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Review Article

Cholangiocarcinoma: the role of genetic and epigenetic factors; current and prospective treatment with checkpoint inhibitors and immunotherapy

Panagiotis Sarantis[1*], Eleftheria Dikoglou Tzanetatou[1*], Evangelia Ioakeimidou[1*], Christos Vallilas[1], Theodoros Androutsakos[2], Christos Damaskos[3,4], Nikolaos Garmpis[3,5], Anna Garmpi[6], Athanasios G Papavassiliou[1], Michalis V Karamouzis[1]

[1]Molecular Oncology Unit, Department of Biological Chemistry, Medical School, National and Kapodistrian University of Athens, 11527 Athens, Greece; [2]Pathophysiology Department, Medical School, National and Kapodistrian University of Athens, 11527 Athens, Greece; [3]N.S. Christeas Laboratory of Experimental Surgery and Surgical Research, Medical School, National and Kapodistrian University of Athens, 11527 Athens, Greece; [4]Renal Transplantation Unit, Laiko General Hospital, 11527 Athens, Greece; [5]Second Department of Propedeutic Surgery, [6]First Department of Propedeutic Internal Medicine, Laiko General Hospital, Medical School, National and Kapodistrian University of Athens, 11527 Athens, Greece. [*]Equal contributors.

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Abstract: Cholangiocarcinoma (CCA) represents 3% of all gastrointestinal cancers worldwide and is the second most common primary liver tumor after hepatocellular carcinoma. CCA is an aggressive tumor that involves the intrahepatic, perihilar and distal biliary tree, with a poor prognosis and an increasing incidence worldwide. Various genetic and epigenetic factors have been implicated in CCA development. Gene mutations involving apoptosis control and cell cycle evolution, histone modifications, methylation dysregulation and abnormal expression of non-coding RNA are the most important of these factors. Regarding treatment, surgical resection, cisplatin and gemcitabine have long been the most common treatment options, but 5-year survival (7-20%) is disappointing. For that reason, inhibitors and small molecules related to specific mutations and molecular pathways have been introduced. Among them, immunotherapy seems to be a promising treatment in CCA, with multiple regimens being under clinical trial studies. The combinatorial therapy of traditional CCA treatment with tyrosine kinase inhibitors and/or immunotherapy seem to be the future, depending on the molecular profile of each patient's tumor.

Keywords: Cholangiocarcinoma, inhibitors, immunotherapy, genetic mutations, epigenetics

Introduction

Cholangiocarcinoma (CCA) represents 3% of all gastrointestinal cancers worldwide and is the second most common primary liver tumor after hepatocellular carcinoma [1]. CCA is an epithelial malignancy originating from transformed cholangiocytes, with preclinical studies suggesting hepatic progenitor cells as possible cells of origin [2]. CCA is classified as intrahepatic (iCCA), perihilar (pCCA), and distal (dCCA). The last two subtypes were grouped as extrahepatic CCAs in the past but are now considered separate entities based on tumor biology and management differences [3, 4]. CCA has shown a rapid increase in incidence alongside a decline in age of presentation [5].

The most common factors leading to CCA development are primary sclerosing or parasitic cholangitis, fibrocellular liver disease, hepatolithiasis and chemical carcinogens [6]. Moreover, inflammatory bowel disease (IBD), hepatitis B (HBV) and C (HCV) virus infections, diabetes mellitus, obesity, and smoking appear to play a key role in CCA development [7].

Surgical resection remains the cornerstone of CCA treatment and should always be attempted when feasible [8]. In patients with CCA, the possibility of surgical excision depends on patient's clinical condition, local extent of the tumor and the presence of metastasis, as well as the functional capacity of the liver [9]. pCCA surgical treatment has substantially improved in the last

15 years, with specialized centers achieving 5-year survival rates of up to 40-50% [10]. However, only 20% of patients with CCA can be surgically treated, mainly due to locally advanced disease or presence of distant metastasis [11].

In the case of advanced CCA, gemcitabine monotherapy is the most commonly used regimen, with a median survival from small, phase II studies being approximately six months, while the addition of cisplatin, in patients in good general condition, can improve survival by 30% compared to gemcitabine monotherapy [12]. In aggressive tumors or those with multinodular infiltration, gemcitabine and oxaliplatin alongside with radiotherapy are preferred [13].

Unfortunately, 5-year survival (7-20%) and tumor recurrence rates after surgical resection are disappointing [1]. In this review, we will focus on the genetic and epigenetic factors that affect the biogenesis of CCA and new therapies that can help improve CCA patients' survival. Inhibitors and small molecules related to mutations and the role of immunotherapy alone or in combination with other therapies will be discussed.

Molecular mechanisms

A number of changes are needed for the transformation of a normal cell to a malignant one. These changes may be gene mutations leading to either loss or gain of function in genes responsible for controlling apoptosis and cell cycle evolution, histone modifications, methylation dysregulation and abnormal expression of non-coding RNA. These lead to unbalanced gene expression and transcription, affecting cell homeostasis and maintaining malignant transformation. These changes include genetic and epigenetic mutations [5, 14].

Genetic alterations

Various mutations in different genes may lead to CCA development. Genes such as those of isocitric dehydrogenase 1 and 2 (IDH1 and IDH2), involved in glucose metabolism by catalyzing the oxidative decarboxylation of isocitrate, producing alpha-ketoglutarate (α-ketoglutarate) and CO_2, may develop function-related mutations [15]. Mutations in the IDH genes lead to abnormal isoforms that transform the isocitrate to 2-hydroxyglutarate (2-HG), a tumor metabolite leading to increased DNA methylation levels, cell proliferation, and angiogenesis [16].

Furthermore, mutations in genes associated with the tyrosine kinase pathway seem to play a significant role in cancer development. Patients with these mutations have a worse overall survival [17]. Phosphorylation-activated PAS proteins (mainly KRAS and NRAS) activate the RAF-MEK-ERK signaling pathway and appear to be responsible for cancer proliferation, differentiation, and metastasis [18]. These mutations, along with BRAF mutations, are found in various cancers such as lung, skin, and gastrointestinal tumors and are commonly used in "targeted therapies". In regard to CCA, KRAS mutations are more commonly found in both iCCAs and extrahepatic CCAs compared with NRAS mutations [16]. On the other hand, BRAF mutations are almost exclusively found in iCCA [19].

The ErbB tyrosine kinase receptor family includes ErbB-1 (HER1/epidermal growth factor receptor, EGFR), ErbB-2 (HER2), ErbB-3 (HER3), and ErbB-4 (HER4). EGFR is activated by binding to its ligands, including epidermal (EGF) and transforming (TGF) growth factors. Upon activation, the inactive receptor is converted to an active homodimer or a heterodimer with another member of the same family (ErbB2/HER2/neu), leading to the activation of several signal transduction catalysts that affect cell proliferation, differentiation, and adhesion, and are stimulated by receptor dimerization [20]. EGFR and HER2/neu overexpression have been detected in a significant proportion of patients with iCCA, whereas EGFR mutations are rare in both iCCA and eCCA [21]. Genetic alterations in the fibroblast growth receptor (FGFR) genes, particularly FGFR2 fusions, have been found in a large proportion of iCCAs, but not in extrahepatic CCAs, confirming the different pathophysiologic features between intra- and extrahepatic CCAs [22].

Another intracellular signaling cascade with a key role in cell cycle regulation is PI3K/Protein kinase B (AKT)/mTOR. This process involves the phosphorylation of AKT, which is then moved to the plasma membrane and leads to several effects, one of which is mTOR activa-

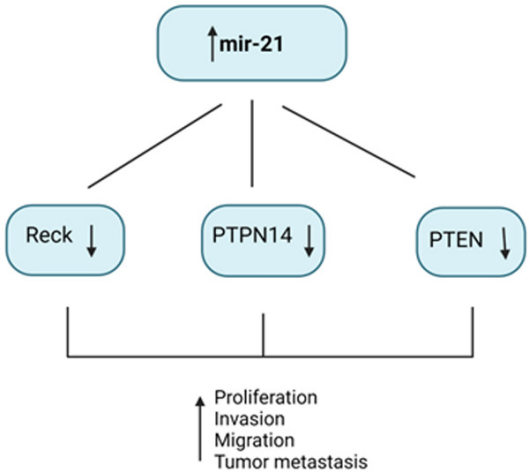

Figure 1. Overexpression of mir-21 leads to an increase in proliferation, migration, and invasion as it regulates the expression of RECK, PTPN14, and PTEN genes, contributing to tumor metastasis.

tion [23]. This process is typically inhibited by the Phosphatase and Tensin Homolog (PTEN) gene, a tumor-suppressor gene. In many cancers this inhibition is impaired, thus mTOR is constantly active leading to proliferation of cancer cells [24]. In both eCCAs and iCCAs PI3K mutations are commonly found; on the other hand, PTEN mutations are rare [25].

As far as chromatin remodeling genes are concerned, in both iCCA and eCCA, mutations in the ARID1A, PBRM1, and BAP genes are detected, indicating that these genes may play a role in CCA pathogenesis [26].

Epigenetic alterations

Micro-RNAs (miRNAs): miRNAs consist of approximately 22 nucleotides of endogenous, non-coding RNA molecules [27]. miRNAs are involved in fundamental cellular processes and regulate different genes and pathways, thus contributing significantly to the heterogeneity of diseases [28]. miRNAs are considered diagnostic biomarkers of gastrointestinal malignancies such as CCA as they are molecules with a simple chemical structure and show biological stability [27, 29]. Various studies have shown that some miRNAs, based on their target genes, act either as oncogenic or tumor suppressor miRNAs [19, 30]. The expression of certain miRNAs seems to be upregulated by interleukin-6 (IL-6), a cytokine associated with chronic inflammation. Since chronic inflammation seems to precede CCA development, these miRNAs could be associated with carcinogenesis [31]. In addition, the ability to regulate the increase in DNA methyltransferase 1 (DNMT1) expression can epigenetically modify the expression of miR-148a and miR-152, which can bind and regulate DNMT1 [32].

Many studies have attempted to elucidate the role of miRNAs in CCA. In some of them miR-21, miR-34c, miR-200b, miR-221 were overexpressed 2.18-3.79 times in cancer cells compared to normal [33, 34]. Mir-21 *in vitro* overexpression can affect many genes, such as MMP/RECK, which is responsible for reduced regulation of its inhibitor. Besides, overexpression of miR-21 leads to an increase in proliferation, migration, and invasion of cancer cells as it regulates the expression of PTPN14 tyrosine phosphatase and PTEN gene, contributing thus to tumor metastases [28]. Moreover, in another study, an increased expression of miR-222 and miR-483-5p was found in patients with CCA compared to PSC [35] (**Figure 1**).

DNA methylation: Epigenetic modifications appear to be an essential factor in the onset and development of CCA [5]. DNA methylation is one of the better-studied epigenetic alterations that can occur in CCA In general, the epigenetic mutation of cell cycle inhibitors and certain tumor suppression genes play a key role in the development and progression of CCA [36]. DNA methylation occurs primarily by modifying cytosine residues at carbon position 5. This modification usually occurs in 5'-C-phosphate-G-3' (CpG) dinucleotides that accumulate in GC regions called CpG islands instead of being evenly distributed [37]. CCA is associated with hypermethylation and inactivation of cell cycle inhibitors, leading to an increase in dysregulated methylation patterns. Moreover, an increase in genomic instability and reactivation of transposable elements is found, as the genomic DNA is less methylated [5].

Furthermore, methylation can also be observed in the promoter regions of tumor suppressor genes (TSG) resulting in gene-silencing [38]. CCA tissue shows a significant reduction in DNA hydroxymethylation compared to non-tumor tissue and several gene promoters involved in Wnt signaling are hyper-methylated [4]. The gene noted in up to 83% of CCA, associated with epigenetic changes, is P16INK4a (a TSG)

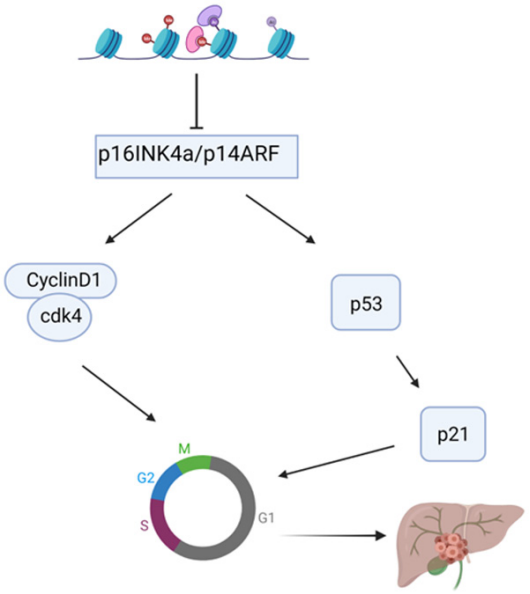

Figure 2. Hypermethylation of p16INK4a/p14ARF leads to inhibition of these genes, so p16INK4a cannot inhibit the Cyclin D1-CDK4 complex and the cell enters S phase. Inhibition of p14ARF leads to loss of p53 function and cell cycle control. The result is the appearance of cholangiocarcinoma.

and found on the human chromosome 9p21. P16INK4a blocks interaction with cyclin D1 as it binds to cyclin-dependent kinase 4 (CDK4). After methylation of the promoter, the activity of P16INK4a stops, CDK4 binds to cyclin D1, and the cell enters into S phase. The main reason for the inactivation of p16INK4a is the methylation of the CpG island [38]. Furthermore, in CCAs, tumor suppression genes and microRNAs, including MLH1, p14, p16, death-related protein kinase (DAPK), miR-370, and mir-376c, are commonly methylated [39]. It has also been shown that in 25% of CCAsp14ARF hypermethylation increases. Normally, p14ARF prevents the degradation of p53 which is a control point that can be lost in CCA [40] (**Figure 2**). Hereditary mutations affect DNA repair genes and are linked to the development of cancer in humans. In summary, in a percentage of 23.6% of CCAs, hypermethylation of the inhibition repair gene promoter hMLH1 is observed [31].

The inflammatory response seems to play an integral role in the occurrence of CCA. In particular, the continuous overexpression of interleukin-6 (IL-6), which is the result of epigenetic silencing of the cytokine 3 (SOCS-3) signaling repressor, plays a dominant role in CCA carcinogenesis. In a variety of experiments, methylation of the SOCS-3 promoter was observed and an attempt was made to enhance its overexpression, which appeared to effectively reduce IL-6 and the corresponding signal transduction cascade [41]. Therefore, the loss of IL-6 in cholangiocarcinoma appeared to contribute to the activity and overexpression of inflammatory molecules found in CCA [42].

Histone modifications: Deacetylation of histones and other cellular proteins has an important role in tumor formation and progression, including CCA. In particular, histone acetylation is a non-reversible, post-translational, modification that is extremely important for the structure and function of chromatin as well as for the regulation of gene expression [43]. Histone deacetylation is favored by the enhanced action of HDAC and thus creates significant DNA structural changes and the transcriptional repression of genes involved in the differentiation and negative regulation of cell proliferation, metastasis, and migration [44].

The balance between histone acetylation, from histone acetyltransferases, and deacetylation, from histone acetylases (HDAC) [45] leads to the reduction of the expression of tumor suppressor genes and plays an important role in cell differentiation, proliferation, and apoptosis in many malignancies [43, 44]. The effects of histone methylation as opposed to histone acetylation vary depending on the targeted amino acid. Regarding CCA, a typical example is the methylation of histone 3 to lysine 27 (H3K27me) by the enhancer of zeste homolog 2 (EZH2), a methylation associated with gene repression. When EZH2 is over-expressed in patients with CCA, it is accompanied by poor prognosis in both iCCA and eCCA [28].

Current treatment-NCCN guidelines V.1.2021

Regarding iCCA, current treatment guidelines are presented below.

After surgical resection with no residual local disease, three therapeutic options are available: observation, chemotherapy with 5-FU + oxaliplatin, capecitabine + oxaliplatin, Gemcitabine + capecitabine, Gemcitabine + cisplatin, or Capecitabine + cisplatin or participation in

a clinical trial with chemotherapy/immunotherapy.

After surgical resection with microscopic margins (R1) or positive regional nodes, treatment options include systemic chemotherapy with the aforementioned agents, Fluoropyrimidine-based Chemoradiation, Fluoropyrimidine-based or gemcitabine-based chemotherapy followed by Fluoropyrimidine-based Chemoradiation, Fluoropyrimidine-based chemoradiation followed by Fluoropyramidine-based, gemcitabine-based chemotherapy, or participation in a clinical trial.

After surgical resection with residual local disease (R2) therapeutic options include 5-FU + Oxalipaltin 5-FU + Cisplatin, Capecitabine + Cisplatin, Capecitabine + Oxaliplatin, Gemcitabine + albumin bound-paclitaxel, Gemcitabine + Capecitabine, Gemcitabine + Oxaliplatin or Gemcitabine + Cisplatin + albumin bound-paclitaxel.

For eCCA, surgery is the preferred therapeutic option. In unresectable cases, possible therapeutic options include chemotherapy with the aforementioned agents, participation in a clinical trial, EBRT with concurrent Fluoropyramidine, palliative EBRT or best supportive care. Finally, for metastatic disease, chemotherapy, participation in a clinical trial or best supportive care can be selected [46].

Prospective treatment approaches

Small molecule-inhibitors

In a targeted treatment approach, agents targeting mutations in molecular pathways associated with CCA are under study.

FGFR: BGJ398, a pan-FGFR inhibitor, reduced the neoplastic potential in a xenograft CCA model [47]. A phase II, multicenter study of BGJ398 in patients with advanced CCA with FGFR genetic alterations demonstrated a remarkable disease control rate of 82% (NCT02150967) [48]. Another phase III clinical trial evaluates BGJ398 versus Cisplatin and Gemcitabine in first-line treatment in patients with advanced CCA (NCT03773302) [49]. JNJ-42756493/erdafitinib is another oral pan-FGFR [50, 51]. In a phase I clinical trial, erdafitinib showed specific anti-tumor activity; however, this included only patients with FGFR mutations [52]. ARQ 087 is an ATP-competitive small molecule kinase inhibitor (SMKI) with antitumor activity in vitro [53]. A multicenter, phase I/II clinical trial enrolled patients with unresectable iCCA, intolerant or not eligible to first-line chemotherapy. Overall response rate (ORR) was 20.7%, and disease control rate was 82.8% (NCT01752920) [54]. Other FGFR SMKIs which have established efficacy in preclinical studies and are currently being assessed in early phase clinical trials include TAS-120 and CH5183284/Debio 1347 (NCT02052778, NCT01948297), Pemigatinib (NCT02924376), Ponatib (NCT02265341), AZD457 (NCT03263637) [16, 55].

EGFR: Regarding EGFR/HER2 mutations are used lapatinib, although the V777L mutation was sensitive to nertatinib [51, 56]. Phase II trials with nertatinib or cetuximab (anti-EGFR antibody) for the treatment of patients with advanced-stage disease showed promising results, which were further confirmed in combination with traditional chemotherapy (gemcitabine and oxaliplatin) [57, 58]. Panitumumab or capecitabine was also tested [16]. Trastuzumab with tipifarnib is currently in an ongoing phase I trial. Additionally, vandetanib, has been tested alone and in combination with chemotherapy in phase I and II trials, with no noteworthy results [59]. Irreversible EGFR inhibitors such as afatinib and dacomatinib may also be effective as therapeutic agents [60, 61].

VEGFR: Bevacizumab, sorafenib, sunitinib, regorafenib, lenvatinib ramucirumab are being successfully used as anti-angiogenic agents [62, 63]. The most promising molecule, bevacizumab, a recombinant anti-VEGF monoclonal antibody, has shown great ability to decrease neoplastic vascularization and tumor development [64]. In a phase II clinical trial, bevacizumab combined with gemcitabine and oxaliplatin showed a median PFS of seven months and median overall survival (OS) of 12.7 months [30]. Furthermore, combinatorial therapy with bevacizumab plus FOLFIRI and erlotinib was tested in phase II clinical trials, achieving a median OS between 9.9-20 months [65]. Sorafenib, an agent that acts additionally on PDGFR and Raf kinases, was tested in a phase II trial, combined with gemcitabine, showing improved PFS and OS ratios [16, 66].

PI3K/AKT/mTOR and RAF/MEK/ERK: Currently, no effective direct inhibitors of KRAS exist, even though many agents are being developed. Preclinical data have shown that dual targeting of the PI3K/AKT/mTOR and RAF/MEK/ERK pathways has synergistic effects in CCA [51]. A phase I trial of the mTOR inhibitor everolimus in combination with gemcitabine and cisplatin in CCA patients demonstrated stable disease in 60% with an acceptable safety grade [67]. Sirolimus, an mTOR inhibitor, is currently under study [68]. Inhibition of MEK is promising for the treatment of advanced CCA. Selumetinib, a selective MEK 1/2 inhibitor, had established efficacy in a phase II trial [69]. Furthermore, other promising MEK inhibitors, including refametinib, trametinib, and MEK162, are being investigated in several clinical trials. Copanlisib and BKM120 (PI3K inhibitors) are under investigation [70]. Finally, MK2206, an AKT inhibitor, used in phase II clinical trial showed a PFS of 1.7 months and an OS of 3.5 months, with no severe side effects (NCT01425879) [71].

IDH1/2: AGI-5198, a selective IDH1 inhibitor, stops the enzyme from producing the oncometabolite 2-HG, resulting in the impaired growth of IDH1-mutant cells [26]. Similarly, AGI-6780, a selective mutant IDH2 inhibitor, triggers differentiation in hematopoietic cell lines [27]. Ivosidenib (AG-120) was evaluated in patients with IDH1-mutant advanced CCA in a phase I clinical trial with the main result being stabilization of the disease [28], while it significantly improved PFS compared with placebo in a phase III study (ClarIDHy) [29]. AG-221, a selective IDH2 inhibitor, is being tested in a clinical trial phase 1/2 (NCT02273739) [30]. Another drug, dasatinib, showed a significant anti-tumor effect *in vivo* [31], while AG-881, a pan-IDH inhibitor is under clinical trial [27].

Chromatin remodeling: Chromatin remodeling is a target of numerous small molecule inhibitors. These include histone deacetylase (HDAC) inhibitors, such as vorinostat and romidepsin and DNA methyltransferase (DNMT) inhibitors, like azacitidine and decitabine [46]. All the above pathways and their inhibitors are represented in **Figure 3**.

Immunotherapy

Since CCA is the second-most common liver cancer, with low survival rate, many drugs are under evaluation as treatment regimens. 602 clinical trials have been scheduled in the field of CCA, with 255 being active or under recruiting (96 clinical trials in phase I, 238 in phase II, 39 in phase III, and 2 in phase IV).

Immunotherapy-based therapeutic approaches have shown significant results against various cancers types [72]. Due to this fact, 56 clinical trials have used immunotherapy in CCA. Fifty-one of them are currently active, combining immunotherapeutic agents (10 drugs) with 62 chemotherapeutic agents and new molecules (14 clinical trials phase I, 41 clinical trials phase II, one clinical trial phase III)-3 completed, 2 terminated.

Immunotherapeutic molecules used include 8 anti-PDL1 agents: Nivolumab (16 clinical trials), Pembrolizumab (12 clinical trials), Durvalumab (7 clinical trials), Camrelizumab (4 clinical trials), Atezolizumab (2 clinical trials), Avelumab (1 clinical trial), Triprilumab and Spartalizumab (1 clinical trial each one) and two anti-CTLA4 ones: Ipilimumab (5 clinical trials), Tremelimumab (3 clinical trials).

Nivolumab is a human programmed death receptor-1 (PD-1) blocking antibody, currently used in 17 clinical trials for CCA treatment. There is an ongoing phase II clinical trial active but not recruiting patients, combining it with an HDAC inhibitor (Entinostat), with no available results yet (NCT03250273) [73]. Also, there is an active phase II clinical trial using a combination with an anti CTLA4 molecule in 818 patients with rare tumors with no results (NCT02834013) [74]. An ongoing phase 1 clinical trial with 138 patients with rare tumors with TPST-1120 with unknown results (NCT03829436) [75]. An active, phase I/II clinical trial will soon begin using Nivolumab with Azacitidine, Gemcitabine, and Cisplatin and FT-2102 in patients with solid tumors, including CCA (NCT03684811) [76]. Another phase II clinical trial is recruiting patients with CCA using Nivolumab with SBRT after induction chemotherapy (NCT04648319) [77] and a phase I, active clinical trial in 75 patients with solid tumors combining Ipilimumab and TRK-950 with chemotherapy (NCT03872947) [78] and Nivolumab and Ipilimumab with radiation therapy in biliary tract cancer patients (NCT028-6638) [79]. There is a phase III clinical trial combining many immunotherapeutic agents

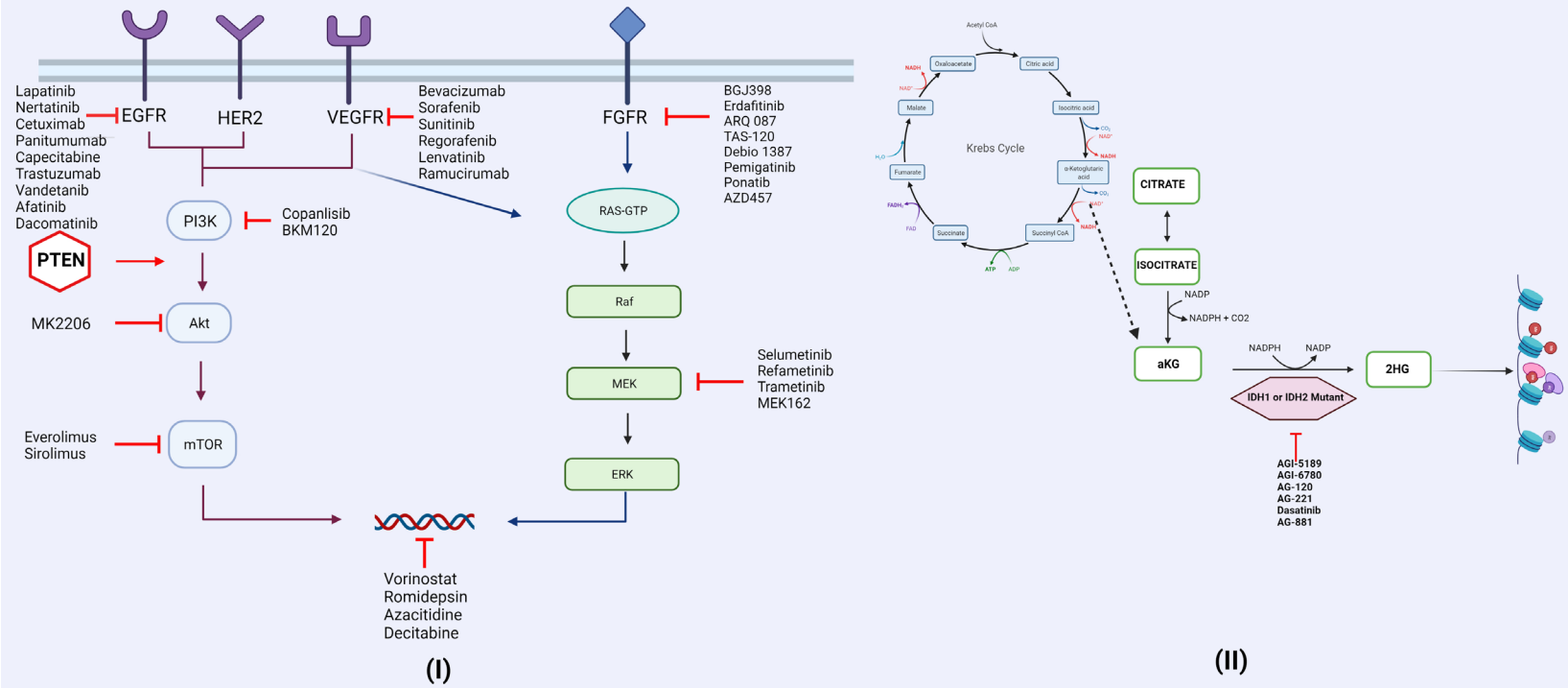

Figure 3. (I) Overview of molecular pathways in the development of cholangiocarcinoma. Also shown, an overview of the small molecules that inhibit these pathways. (II) Mutations in the IDH genes make abnormal isoforms that transform the isocitrate to 2-hydroxyglutarate (2-HG), a tumor metabolite leading to increased DNA methylation levels.

Table 1. Immunotherapy using Nivolumab combined with other drugs/molecules

NIVOLUMAB	NCT	Phase
+ Entinostat	NCT03250273	II
+ Ipilimumab	NCT02834013	II
+ TPST-1120	NCT03829436	I
+ FT-2102 + Azacitidine + Gemcitabine and Cisplatin	NCT03684811	I/II
+ SBRT	NCT04648319	II
+ Pembrolizumab + Ipilimumab + chemo + TRK 950	NCT03872947	I
+ pembrolizumab ++ atezolizumab + ipilimumab	NCT04157985	I
monotherapy	NCT02829918	II
+ DKN-01	NCT04057365	II
+ chemotherapy	NCT04172402	II
+ Nanoliposomal-Irinotecan + chemotherapy	NCT03785873	I/II
+ Radiation	NCT02866383	II
+ Rucaparib	NCT03639935	II
+ Ipilimumab	NCT03695952	N/A
+ Ipilimumab + Chemotherapy	NCT03101566	II
monotherapy	NCT02829918	II

(pembrolizumab, nivolumab, atezolizumab, ipilimumab) in 578 participants with advanced solid tumors (NCT04157985) [80]. A phase II clinical trial will recruit patients with advanced refractory biliary tract cancers using Nivolumab as monotherapy (NCT02829918) [81] (NCT02829918) [82], or with DKN-01 in previously treated patients with advanced biliary tract cancer (NCT04057365) [83]. In the next few months, a phase II clinical trial will commence combining Nivolumab with Gemcitabine and TS-1 as first-line treatment in patients with advanced biliary tract cancer (NCT04172402) [84]. Furthermore, an ongoing phase I/II clinical trial using Nanoliposomal-Irinotecan and chemotherapy as second-line therapy in 40 patients with advanced biliary tract cancer is underway since December 2018; no results are known yet (NCT03785873) [85]. A phase II clinical trial is under recruitment phase of patients with advanced unresectable biliary tract cancer using the combination of Nivolumab/Ipilimumab only (NCT03695952) [86] with chemotherapy (NCT03101566) [87]. Rucaparib, a PARP inhibitor, combined with Nivolumab is being tested in 35 patients with advanced or metastatic biliary tract cancer, in a phase II clinical trial (NCT03639935) [88] (**Table 1**).

Pembrolizumab, a human programmed death receptor-1 (PD-1) blocking antibody, currently is used in 12 clinical trials. Pembrolizumab was used as monotherapy in a phase II clinical trial, in 1595 patients with advanced solid tumors (NCT02628067) [89] and a phase II clinical trial in 33 patients with metastatic biliary cancer or as a second-line treatment (NCT03110328) [90]. Pembrolizumab is also combined with Nivolumab, Ipilimumab, TRK 950, and chemotherapy (NCT03872947) [78], in combination with capecitabine and oxaliplatin in 11 patients with advanced biliary tract cancer (NCT03111732) [91] with Cisplatin/Gemcitabine to 50 patients with biliary cancer in phase II clinical trial (NCT03260712) [92] and with Gemcitabine/Cisplatin in phase III clinical trial, with 1048 patients with biliary tract carcinoma (NCT04003636) [93]. Pembrolizumab is combined with lenvatinib in a phase II clinical trial with 40 participants with advanced CCA (NCT04550624) [94] and a phase II clinical trial with 50 patients with advanced hepatobiliary tumors (NCT03895970) [95]. In an active, phase II, clinical trial Pembrolizumab in conjunction with Olaparib is being tested in 29 patients with bile duct cancer (NCT04306367) [96]. XmAb®22841 molecule is given with Pembrolizumab in a phase II clinical trial, including 242 patients with advanced solid tumors (NCT03849469) [97]. Allogeneic NK Cell ("SMT-NK") in combination with Pembrolizumab is used in an active, phase I clinical trial with 40 patients with advanced biliary tract cancer, but no results are available yet (NCT03937895) [98]. There is an active but not recruiting phase II clinical trial that will combine Pembrolizumab with Sargramostim (Gm-GSF) in biliary cancer (NCT02703714) [99]. Finally, a phase I clinical trial that will start recruiting patients will combine Pembrolizumab with Cyramza in patients with solid tumors (NCT02443324) [100] (**Tables 2**, **3**).

Durvalumab is an anti-PD-L1 monoclonal antibody, used in a considerable number of clinical trials in combination with other chemothera-

Table 2. Immunotherapy using Pembrolizumab combined with other drugs/molecules

PEMBROLIZUMAB	NCT	Phase
+ Lenvatinib	NCT04550624	II
	NCT03895970	II
+ Olaparib	NCT04306367	II
monotherapy	NCT02628067	II
+ XmAb®22841	NCT03849469	I
+ Chemotherapy + Nivolulab + Ipilimumab + TRK950	NCT03872947	I
+ SMT-NK	NCT03937895	I/II
+ Sargramostim	NCT02703714	II
+ Chemotherapy	NCT03111732	II
+ Chemotherapy	NCT03260712	II
+ Ramucirumab	NCT02443324	I
+ Chemotherapy	NCT04003636	III
+ monotherapy	NCT03110328	II

Table 3. Immunotherapy using Ipilimumab combined with other drugs/molecules

IPILIMUMAB	NCT	Phase
Nivolumab	NCT02834013	II
+ Pembrolizumab + Nivolumab + chemo	NCT03872947	I
+ pembrolizumab ++ atezolizumab + Nivolumab	NCT04157985	I
+ Nivolumab	NCT03695952	N/A
+ Nivolumab + Chemotherapy	NCT03101566	II

Table 4. Immunotherapy using Durvalumab combined with other drugs/molecules

DURVALUMAB	NCT	Phase
+ Gem/Cis	NCT04308174	II
+ Tremelimumab	NCT04238637	II
	NCT03704480	II
+ Tremelimumab + bevacizumab + Chemo	NCT03937830	II
+ AZD6738 + camrelizumab	NCT04298008	II
+ SNDX-6352	NCT04301778	II
+ Tremelimumab + Chemotherapy	NCT03473574	II

peutic agents. In a phase II, randomized, clinical trial with 45 patients with resectable biliary tract cancer, Durvalumab is used as a neoadjuvant with or without Gemcitabine/Cisplatin, with no results yet (NCT04308174) [101]. In a phase II clinical trial recruiting patients, durvalumab plus tremelimumab are used in patients with advanced intrahepatic biliary tract cancer (NCT04238637) [102] with or without paclitaxel as second-line treatment after failure of platinum-based chemotherapy (NCT03704480) [103]. In the next few months, a new study will combine durvalumab/tremelimumab with gemcitabine/cisplatin in patients with advanced cholangiocarcinoma (NCT03473574) [104]. In a recruiting, phase II clinical trial durvalumab is combined with Ceralasertib (AZD6738), an RTK inhibitor, in 26 patients with biliary tract cancer. Also, durvalumab is used with Axatilimab (an anti-CSF-1R monoclonal antibody) in phase II in 30 patients as second-line treatment after chemo or radioembolization in patients with intrahepatic cholangiocarcinoma (NCT04301778) [105] (**Table 4**).

Camrelizumab is an anti-PD-1 immune inhibitor, used in a phase II clinical trial with apatinib, a TKI inhibitor, in 20 patients with advanced biliary tract cancer, with no results available yet (NCT04642664) [106] and in two, phase II, clinical trials with chemotherapy and radiotherapy in patients with CCA (NCT04333927) [107] or with radiotherapy alone in unresectable iCCA (NCT03898895) [108].

Atezolizumab is a monoclonal antibody against PDL-1. It is used in phase II clinical trial combined with bevacizumab and chemotherapy in 150 patients with untreated advanced biliary tract cancer, with no results yet (NCT04677504) [109], as well as with many other molecules such as pertuzumab, trastuzumab, and cobimatinib with no results yet (NCT02091141) [110].

Avelumab is a monoclonal antibody against PDL-1. It is used in an ongoing phase I/II clinical trial combined with Nedisertib and radiation

Table 5. Immunotherapy using various immunotherapy agents combined with other drugs/molecules

OTHER AGENTS	NCT	Phase
anti-PD-1 antibody + nab paclitaxel	NCT04004234	I/II
anti-PD-1 antibody + anti-CTLA-4 antibody + INT230-6	NCT03058289	I/II
Triprilumab + Chemotherapy	NCT04413734	II
Spartalizumab	NCT04802876	II
Camrelizumab		
+ chemotherapy + Radiotherapy	NCT04333927	II
	NCT03898895	II
+ apatinib	NCT04642664	II
Atezolizumab		
+ bevacizumab + chemotherapy	NCT04677504	II
+ Cobimetinib + molecules	NCT02091141	II
Avelumab		
+ Nedisertib + RT	NCT04068194	I/II

therapy for advanced solid tumors in 30 patients (NCT04068194) [111]. Spartalizumab is also a PDL-1 inhibitor, used in an ongoing phase II clinical trial in 141 patients with multiple cancer types, including CCA (NCT04802876) [112]. Finally, in another ongoing clinical trial an anti-PDL-1 agent (which??) is combined with chemotherapy and nab-paclitaxel in 20 participants with advanced or metastatic biliary tract cancer, (NCT04004234) [113] (**Table 5**).

Conclusion

CCA is a high-mortality cancer with an increasing incidence worldwide. Due to the lack of specific symptoms, CCA is diagnosed at a late stage, giving little room for successful treatment. For this reason, it is necessary to understand the molecular pathogenesis of the disease in order to identify new therapeutic agents and strategies. Due to the molecular complexity of the disease, gene fusions and mutations need further investigation in functional studies and clinical trials, in order to find accurate non-invasive biomarkers for the diagnosis and assessment of prognosis in patients with CCA. Most clinical trials are ongoing and thus few data are currently available. Since only 30% of ongoing clinical trials recruit only CCA patients, more CCA exclusive clinical trials must be developed. Combination therapy of traditional CCA treatment with inhibitors and immunotherapy is likely to be the best option in the future, depending on the molecular profile of each patient's tumor; thus contributing to the emergence of personalized medicine.

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Disclosure of conflict of interest

None.

Address correspondence to: Michalis V Karamouzis, Department of Biological Chemistry, Medical School, National and Kapodistrian University of Athens, 75, M. Asias Street, 11527 Athens, Greece. Tel: +30-210-746-2508/9; Fax: +30-210-746-2703; E-mail: mkaramouz@med.uoa.gr

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