antigens from an individual tumor prior to vaccine therapy.

The efficacy of a personalized vaccine containing seven non-mutated tumor-associated epitopes, previously shown to be immunogenic, was recently reported in a patient with advanced iCCA repeatedly treated with surgery for recurrent disease. The vaccine contained four HLA-A03 restricted short peptides and three promiscuous HLA-DR long peptides [84]. Interestingly, in this case report, 41 months after the first vaccination, follow-up imaging did not show any evidence of tumor recurrence, neither at intra- nor extrahepatic sites. T-cell responses and CD3-positive cell infiltration in tumor tissues (surgically removed after the beginning of the vaccination program) confirm the efficacy of an antitumor surveillance induced by the vaccine, and underscore the role of this strategy to prevent the dissemination of further metastases [84].

Also, personalized approaches targeting tumor neoantigens have been successfully pursued in high-risk melanoma patients after surgical resection in the frame of a phase 1 trial [85].

5.3. Adoptive cell therapy

TILs that recognize specific mutations of an individual tumor can be extracted from a tumor biopsy or peripheral blood. In addition, they can be tested for antitumor activity and expanded to very large numbers *ex vivo*, before they are reinfused into cancer patients.

Historically, studies performed in melanoma patients have supported the most promising evidences on cell transfer therapy as a tool to induce disease regressions in a context of advanced metastatic cancer [86].

In BTC, the experiences with adoptive cell therapy (ACT) are limited to case reports or small case series of patients treated in single-arm phase II studies. Tran and colleagues reported on a patient with metastatic cholangiocarcinoma treated with TILs and co-cultured antigen-presenting cells that were transfected with an *in vitro* transcribed RNA generated from tandem minigene products that encode mutated antigens. Following the adoptive transfer of TILs containing $CD4^+$ erbb2 interacting protein (ERBB2IP) mutation reactive T cells, interleukin-2 was administered to increase T-cell proliferation and function. Seven

months after initial treatment, metastatic sites responded to treatment and a 30% reduction in size was observed. Disease stabilization took place for 13 months, then lung progressive disease was detected [87]. Indeed, an important message from this study was that endogenous TH1 responses can be mounted against specific mutations detected in epithelial cancers. Given that the low frequencies of tumor-reactive T cells are thought to be an obstacle to the development of immunotherapies in gastro-intestinal cancers [88], results gained by Tran et al. overall suggest that an effective ACT can take place with the expansion of highly enriched mutation-reactive T cells.

In an adjuvant setting, an autologous dendritic cell vaccine given in combination with ACT was tested after surgery for iCCA. Among 36 patients that were treated with adjuvant adoptive transfer of T cells plus dendritic cell vaccine an OS of 31.9 months was detected and favorably compared with 17.4 months in the surgery only group of 26 patients ($P = 0.022$) [89].

5.4. Immune checkpoint inhibitors

Immune checkpoints are a class of receptors that include, among the others, PD-1, PD-L1, and CTLA-4. The physiological role of these pathways is to prevent excessive inflammation, although they can be hijacked by tumor cells to establish a condition of immune evasion. Although the exact mechanisms underlying the immune escape of BTC are still to be elucidated, there is strong evidence that the PD-1/PD-L1 pathway plays a crucial role in the development of a tolerant tumor microenvironment. PD-L1 expressed on tumor cells, antigen presenting cells and stroma interacts with PD-1 on T cells, and their binding initiates T cell exhaustion [90].

Therefore, these molecules represent attractive immunotherapeutic targets. The development of monoclonal antibodies that block the binding between the receptors and their ligands, commonly called immune checkpoint inhibitors, has paved the way to the approval and wide introduction of these agents for the treatment of several tumor types, such as melanoma, Hodgkin lymphoma, non-small cell lung cancer, urothelial carcinoma, renal cell carcinoma, Merkel cell carcinoma, squamous cell carcinoma [91]. Ongoing studies of checkpoint inhibitors, as single agents or in combination, will hopefully allow to identify novel indications, improve antitumor activity, treatment selection and sequencing, and address therapy resistance.

In BTCs, the efficacy and the potential complications associated with immunotherapy may depend on clinical and epidemiological factors. Conditions such as liver-fluke disease, viral hepatitis B and C, and bacterial pyogenic cholangitis, as well as autoimmune diseases, such as PSC and inflammatory bowel disease, are included amongst the best-established risk factors for BTC. Notably, there is evidence that in other cancer types associated with viral infections, such as head-and-neck cancer, Hodgkin lymphoma, Merkel cell carcinoma, and hepatocellular carcinoma (HCC), immune checkpoint inhibitors have shown interesting clinical activity, and that this relationship could be mediated, at least in part, by the presentation of non-self or neoantigens [92,93]. Of note, immune checkpoint inhibitors have proven safe in patients with HCC and underlying chronic infections, whereas clinical trials of immunotherapy have systematically excluded patients with underlying autoimmune diseases.

Importantly, high tumor mutational burden (TMB), overexpression of tumor-specific neoantigens and immune-related genes are associated with a worse prognosis and these patients with BTC may potentially benefit from treatment with checkpoint inhibitors [94]. Also, preliminary data have shown upregulation of the PD-1/PD-L1 pathway in BTCs and a correlation between this upregulation and worse clinical outcomes, making targeting checkpoint pathways a potential novel therapeutic strategy for patients with BTC. Nakamura et al. showed that in 45% of BTCs there is an increase in the expression of immune checkpoint molecules, and that patients with hypermutated tumors and overexpression of CTLA-4 and PD-L1 have the worst prognosis [6]. Gani et al. observed PD-L1 expression on tumor cells in 72% of tumor samples of resected iCCA, and this expression was associated with 60% reduction in OS compared to negative tumors [95]. Sabbatino et al. reported PD-1 and PD-L1 expression in 100% of 27 resected iCCA samples with evidence of antitumor T-cell mediated immune response. Interestingly enough, PD-1 was expressed on tumor-infiltrating lymphocytes and not on tumor cells, while in 8 samples (30%) PD-L1 was expressed in tumor cells in addition to tumor-infiltrating leukocytes and macrophages [65]. Inhibition of additional receptors such as T-cell immunoglobulin and mucin domain-containing 3 (TIM3), indoleamine 2,3-dioxygenase (IDO), and lymphocyte activation gene 3 (LAG3) is being assessed in clinical trials, however, there is still a lack of data in BTC [96].

Finally, BTCs are surrounded by stromal cells including cancer-associated fibroblasts, endothelial cells, and TAMs, producing interleukins, growth factors, and cytokines, that can promote tumor-cell growth and invasiveness, and modulate anticancer immune responses. Therefore, targeting these cells, also in combination with checkpoint inhibition, can be an attractive therapeutic approach for patients with BTC [97,98].

5.4.1. Preliminary clinical data

Clinical data of immune checkpoint inhibitors in BTC are limited and come from basket/multicohort trials, mostly reported in abstract form. The KEYNOTE-028 trial (NCT02054806) assessed the safety and efficacy of pembrolizumab (*anti*-PD-1) at the dose of 10 mg/kg IV every two weeks in patients with several cancer types, including a small cohort of patients with PD-L1-positive BTC. PD-L1 positivity was defined as staining in ≥1% of tumor cells or PD-L1-positive bands in stroma, assessed at a central lab by immunohistochemistry. Eighty-nine patients with BTC were screened, 37 patients (42%) had PD-L1-positive tumors, and 24 patients (20 with CCA and four with GBC) received treatment. Thirty-eight percent had received more than three previous lines of therapy. Overall response rate (ORR) was 17% (three patients with CCA and one patient with GBC), duration of response ranged from 5.4 + to 9.3 + months, four patients (17%) had stable disease, and treatment duration was longer than 40 weeks. Median progression-free survival (PFS) was not reached. Grade 3 adverse events (AEs) were observed in 17% of patients. No grade ≥4 AEs or relevant liver toxicity were reported. No information about the mismatch repair (MMR) proteins or microsatellite status was reported [99].

Based on these results, 100 patients with BTC, progressing on or intolerant to standard therapy, were enrolled in the KEYNOTE-158 trial (NCT02628067). Pembrolizumab was administered at the dose of 200 mg IV every three weeks. Primary endpoint was ORR according to RECIST 1.1 [100], secondary endpoints were duration response, safety, PFS, OS. PD-L1 positivity was defined as a combined positive score (CPS) ≥1 (number of PD-L1-positive cells, including tumor cells, lymphocytes and macrophages, in relation to total tumor cells). Partial responses were observed in six of 104 enrolled patients (ORR 6%), four in 61 PD-L1-positive patients (7%), and one in 34 PD-L1-negative patients (3%). Nine patients were not evaluable for PD-L1 expression. Median duration of response was not reached (range 6.2–15.7 + months), two patients had a duration of response ≥15 months. Overall, 17 patients (16%) had stable disease, six patients (10%) in the PD-1-positive group, and 11 patients (32%) in the PD-1-negative group. Median PFS was 2 months (95% CI 1.9–2.1) and median OS 9.1 months (95% CI 5.6–10.4). Fifty-five percent of the patients had treatment-related AEs (TRAEs), such as fatigue (14%), rash (12%), pruritus (9%); grade 3–5 AEs were reported in 13% of the patients, and immune-mediated AEs in 16% of the patients. Six patients discontinued due to treatment-related AEs [101].

A further trial (NCT02829918) evaluated nivolumab (*anti*-PD-1 antibody) in 34 patients with previously treated BTC (iCCA 64.7%, extrahepatic CCA [eCCA] 2.9%, GBC 32.4%). Patients received nivolumab at the dose of 240 mg IV every two weeks for 16 weeks and then

480 mg IV every four weeks until disease progression or unacceptable toxicity. Primary endpoint was ORR according to RECIST 1.1 [100], secondary endpoints were PFS, OS and safety. ORR was 17% (partial response in five of 29 evaluable patients), stable disease 38% (11 patients), for a disease control rate (DCR) of 55%. Four responding patients were MSS and two of them had durations of response > 12 months. Median PFS was 3.5 months (95% CI 2.1–7.6), median OS was not reached, and 6-month OS was 76.3%. Most frequently observed TRAEs were fatigue (24%) and elevated AST and ALT (15% and 11%). Grade 3 TRAEs were reported in 21% of the patients, including elevated bilirubin (3%) and alkaline phosphatase (6%). No grade ≥4 AEs were reported. A tissue biomarker analysis is ongoing [102].

A recently published phase I trial (NCT02443324) assessed safety and efficacy of pembrolizumab plus ramucirumab, an anti-vascular endothelial growth factor receptor 2 (VEGFR-2) monoclonal antibody, in 26 patients with previously treated BTC, based on preclinical data suggesting that simultaneous blockade of VEGFR-2 and PD-1/PD-L1 might enhance antitumor activity. Forty-six percent of the patients had PD-L1-positive tumors defined as CPS ≥1 and only a minority of patients were tested for microsatellite status. Pembrolizumab was administered at the dose of 200 mg IV on day 1 and ramucirumab at the dose of 8 mg/kg IV on days 1 and 8 every three weeks. Grade 3 TRAEs, most commonly hypertension, were reported in 35% of the patients. One grade 4 TRAE of neutropenia was observed, no treatment-related deaths were reported. Response rate was 4% (one patient with PD-L1-positive, MSS eCCA), stable disease 35% and DCR 50%. Median PFS was 1.6 months with no difference according to PD-L1 expression. Median OS was 6.4 months overall, 11.3 months in PD-L1-positive patients and 6.1 months in PD-L1-negative patients [103].

Patients with mismatch repair (MMR) deficient (dMMR)/MSI BTC have shown responsiveness to treatment with checkpoint inhibitors [104–106]. In a phase II trial (NCT0187651), among four patients with dMMR/MSI BTC (three patients with Lynch syndrome and ampullary tumors, one with bile duct tumor) treated with pembrolizumab, two patients (one ampullary, one bile duct) achieved a partial response (ORR 50%) [104]. With more patients enrolled in the trial, treatment with pembrolizumab achieved one complete response (25%) and three disease stabilizations (75%), for a DCR of 100%, among four patients with dMMR CCA, and one complete response (25%) and one disease stabilization (25%), for a DCR of 50%, among four patients with dMMR ampullary tumor [105].

These preliminary clinical data suggest a potential role for immunotherapy in BTC patients and highlight the importance of biomarker analyses with the aim to identify and select patients who are most likely to benefit from thiszfects of current treatments on T cells, inhibitory cells, and checkpoint signals and to assess potential synergistic activity with simultaneous inhibition of different pathways [96].

5.4.2. Potential biomarkers of response

A crucial point to improve the activity of immune checkpoint inhibitors is the possibility to select patients according to predictive biomarkers. Unfortunately, none of them has been identified so far in BTC. The most studied biomarkers are PD-L1, MMR proteins/microsatellite status, and TMB. PD-L1 expression across various tumor types has been found to correlate with poor prognosis, better prognosis, or no effect, and has been associated with sensitivity to checkpoint inhibitors in some malignancies, although conflicting results have been observed in other tumors. Data about PD-L1 expression in BTC rely on small series, and PD-L1 expression has been reported in 9–72% of tumors and on 46–63% of immune cells in the tumor microenvironment, thus suggesting that, although ranges are wide, checkpoint inhibition could have a potential role in a relevant percentage of BTC patients [63,95,99]. However, further studies on the role of PD-L1 expression as a clinically meaningful biomarker for response to *anti*-PD-1/PD-L1 therapies are warranted to better understand the role of other important factors such as expression on tumor cells versus immune cells or both, in primary tumors versus metastatic lesions, primary tumor site, prior and concomitant treatments, methodology and cut-off used.

The presence of dMMR/MSI, characterized by the expression of neoantigens that activate antitumor T-cell response, has been associated with a high rate of durable responses to checkpoint inhibitors across various tumor types. Notably, pembrolizumab is the first drug approved by the United States Food and Drug Administration for previously treated patients with advanced dMMR/MSI solid tumors, independently of primary tumor site, therefore including also BTC. Importantly, one of the genetic risk factors for BTC includes Lynch syndrome, characterized by dMMR/MSI and, in general, dMMR has been estimated to occur in 5–10% of patients with BTC [107]. Recently, Kunk et al. presented a study of 99 patients with BTC and showed a dMMR rate of 20%, associated with worse survival compared to patients with pMMR BTC (19 vs 28 months; P = 0.07). Of note, PD-L1 positivity was observed only in pMMR tumors (P = 0.01) [108].

Tumor mutational burden has been shown to correlate with durable clinical benefit in patients with different tumor types treated with immune checkpoint inhibitors. A whole-exome sequencing study of 232 BTCs demonstrated a median of 39 somatic nonsynonymous mutations in iCCA and of 35 somatic nonsynonymous mutations in eCCA. Also, 3–6% of BTCs had a mutation rate > 10/Mb (3% in iCCA, 6% in eCCA and GBC), and a median number of 641 nonsilent mutations, classifying them as hypermutated. Of note, 36% of these tumors were concomitantly dMMR/MSI [6,109]. A retrospective study of 309 patients with BTC, identified TMB of ≥6 mutations/Mb in 19% of cases, even if high TMB (defined as ≥20 mutations/Mb) was observed only in 3% of cases [110]. A recent study of 422 tissue blocks of BTC (270 iCCA, 60 eCCA, 92 GBC) to assessed TMB classified them into three groups: high (≥20 mutations/Mb), intermediate (6–19 mutations/Mb) and low (< 6 mutations/Mb). The frequency of the three groups differed significantly according to primary tumor site. Patients with eCCA and GBC had significantly higher TMB compared to patients with iCCA (TMB high or intermediate: 18% eCCA, 22% GBC, 13% iCCA; $P = 0.04$) [111].

Overall, these data suggest that checkpoint inhibitors may be a promising therapeutic approach for BTC patients with dMMR/MSI and/or high TMB.

6. Conclusions

Recent advances in the genetic and epigenetic profiling are changing the therapeutic landscape of many types of cancer, including CCA. However, the road towards a precision medicine in CCA is hampered by the wide heterogeneity of the tumor, the complexity of the tumor microenvironment, and the uncertainties on the genomic profile. Indeed, crucial molecular fingerprints addressing therapeutic decisions, are still lacking in CCA. Immunotherapy might actually make the difference, but only once the real involvement of MSI and MMR deficiency in CCA has been better clarified.

While precision medicine in CCA still offers no guarantee, matching therapies with molecular alterations is helping redefine clinical trials and leading to better treatment options. Next steps should include further functional studies and clinical trials, which patients should be include based on specific CCA subtypes and stratified according to their genetic, epigenetic and metabolic drivers. Further, efforts to develop and validate an integrated systems biology-based approach should be supported and encouraged. Finally, the tumor microenvironment and immune response are still largely unknown and further translational efforts are needed to improve the pipeline for developing new therapeutic strategies in CCA.

Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) induces inhibitory signals that suppress T-cell priming promoted by antigen-presenting cells, such as dendritic cell, mostly in lymphoid organs. Likewise, the programmed cell death 1 ligand 1 (PD-L1) is commonly expressed by tumor cells and immune cells populating tumor

microenvironment, including tumor-associated macrophages. Cancer-associated fibroblasts (CAFs) form a tight shell around the malignant bile ducts. Tregs cells represent an additional mechanism leading CCA tolerance producing anti-inflammatory cytokines and inhibiting Natural Killer (NK) antitumor activity.

In cholangiocarcinoma, antibodies targeting receptor programmed cell death protein 1 (PD-1), such as pembrolizumab or nivolumab, can stimulate T cells that recognize tumor cells, thereby potentiating overall anticancer immune responses. Though largely investigational, also intravenous adoptive transfer of tumor-infiltrating lymphocytes (TILs) has been reported to be effective in patients with cholangiocarcinoma. Together with immunotherapy, modulation of the CCA-CAF crosstalk may represent a valid therapeutic prospective, as indicated by the inhibition of the PDGFR-β (imatinib mesylate).

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