


# Focusing on the Immune Cells: Recent Advances in Immunotherapy for Biliary Tract Cancer

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**Abstract:** Biliary tract cancer (BTC) represents a challenging malignancy characterized by aggressive behavior, high relapse rates, and poor prognosis. In recent years, immunotherapy has revolutionized the treatment landscape for various cancers, but its efficacy in BTC remains limited. This article provides a comprehensive overview of the advances in preclinical and clinical studies of immunotherapy for BTC. We explore the potential of immune checkpoint inhibitors in reshaping the management of BTC. Despite disappointing results thus far, ongoing clinical trials are investigating the combination of immunotherapy with other treatment modalities. Furthermore, research on the tumor microenvironment has unveiled novel targets for immunotherapeutic interventions. By understanding the current state of immunotherapy in BTC and highlighting future directions, this article aims to fuel further exploration and ultimately improve patient outcomes in this challenging disease.

**Keywords:** biliary tract cancer, cancer immunotherapy, tumor microenvironment, immune checkpoint inhibitors, clinical trials

## Introduction

Biliary tract cancers (BTCs) encompass a diverse group of adenocarcinomas characterized by genetic variability and a grim prognosis. They are categorized based on their anatomical origin into intrahepatic cholangiocarcinoma (iCCA), extrahepatic cholangiocarcinoma (eCCA, including perihilar CCA (pCCA) and distal CCA), and gallbladder cancer (GBC).<sup>1</sup> The global incidence of BTC varies, with higher rates observed in Asian countries like Korea, Thailand, and China.<sup>2</sup> This disparity may arise from multifaceted risk factors such as liver disease, lifestyle choices, age, gender, and hereditary factors. Additionally, although BTC was previously considered a rare tumor, accounting for approximately 3% of all gastrointestinal tumors,<sup>3</sup> these years have witnessed a rising trend of its incidence,<sup>4</sup> especially among younger adults.<sup>5,6</sup>

Surgical resection remains the sole potentially curative treatment option for BTC.<sup>7</sup> Unfortunately, due to its asymptomatic clinical course, most patients are diagnosed only after the disease has metastasized extensively.<sup>8</sup> Consequently, merely 20–30% of patients qualify for surgical intervention.<sup>9,10</sup> Moreover, despite initial eligibility for surgery, 40–85% of patients experience disease recurrence either during or after radical resection,<sup>11,12</sup> resulting in a high case–fatality ratio and a median overall survival (OS) of less than 12 months.<sup>13</sup> Limited data exist regarding the impact of adjuvant therapy on biliary tract cancer (BTC); however, certain clinical trials have demonstrated the potential of this treatment to offer long-term benefits to BTC patients.<sup>14</sup> Despite a recent meta-analysis of the French PRODIGE-12 and Japanese BCAT Phase III studies showing no significant enhancement in relapse-free survival (RFS) or overall survival (OS) with adjuvant therapy,<sup>15–17</sup> the UK BILCAP phase III trial, which randomly assigns patients with BTC after resection to receive oral capecitabine or observation, reports a clinically meaningful prolonged median OS for capecitabine group.<sup>18</sup> In a French single-center study, patients with locally advanced iCCA who receive surgery following adjuvant chemotherapy have similar short- and long-term results to patients with initially resectable iCCA treated by surgery alone, indicating that this therapeutic option may facilitate patients with initially unresectable disease.<sup>19</sup> Of note, recent Phase II trials also indicate promise for Teysuno in resected BTC patients.<sup>20,21</sup>

For advanced BTC, the cisplatin-gemcitabine (GemCis) combination stands as the internationally accepted first-line treatment regimen, endorsed by studies like UK ABC-02 and Japanese BT22.<sup>22,23</sup> Further refinement includes the GCS

triplet (cisplatin, gemcitabine, and S1) tested in the KHBO1401 trial.<sup>24</sup> However, the addition of albumin-bound paclitaxel to GemCis in the SWOG 1815 trial did not significantly enhance median OS.<sup>25</sup>

Advancements in understanding BTC's genetic diversity have identified potential therapeutic targets. For instance, ivosidenib targeting Isocitrate dehydrogenase 1 (IDH1) and pemigatinib against the Fibroblast Growth Factor Receptor (FGFR) pathway represent notable breakthroughs.<sup>26–28</sup> HER2 overexpression, amplification, or mutation can occur in GBC (19%), eCCA (17.4%), and is rarer in iCCA (4.8%).<sup>29</sup> The HERIZON-BTC-1 phase II trial investigates the application of zanidatamab, a bispecific antibody targeting two distinct HER2 epitopes, in patients with HER2-amplified BTC. The trial reports an impressive objective response rate (ORR) of 41.3% and observes grade 3 treatment-related adverse events in 16% of patients.<sup>30</sup> These findings support the potential of zanidatamab as a future treatment option for HER2-positive biliary tract cancer.

In addition to the emerging therapeutic options mentioned above, immunotherapy, which includes immune checkpoint inhibitors (ICI), adoptive cell therapy (ACT), cancer vaccines, and chimeric antigen receptor T cell (CAR-T cell) therapy, has revolutionized cancer treatment by enhancing the immune system's ability to recognize and attack cancer cells.<sup>31</sup> The TOPAZ-1 trial, which randomly assigns advanced BTC patients to receive durvalumab or placebo in combination with GemCis, followed by durvalumab or placebo monotherapy, demonstrates superior OS in favor of the durvalumab and GemCis triplet.<sup>32</sup> This combination has thus become a first-line treatment option for advanced BTC. Despite these advancements, only a minority of BTC patients currently benefit from immunotherapy.<sup>33,34</sup> In conclusion, while significant progress has been made in understanding and treating BTC, challenges remain in improving early detection, expanding treatment options, and personalizing therapies based on molecular profiles. Ongoing research and clinical trials continue to explore new avenues, offering hope for further advancements in BTC management. Herein, we will describe recent results and future directions of immunotherapy in BTC.

## Immune Cells in Tumor Microenvironment (TME) of BTC

BTCs are characterized by desmoplastic tumors with a dense TME. This TME comprises various components, including nonmalignant cells, lymphoid tissue, blood vessels, nerves, intercellular components, and metabolites. It develops within the tumor lesion and the internal environment of tumor cells, influenced by the recruitment and adaptation of normal cells surrounding the mutated ones. Cancer cells actively shape the TME to promote tumor development, contributing to its dynamic heterogeneity.<sup>35,36</sup> Within the TME, immune cells play a crucial dual role in tumor biology, affecting initiation, progression, and metastasis. For instance, CD4+ and CD8+ T cells exhibit potent antitumor activity through distinct mechanisms, such as direct cytotoxicity and cytokine release. Conversely, immunosuppressive cells like regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) are also present, dampening immune responses and facilitating tumor immune evasion.<sup>37</sup> The intricate interplay between these immune cell subsets within the TME presents both challenges and opportunities for immunotherapy. Immune cells respond dynamically to intrinsic and extrinsic stimuli, influencing tumor behavior and response to treatment. Harnessing these interactions holds promise for developing novel immunotherapeutic strategies tailored to combat BTC effectively.

## Adaptive Immune Cells

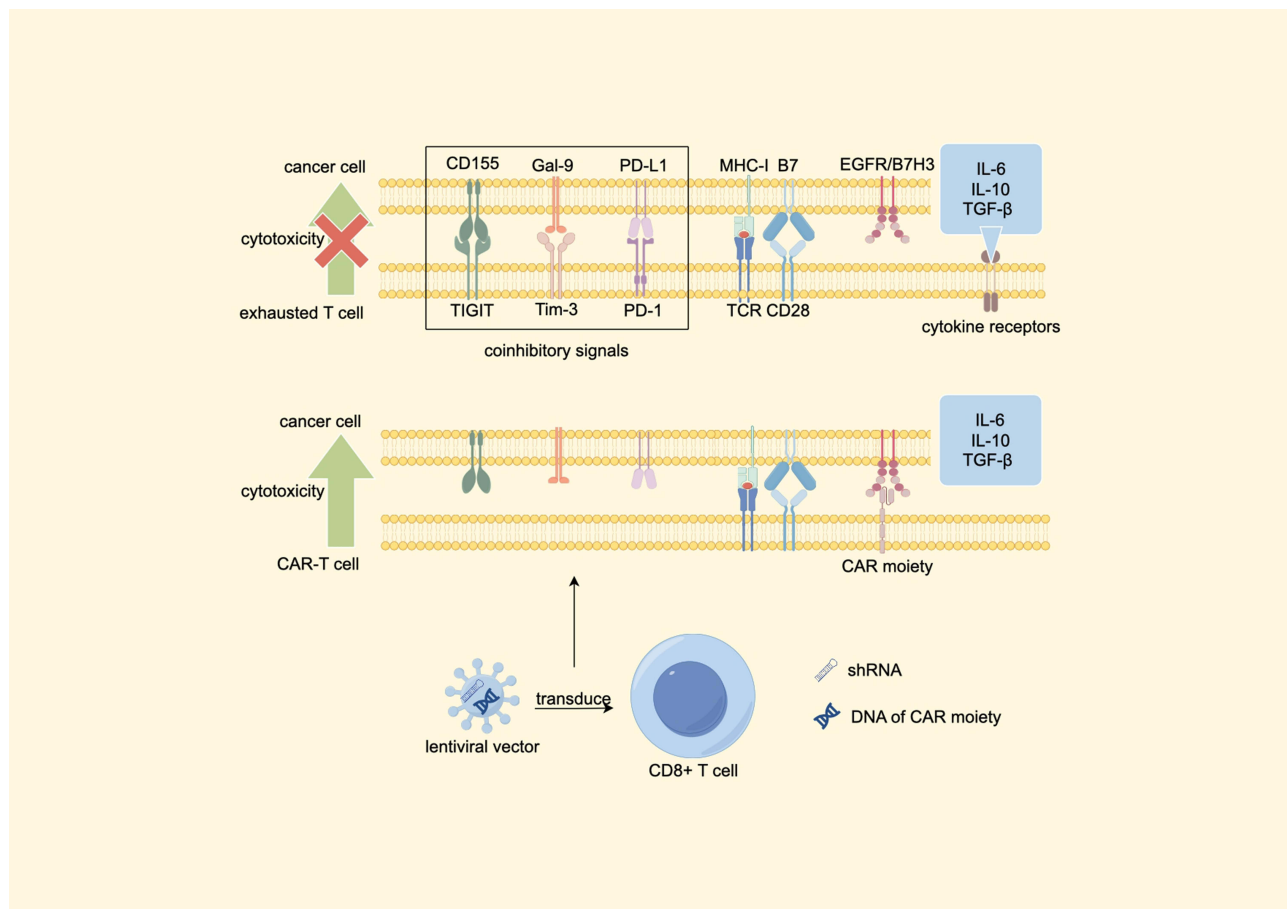
### CD8+ T Cell

CD8+ T cells, originating in the red bone marrow, possess a remarkable ability to selectively recognize tumor antigens and induce apoptosis in target cells by releasing cytotoxic substances like perforin and granzyme. This mechanism is pivotal in antitumor responses and forms the cornerstone of immunotherapy.

When CD8+ T cells become overactivated, several immune-checkpoint molecules including programmed cell death protein 1 (PD-1), cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), and T cell immunoglobulin and ITIM domain (TIGIT) can dampen immune responses. These mechanisms can also contribute to creating an immunosuppressive TME.<sup>38</sup> Research by Qiao et al demonstrate that downregulating PD-1, TIGIT, T cell immunoglobulin mucin domain-containing protein 3 (Tim-3), and certain immunosuppressive cytokine receptors within CAR-T cells significantly enhances their efficacy in suppressing BTC.<sup>39</sup> Additionally, the PD-1-CD28 switch receptor (SR) has shown the ability to convert the immunosuppressive signals from PD-1/PD-L1 interactions into stimulatory signals via CD28. CAR-T cells

engineered to include SR exhibit enhanced cytotoxicity against targeted tumor cells.<sup>40</sup> The mechanism of CAR-T cells with reduced immune-checkpoints and immunosuppressive cytokine receptors is shown in Figure 1.<sup>39</sup>

Several gene mutations that influence the activity and recruitment of CD8<sup>+</sup> T cells are highlighted as promising therapeutic targets for treating BTC. For instance, IDH1 mutations, which generate (R)-2-hydroxyglutarate [(R)-2HG], can hinder the accumulation of CD8<sup>+</sup> T cells.<sup>41</sup> (R)-2HG also restricts glycolysis and mitochondrial function in CD8<sup>+</sup> T cells, thereby impairing cytokine production in cholangiocarcinoma (CCA). IDH1 inhibitors can counteract this mechanism and promote the recruitment and activation of CD8<sup>+</sup> T cells. However, these inhibitors may also enhance the recruitment of regulatory T cells (Tregs) and the expression of CTLA-4. Therefore, combination therapy involving IDH1 and CTLA-4 inhibitors is utilized to achieve nearly complete tumor regression and sustained immunological memory.<sup>42</sup> Furthermore, the endoplasmic reticulum stress (ER) response gene, X-box binding protein 1 (XBP1), is found to be upregulated in CD8<sup>+</sup> T cells within the TME of BTC, particularly in those with low levels of PD-1 expression. In contrast, the rest of the CD8<sup>+</sup> T cells exhibit the opposite pattern. Via using the specific inhibitors of XBP1 (4 $\mu$ 8C), researchers report a downregulation of TIGIT in tumor tissue, suggesting the potential benefits 4 $\mu$ 8C have on BTC patients who are refractory to PD-1 blockers.<sup>43</sup> In addition, Liu et al report a negative correlation between the expression of B-cell-specific Moloney murine leukemia virus integration site 1 (BMI1) and CD8 in CCA samples. Subsequent investigations validate the effectiveness of BMI1 inhibitors in inducing the transcription of CD8<sup>+</sup> T cell-recruiting



**Figure 1** Lentiviral vectors carrying EGFR or B7H3 specific CAR moiety, together with shRNAs targeting immune checkpoints and cytokine receptors, are designed to transduce primary human CD8<sup>+</sup> T cells into CAR-T cells with decreased expression of PD-1, Tim-3, TIGIT and cytokine receptors. These CAR-T cells revealed an enhanced cytotoxicity.<sup>39</sup>

**Note:** By Figdraw.

**Abbreviations:** EGFR, Epidermal growth factor receptor; B7H3, B7 homolog 3 protein; shRNAs, Small hairpin RNAs; CAR T cell, Chimeric Antigen Receptor T Cell; TIGIT, T cell immunoglobulin and ITIM domain; Tim-3, T cell immunoglobulin and mucin domain-containing protein 3; PD-1, programmed cell death protein; TGF- $\beta$ , transforming growth factor- $\beta$ ; Gal-9, galectin-9; TCR, T cell receptor; MHC-I, major histocompatibility complex-I.

chemokines such as C-C motif ligand 5 (CCL5) and C-X-C motif ligand 9 (CXCL9).<sup>44</sup> Through investigating tumor tissues from patients treated by Gemcitabine, Oxaliplatin, Lenvatinib, and anti-PD1 antibody (GOLP) therapy, Lu et al report that GZMB+ CD8+ T cell, GZMK+ CD8+ T cell and proliferating CD8+ T cell are the three most active CD8+ subtypes in response to GOLP treatment. Intriguingly, these three clusters share high clonal similarities, suggesting common origins. Further cell trajectory analysis reveals that proliferating CD8+ T cell is at a naive stage with a relatively high exhausted score and low cytotoxic score, while GZMK+ CD8+ T cell is terminally differentiated with the highest cytotoxic score and lowest exhausted score. GZMB+ CD8+ T cell fall in an intermediate stage. CD5L+ macrophage can promote macrophage M2 polarization.<sup>45</sup> The interaction of CD5L+ macrophages with GZMB+ CD8+ T cells may contribute to their exhausted status, potentially leading to resistance to GOLP therapy in iCCA patients.<sup>46</sup> According to the study carried out by Li et al, about 45.6% of their iCCA patients have been infected by hepatitis b virus (HBV). They report an upregulation of the expression of CD3, CD4, and CD8 in HBV infected iCCA patients, showing that their immune system is activated. Meanwhile, TNFSF9, which suppress the expression of IL-12 and subsequently attenuates the cytotoxicity of CD8+ T cells,<sup>47</sup> is higher in HBV-negative iCCA patients. After inhibiting the expression of TNFSF9, there is a significant decrease in cell viability in HBV-negative iCCA organoids. The altered TME observed in HBV-positive iCCA patients may explain their better overall survival compared to HBV-negative patients.<sup>48</sup>

It is well established that CD8+ T cells confer significant prognostic value in BTC. A high density of CD8+ T cells, especially those in the tumor margin,<sup>49</sup> correlates with improved OS in BTC patients.<sup>50</sup> Moreover, the infiltration of PD-1–eomesodermin (EOMES)–CD8+ and Granzyme-B+CD8+ T cells is associated with a favorable clinical outcome in BTC patients. Notably, the prognostic impact of PD-1-EOMES-CD8+ T cells is predominantly observed within tumor areas rather than peripheries.<sup>51</sup> As a marker of activated CD8+ T cells in ovarian cancer,<sup>52</sup> a high level of CXCL13 expression are found to be an indicator of “hot” TME, thus leading to a better prognosis in GBC patients receiving immunotherapy.<sup>53</sup> Tissue-resident CD103+CD8+ T cells exhibit persistent antitumor effects within tumor tissues and may serve as prognostic predictors. Patients with higher infiltration of these T cells tend to benefit more from ICI therapies. This unique activity may be attributed to interactions between E-cadherin produced by cancer cells and CD103.<sup>54</sup> C-reactive protein (CRP), produced by the liver in response to inflammation, negatively correlates with the prognosis of iCCA patients.<sup>55</sup> The lymphocyte-to-CRP ratio (LCR), which combines lymphocyte count and CRP level, is validated as a prognostic marker across various cancers.<sup>56–59</sup> Similarly, iCCA patients with unresectable tumors often exhibit lower LCR values along with reduced CD8+ T cell densities.<sup>60</sup>

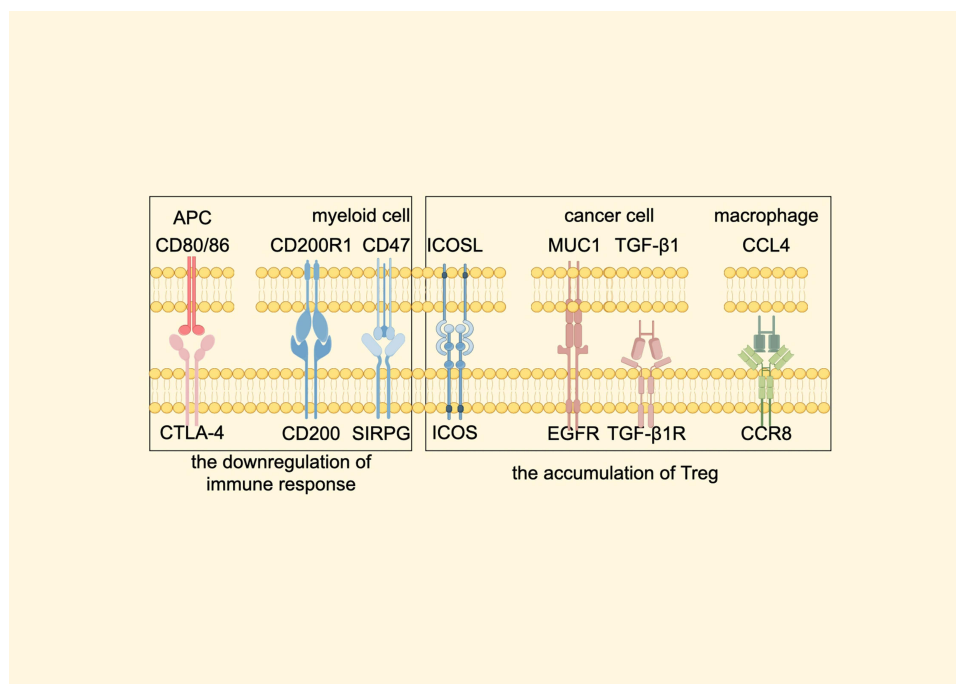
### Helper T Cell (Th Cell)

The interaction between MHC-antigen-peptide complex and T cell receptor (TCR), alongside co-stimulatory signals and specific cytokine milieu, drives the differentiation of naive CD4+ T cells into distinct functional subsets through activation of multiple signaling pathways. Once activated, Th1 cells exert cytotoxic effects on CD8+ T cells and directly target tumor cells by secreting interferon (IFN)- $\gamma$ , tumor necrosis factor (TNF)- $\alpha$ , and interleukin (IL)-2. In line with their antitumor activity, low expression of CD4 in both the tumor center and the margin is correlated with reduced OS.<sup>61,62</sup> Various strategies aimed at enhancing the expression of costimulatory molecules or suppressing immune checkpoints on CD4+ T cells are proven effective as supplements to traditional ICIs. *Listeria monocytogenes* (Lm) serves as an antigen provider for MHC class I or II-dependent presentation, contingent on whether Lm undergoes lysosomal degradation. This process stimulates cytotoxic lymphocytes and promotes cell-mediated immunity.<sup>63</sup> By deleting virulence factors internalin B and actA, the double-deficient strain *L. monocytogenes*  $\Delta$ actA/ $\Delta$ inlB (LmA1) exhibits reduced off-target toxicity while maintaining comparable antitumor responses to wild-type Lm.<sup>64</sup> Building upon this research, LmA1O, a derivative of LmA1 delivering the model antigen Ovalbumin (Ova), is shown to induce significant Th1 responses, reduce the expression of immune checkpoint molecules on CD4+ and CD8+ T cells, and prolong survival in mice with CCA and Hepatocellular carcinoma (HCC) expressing Ova.<sup>65</sup> Additionally, the CD40 agonist and mitogen-activated protein kinase/ERK kinase (MEK) inhibitor can also enhance the activation of CD4+ and CD8+ T cells via various mechanisms.<sup>66,67</sup>

## Regulatory T Cell (Treg)

The Forkhead box protein P3 (FoxP3) expressing Tregs dampen overactivated immune responses, while the crosstalk between Tregs and several other cells in TME can impede antitumor immune responses and further induce the accumulation of Tregs. Recombinant Human Signal-Regulatory Protein gamma (SIRPG) and CD200, known suppressors of immune responses,<sup>68,69</sup> are upregulated in the intratumoral Tregs.<sup>70</sup> Meanwhile, the interaction between Inducible T cell CO-Stimulator (ICOS) and ICOSL, pivotal in Treg accumulation,<sup>71</sup> is notably strengthened in tumoral tissues.<sup>70</sup> Several other interplay between Tregs and TME is shown in Figure 2.<sup>70,72,73</sup>

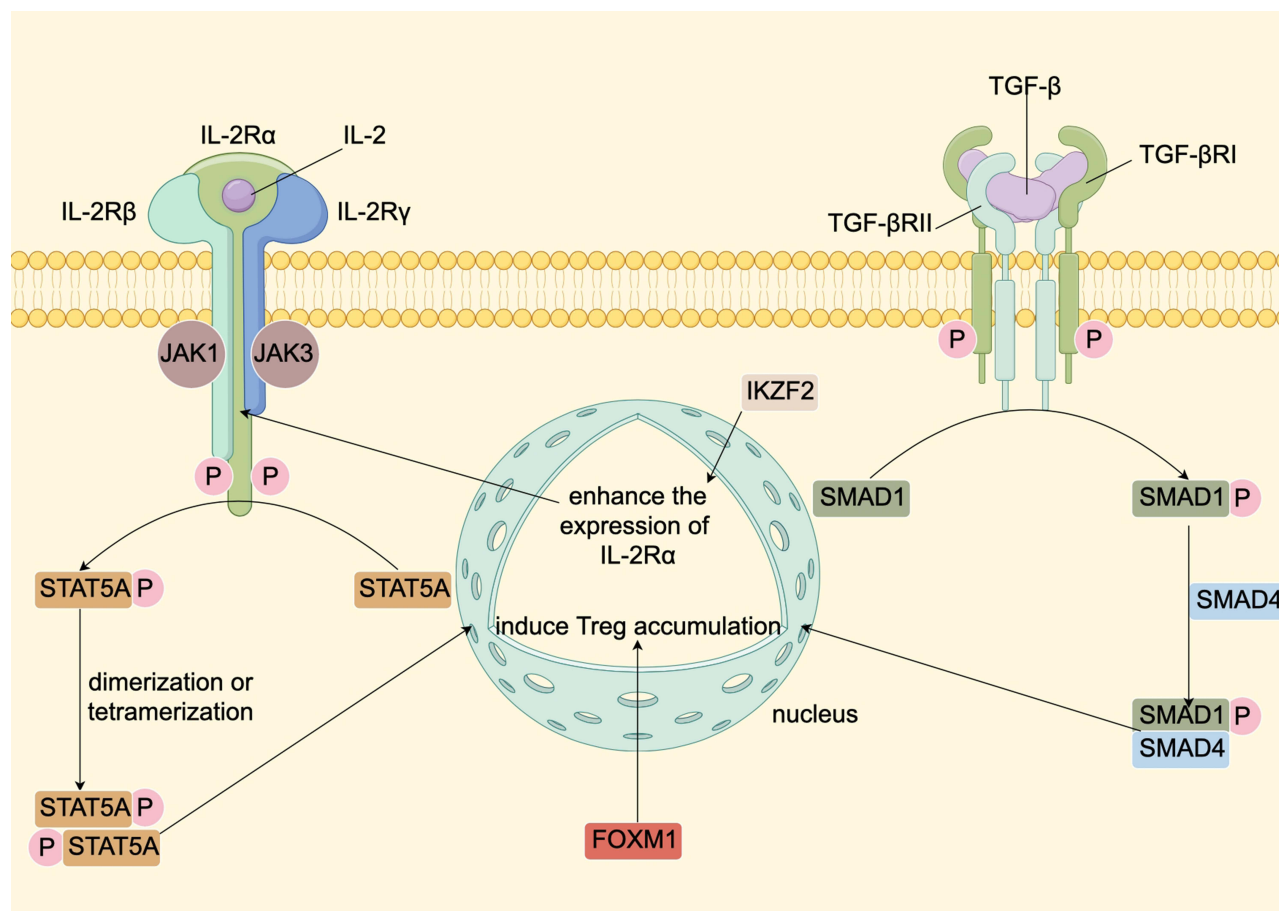
Upon infiltration into the TME of BTC, Tregs demonstrate heightened expression of several transcription factors (TFs). Forkhead box M1 (FoxM1), a member of the Forkhead box O (FOXO) family, binds to the FoxP3 promoter, thereby enhancing its transcription in CCA.<sup>74</sup> Similarly, IKZF2 increases the expression of FoxP3 and effector cytokines by upregulating the IL-2R $\alpha$ -STAT5B pathway.<sup>75</sup> NVP-DKY709, a selective molecular glue degrader of IKZF2, diminishes Treg immunosuppressive activity and restores cytokine production in exhausted T-effector cells.<sup>76</sup> Interferon regulatory factor 4 (IRF4) modulates genes critical for Treg differentiation and chemokine receptors expression and is more abundant in patients with advanced cancer.<sup>77</sup> Signal transducers and activators of transcription 5A (STAT5A), activated by IL-2, binds to promoter and enhancer elements to induce FoxP3 expression.<sup>78</sup> SMAD-1, a component of the TGF- $\beta$ 1 signaling pathway, facilitates Treg induction.<sup>79</sup> Enhancer of zeste homolog2 (EZH2) activity, inhibitable to disrupt Treg transcriptome, is also elevated in intratumoral Tregs.<sup>80</sup> Meanwhile, mesenchyme homeobox 1 (MEOX1) transduction leads to increased accessibility of multiple genes involved in immunosuppression in Tregs. The upregulation of the aforementioned TFs provides insights into the transcriptional and epigenetic landscape of tumor-infiltrating Tregs.<sup>70</sup> The TFs upregulated in the intra-tumoral Tregs and the pathways in which they are involved are shown in Figure 3.<sup>74,75,78,79</sup>



**Figure 2** The crosstalk between Tregs and other components of TME which is found enhanced in biliary tract cancer. The interaction between CD80/86 and CTLA-4 is important for the Treg-mediated inhibition of immune responses.<sup>70</sup> Through combining to their ligands, MUC1, TGF- $\beta$ 1 (secreted by cancer cell) and CCL4 (secreted by macrophage) can lead to the accumulation of Tregs.<sup>72,73</sup>

**Note:** By Figdraw.

**Abbreviations:** Treg, regulatory T cell; TME, tumor microenvironment; APC, Antigen-presenting cells; CCR8, Chemokine receptor 8; EGFR, Epidermal growth factor receptor; TGF- $\beta$ 1, Transforming growth factor- $\beta$ 1; ICOS, Inducible T cell CO-Stimulator; MUC1, Mucin1; CTLA-4, Cytotoxic T lymphocyte-associated antigen-4; SIRPG, Recombinant Human Signal-Regulatory Protein gamma.



**Figure 3** TFs upregulated in the intra-tumoral Tregs and the pathways in which they are involved. Upon binding of IL-2 and IL-2R, Jak1 coupled to the IL-2R $\beta$  and Jak3 on the IL-2R $\gamma$  will phosphorylate and activate each other. The activated Jak1 and Jak3 can in turn phosphorylate the tyrosine on the IL-2R $\beta$ , introducing a binding site for the STAT5A protein. The bound STAT5A protein is activated, generating dimers or tetramers and transferring to the nucleus, increasing FoxP3 transcription.<sup>78</sup> IKZF2 can induce the expression of IL-2R $\alpha$  and enhance the activity of IL-2R $\alpha$ -STAT5B pathway.<sup>75</sup> TGF- $\beta$  dimers can interact with TGF- $\beta$ RI and TGF- $\beta$ RII, inducing TGF- $\beta$ RII to phosphorylate TGF- $\beta$ RI and activating it. The activated TGF- $\beta$ RI is able to phosphorylate SMAD1, which has a strong affinity for SMAD4. The phosphorylated SMAD1/SMAD4 complex enters the nucleus and binds to transcription promoters to induce DNA transcription, leading to the Treg accumulation.<sup>79</sup> In addition, FOXM1 can play a similar role to STAT5A and SMAD1.<sup>74</sup>

**Note:** By Figdraw.

**Abbreviations:** TF, Transcription factor; Jak1, Janus kinase 1; STAT5A, Signal transducers and activators of transcription 5A; FoxM1, Forkhead box M1; TGF- $\beta$ , Transforming growth factor- $\beta$ ; FOXM1, forkhead box protein M1.

Intra-tumoral tertiary lymphoid structures (TLSs) serve as specialized sites for T cell activation and antigen presentation, correlating with improved prognosis in various solid tumors.<sup>81</sup> Conversely, peri-tumoral TLSs negatively impact overall survival in iCCA patients, potentially due to higher CD4+Bcl6+ follicular helper T cell (Tfh) and CD4+Foxp3+ Treg cell densities. Intriguingly, Treg cell frequencies within intra-tumoral TLSs escalate with increased peri-tumoral TLS density, suggesting a mechanism for inducing an immunosuppressive intratumoral environment.<sup>82</sup> Deemed as a blocker of leukocyte associated immunoglobulin like receptor 1 (LAIR-1),<sup>83</sup> LAIR-2 demonstrates efficacy in restoring exhausted CD8+ T cells in lung cancer.<sup>84</sup> Nevertheless, LAIR-2 is predominantly expressed by Tregs and CD8+GZMB+ T cells in CCA, highly associated with various immune inhibitory and stimulatory markers, thereby serving as a marker for T cell exhaustion.<sup>85</sup> Although Tregs accumulating at the tumor center typically signal poor prognosis in BTC patients,<sup>86</sup> TregIII, one of the three Treg subtypes,<sup>87</sup> exhibits a significant negative correlation with iCCA recurrence post-surgery. Higher TregIII infiltration is also linked to reduced perineural invasion.<sup>88</sup>

## B Cell

B cells, activated by the stimulatory signals from antigens and co-stimulatory signals from the interaction between CD40 and CD40L, play a crucial role in augmenting innate and adaptive immune responses through antibody production,

antigen delivery, and the release of cytokines and cytotoxic effector molecules. Early B cell Factor 1 (EBF1), involved in B cell differentiation, has emerged as a potential tumor suppressor in various cancers,<sup>89–92</sup> including CCA. Oxidative stress is reported to decrease the expression of EBF1, possibly facilitating CCA development.<sup>93</sup> This downregulation is likely mediated by DNA hypermethylation in the EBF1 promoter, with high methylation rates correlating with poorer prognosis.<sup>94</sup> Elevated infiltration levels of B cells in CCA are associated with improved prognosis, and the expression of pronociceptin (PNO) by B cells holds similar prognostic significance.<sup>85</sup> Unlike T cells, the specific pathogenic role of B cells in BTC remains unclear. Further exploration of B cell functions and their interactions with other immune cells within the BTC tumor TME holds promise for extending the efficacy and durability of immunotherapy strategies.

## Innate Immune Cells

### Dendritic Cell (DC)

Dendritic cells (DCs), functioning as antigen-presenting cells capable of producing various cytokines and fostering different T cell responses against diverse pathogens, wield significant influence over both anti- and pro-tumor activities, profoundly impacting clinical outcomes. DCs incubated with lysates of cancer cells are capable of generating a broad spectrum of tumor-associated antigens (TAAs), thereby enhancing their cytotoxic potential.<sup>95</sup> Investigations into this mechanism reveal promising therapeutic avenue. Honokiol, a pleiotropic compound isolated from magnolia species known for inducing apoptosis in cancer cells, shows potential in this context.<sup>96</sup> DCs loaded with tumor cell lysates from honokiol-treated CCA cells exhibit enhanced IL-12 secretion, facilitating heightened activation of CD8<sup>+</sup> T cells and Th cells.<sup>97</sup> Furthermore, DCs pulsed with tumor lysates and transduced with adenoviruses encoding human CD40L (Ad-hCD40L) exhibit elevated expression of both soluble (s)CD40L and membrane-bound (m)CD40L. These engineered DCs also show increased levels of costimulatory markers and cytokines. Co-culturing these DCs with cytokine-induced killer (CIK) cells results in augmented proliferation and cytotoxicity. Interestingly, soluble CD40L appears to induce predominantly Th2 cytokines, whereas membrane-bound CD40L predominantly induces Th1 cytokines.<sup>98</sup> Neoantigens derived from common mutations like TP53, KRAS, and RNF43 in CCA can also stimulate DCs during differentiation, enhancing their ability to selectively target cancer cells and activate T cells.<sup>99</sup> Conversely, interactions between tumor associated macrophages (TAMs), CCA cells, and DCs via IL-10 and Transforming Growth Factor Beta receptor (TGF- $\beta$ R) signaling contribute to reduced expression of MHC class II molecules, chemokine receptors, co-stimulatory molecules, and cytokines on DCs.<sup>100</sup> Subsequent studies employing lentiviral short-hairpin RNAs to knock down TGF- $\beta$ RII and IL-10RA mRNA in DCs have demonstrated improved enhancement of effector T cell cytotoxicity.<sup>101</sup>

Consistent with its activity in inducing an immunosuppressive TME in various tumors,<sup>102</sup> the accumulation of peritumoral Plasmacytoid Dendritic Cells (pDCs) is reported as a predictor of poor prognosis in iCCA patients and are positively correlated with peritumoral Treg abundance.<sup>103</sup> Moreover, the high expression of four m6A-related mRNAs (AIP, CEBPB, SDC1, and VPS25), associated with adverse outcomes in CCA patients, correlates with increased infiltration of resting DCs, suggesting a possible contribution to poor prognosis in the high m6A-related mRNA risk group.<sup>104</sup> In addition, CD1a, a transmembrane glycoprotein expressed by immature DCs,<sup>105</sup> has been linked to improved survival in GBC patients following surgery.<sup>106</sup>

### Natural Killer (NK) Cell

NK cells are cytotoxic lymphocytes of the innate immune system with potent activity against virally infected and/or transformed cells via direct cell killing and production of pro-inflammatory cytokines.<sup>107</sup> A plethora of methods to augment the cytotoxicity and longevity of NK cells are currently under clinical investigation. For instance, natural compounds are capable of sensitizing cancer cells to be susceptible to NK cells and display less side effects compared to traditional chemotherapy. Cordycepin, a bioactive compound from *Cordyceps militaris* extract, possesses anti-tumor properties. Suthida et al demonstrated that cordycepin upregulates mRNA expression of DR4 and DR5 on intrahepatic cholangiocarcinoma (iCCA) cells, enhancing the antitumor immune response and inducing NK cell cytotoxicity.<sup>108,109</sup> Genistein, an isoflavone derived from soybean products, is well documented for its anti-tumor effect, similarly upregulating DR4 and DR5 expression on iCCA cells.<sup>110</sup>

NK cell activity can also be modulated through alternative mechanisms. MHC class I polypeptide-related sequence A (MICA) and MHC class I polypeptide-related sequence B (MICB) are expressed in iCCA tissues under cellular stress and serve as ligands for NK group 2 member D (NKG2D), pivotal for NK cell activation and subsequent cancer cell elimination. However, endoplasmic reticulum protein 5 (ERp5) can bind to the MICA/B  $\alpha$ -3 domain and facilitate their cleavage. The monoclonal antibody 7C6 competes for binding to the MICA/B  $\alpha$ -3 domain, thereby preventing the loss of cell surface MICA/B and enhancing NK cell cytotoxicity in peripheral and tumor-infiltrating NK cells from iCCA patients.<sup>111,112</sup> Globo H ceramide (GHCer), a tumor-associated carbohydrate antigen, promotes tumor angiogenesis and immune evasion.<sup>113</sup> Existing research demonstrate that high Globo H expression is correlated with poor clinical outcomes in rats bearing iCCA. After the administration of anti-Globo H VK9, boosted cytotoxicity of NK cells and suppressed tumor growth are witnessed.<sup>114</sup> High mesenchymal–epithelial transition (MET) expression is discovered in 53.1% (135 of 254) of resected samples from CCA patients in a Chinese cohort study. High MET expression is also found to be correlated with short OS in CCA patients.<sup>115</sup> Therefore, CAR-NK cells containing humanized single-chain antibody fragment (scFv) targeting MET are designed and has revealed its potent activity to kill specific cancer cells.<sup>116</sup> In addition, Fukuda et al's immunohistochemical analysis reveals that high endogenous expression of CXCL9, a chemoattractant for activated NK cells, is associated with favorable postoperative survival in iCCA patients.<sup>117</sup>

## Neutrophil

Neutrophils, as versatile phagocytes involved in both tumor-promoting and tumor-suppressing roles, play complex and evolving roles in cancer biology. Zhou et al utilize whole-exome sequencing to identify SLIT2 mutations specifically in relapsed intrahepatic iCCA, where SLIT2 inactivation enhances neutrophil chemotaxis and contributes to metastasis.<sup>118</sup> In early GBC, upregulation of proteins associated with neutrophil degranulation (MPO, PRTN3, S100A8) is observed, while proteins involved in extracellular matrix (ECM) organization (COL14A1, COL1A2, COL6A1) are downregulated. This suggests that neutrophil infiltration is promoted in early GBC stages, with neutrophil degranulation facilitating ECM degradation and cancer cell invasion.<sup>119</sup> Interaction between GBC cells and neutrophils increases the expression of OLR1, a gene mediating oxidized low-density lipoprotein (oxLDL) uptake in neutrophils. This uptake leads to lipid peroxidation and ferroptosis in neutrophils, enhancing their pro-metastatic effects.<sup>120,121</sup> Multiplex immunofluorescent staining of iCCA tissues reveals co-distribution and strong correlation in local densities between tumor-associated neutrophils (TANs) and TAMs. Co-cultured TANs and TAMs secrete Oncostatin M (OSM) and IL-11, which promote iCCA tumor growth and metastasis. TANs express TAM chemoattractants (CCL2, CCL5, CSF1), while TAMs express chemokines (CXCL8, CSF3) that induce TAN infiltration, suggesting reciprocal crosstalk between these cell types.<sup>122</sup>

Sciellin (SCEL), a precursor of the cornified envelope, is demonstrated to be overexpressed in GBC tissues and to enhance carcinogenesis both *in vitro* and *in vivo* by stabilizing EGFR expression. Improved EGFR expression also induce the generation of IL-8, leading to the release of chromatin and granular proteins from polymorphonuclear neutrophils (PMN) and the formation of neutrophil extracellular traps (NETs), an extracellular fibrillar matrix that promotes the motility and migration ability of GBC cells.<sup>123,124</sup> Meanwhile, it is found that the binding of platelets to cancer cells can also increase the NET formation in iCCA,<sup>125</sup> which may consequently lead to adverse prognostic effects in CCA patients.<sup>126</sup> Through its interplay with  $\alpha$ V integrin, NET can increase the activity of NF $\kappa$ B, and consequently enhance the expression of vascular endothelial growth factor (VEGF), inducing the proliferation, angiogenesis, and metastasis in CCA. Interestingly, It is NET-DNA, rather than NET protein, that can induce tumor growth.<sup>127</sup>

Neutrophil infiltration levels in tumor regions negatively correlate with OS in patients with GBC post-cholecystectomy.<sup>128</sup> The neutrophil-to-lymphocyte ratio (NLR), reflecting circulating blood neutrophil/lymphocyte counts, emerges as a promising prognostic factor for tumor metastasis and surgical infectious complications in BTC patients.<sup>129–132</sup> Low albumin levels are also associated with significantly shortened survival, suggesting albumin as a potential prognostic biomarker for BTC patients.<sup>133</sup> To further optimize NLR's efficiency as a biomarker, the cachexia index (CXI), incorporating skeletal muscle index (SMI), albumin levels, and NLR, has been developed and shown to independently predict OS.<sup>134</sup> Meanwhile, multiple NLR-based models have been devised to predict long-term outcomes in BTC patients.<sup>135–138</sup>



## Macrophage

Macrophages, as pivotal phagocytic innate immune cells, exhibit distinct polarization states into pro-inflammatory M1 macrophages or anti-inflammatory M2 macrophages. During cancer progression, M1 TAMs initially dominate but are gradually supplanted by M2 TAMs.<sup>139</sup> Therapeutic strategies aimed at reducing M2 TAM polarization have thus emerged as promising anti-cancer approaches. Increased expression of secreted midkine (MDK) in GBC with epidermal growth factor receptor (ErbB) mutations promotes M2 TAM polarization via crosstalk with Low-Density Lipoprotein Receptor-Related Protein 1 (LRP1), highlighting MDK as a potential immunotherapy target.<sup>140</sup> Yes-associated protein (YAP) is a transcriptional regulator which can activate various oncogenic pathways/target genes.<sup>141</sup> Targeting at YAP, verteporfin is identified to induce a higher percentage of M1 TAMs.<sup>142</sup> Meanwhile, IGF2BP3 and leptin, involved in the GBC cells-derived exosomes, are also found to increase the proportion of M2-polarized TAMs.<sup>143,144</sup> Furthermore, the TME of KRAS-mutated BTC is characterized by a higher M1 macrophage activation and interferon- $\gamma$  expression.<sup>145</sup> The metabolic pathways of M1 and M2 macrophages are different. M1 macrophages prefer glycolytic metabolism, while M2 macrophages are in fond of mitochondrial respiration.<sup>146</sup> Immune responsive gene 1 (IRG1), encoding itaconate, can break the TCA cycle and restrain M2 macrophage polarization.<sup>147</sup>

Interactions between TAMs and the TME critically influence tumor progression. Yang et al demonstrate that TGF $\beta$ 1 derived from M2 macrophages upregulates the expression of aPKC $\zeta$ , which consequently activates NF- $\kappa$ B signaling pathway, leading to epithelial–mesenchymal transition (EMT) and immunosuppression in CCA. Reciprocally, macrophages are recruited by CCA cells via the secretion of CCL5.<sup>148</sup> It is revealed that the Macrophage migration inhibitory factor (MIF) can promote cancer metastasis and progression.<sup>149</sup> After the application of ICIs, MIF-CD74 signaling between cancer cells and TAMs is found to be enhanced. In addition, M1 gene signatures are found to be enriched in ICIs treatment-naïve TAMs, whereas M2 gene signatures and SPP1+ TAMs, which is reported to be correlated with poor prognosis, are enriched in TAMs after ICIs treatment.<sup>150,151</sup> These findings may help explain the limited efficacy of immune checkpoint inhibitors (ICIs) in iCCA. High-throughput RNA-sequencing by Luo et al identifies elevated miR-183-5p in exosomes from iCCA cells, inhibiting phosphatase and tensin homolog (PTEN) to increase PD-L1 expression on macrophages.<sup>152</sup> Involved in the exosomes from TAMs, Circ\_0020256 is also reported to promote the proliferation, migration and invasion of cancer cells by interacting with its intra-cellular microRNA target, miR-432-5p.<sup>153</sup>

As a main cause of malignant biliary diseases, Pancreaticobiliary reflux (PBR) is featured by a high-amylase bile.<sup>154</sup> To figure out how high-amylase bile promotes CCA progression, Wu et al measured the concentration of inflammatory factor in the tumor tissue after treatment of high-amylase bile and report an upregulation of IL-8 derived from macrophage.<sup>155</sup> IL-8 has been demonstrated to induce metastasis and tumor growth in iCCA via activating PI3K/AKT signaling pathway.<sup>156</sup> From chronic cholecystitis to GBC, the expression of Olfactomedin 4 (OLFM4) is gradually upregulated in the tumor tissue and is negatively correlated with survival in GBC patients. OLFM4 depletion can sensitize GBC cells to cis in vivo.<sup>157</sup> Via ligand-receptor pairs such as ICAM1-integrin, CCL15-CCR1 and AREG-MMP9, OLFM4+epithelium can interact with TAM intensively. Furthermore, OLFM4 is demonstrated to induce the expression of PD-L1, thus impairing the function of T cells.<sup>158</sup> Dermatotontin (DPT) is an extracellular matrix (ECM) protein rich in tyrosine. It is established that the expression of DPT is positively associated with the immune infiltration and the survival of CCA patients. The further study reveals DPT's activity to stimulate macrophages to release CCL19, which may increase the immune infiltration in CCA TME.<sup>159</sup>

## Myeloid-Derived Suppressor Cell (MDSC)

As stated by their name, MDSCs are immature myeloid cells with immunosuppressive activities. Guided by cytokines such as IL-6 and GM-CSF, MDSCs presence in resectable tumor regions is correlated with the aggressiveness of BTCs. It is reported that Anti-CSF-1R, a TAM blockade, fails to delay CCA progression, likely due to the compensatory upregulation of the expression of granulocytic MDSC (G-MDSC) chemoattractant CXCL2 by cancer associated fibroblasts (CAFs). Combined inhibition of MDSCs and TAMs has proven significantly more effective than monotherapy in subsequent studies.<sup>160</sup> Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), expressed predominantly on immune cells, can induce cell death by correlating with its receptor such as DR4 and DR5.<sup>108</sup> It is reported that TRAIL reduces the abundance of MDSCs by promoting MDSCs apoptosis in breast cancer.<sup>161</sup> However, MDSCs in CCA are

resistant to TRAIL-mediated apoptosis due to increased expression of cellular FLICE inhibitory protein, an inhibitor of proapoptotic TRAIL signaling. Moreover, TRAIL can enhance the activation of NF- $\kappa$ B, thus promoting proliferation of MDSCs in CCA.<sup>162</sup> MDSC recruitment by CCL2 from CAFs is another mechanism identified. Both the knockdown of fibroblast activation protein (FAP) and high tumor clostridia abundance are able to impair this mechanism.<sup>163,164</sup> Additionally, CAFs are demonstrated to promote the activity of MDSCs through the secretion of IL-6 and IL-33. Hyperactivated 5-lipoxygenase (5-LO) metabolism is mediated by these two cytokines in MDSCs. 5-LO consequently induces the synthesis of leukotriene B4 (LTB4), which interacts with leukotriene B4 receptor type 2 (BLT2) to facilitate the iCCA tumor progression, emphasizing this pathway as a potential target for immunotherapy.<sup>165</sup>

## Cancer Associated Fibroblast (CAF)

Mainly originating from hepatic stellate cells (HSCs) and portal fibroblasts (PFs) in BTC,<sup>166,167</sup> cancer associated fibroblasts (CAFs) are a very heterogeneous group of cells. The myofibroblastic CAFs (myCAFs) and the inflammatory CAFs (iCAFs), the two most abundant CAF subtypes in the CCA TME, are demonstrated to promote the progression of iCCA through distinct mediator. An upregulation of hyaluronan synthase 2 (Has2) are witnessed in myCAFs, while iCAFs can modulate the growth of carcinoma via the interplay between hepatocyte growth factor (HGF) and mesenchymal to epithelial transition factor.<sup>168</sup> Intriguingly, according to the transcriptional similarity between myCAFs and iCAFs, iCAFs might represent an inflamed immunologically activated population of myCAFs rather than an independent subtype.<sup>169</sup>

As a major cellular component of desmoplastic stroma of BTC, CAFs are activated myofibroblasts that express a wide range of factors, thus playing a pivotal role in BTC growth and progression.<sup>170</sup> Alpha smooth muscle actin ( $\alpha$ -SMA) is one of the specific markers of CAFs. Accordingly, patients with high  $\alpha$ -SMA expression in the tumor stroma have shorten survival.<sup>171</sup> Due to strong cell–cell interaction between CAFs and other cell types in TME, the number of CAFs and other cells can also help predict the prognosis of BTC patients.<sup>169</sup> Expressed by CAFs, transgelin-2 (TAGLN2) is witnessed to be upregulated in the serum of CCA patients and can be used as a diagnostic marker for BTC. The silenced TAGLN2 expression sensitizes BTC cells to gemcitabine and other chemotherapeutic drugs.<sup>172</sup> Furthermore, high expression of IL-33 in CAFs is correlated with better two-year survival of patients with BTC,<sup>173</sup> while the matricellular protein periostin is a poor prognostic factor in post-resected BTC.<sup>174</sup>

Yan et al find that CAF-derived Platelet-derived growth factor (PDGF)-BB is a regulator of lymph node metastasis in CCA. Via correlating with its receptor PDGFR- $\beta$  on lymphatic endothelial cells (LECs), PDGF-BB can upregulate lymphangiogenesis. Anti-PDGF-BB antibodies and PDGFR inhibitor STI571 significantly inhibit the activation of GSK3 $\beta$  and P65, leading to the suppression of tumor growth, revealing PDGF-BB/PDGFR- $\beta$ -GSK3 $\beta$ /P65 axis as a potential therapeutic target in CCA.<sup>175</sup> CXCR4 is increased tremendously in CAFs, and this overexpression is reported to contribute to the resistance to bortezomib of CCA tissue. Moreover, the combination of CXCR4 and bortezomib sensitizes CCA to anti-PD-1 treatment in the further study.<sup>176</sup> Polo-like kinase 1 (PLK1)'s ability to phosphorylate glucose-6-phosphate dehydrogenase (G6PD) and to transform hepatic stellate cell into CAFs phenotype allow it plays a dual role in the regulation of both tumor cells and CAFs. The sigma receptor, which is increased in tumor cells and CAFs, has high affinity with anisamide-derived ligands, making it an ideal target for targeted drug delivery system loaded with PLK1 inhibitor.<sup>177,178</sup> Thrombospondin 4 (TSP-4) is highly expressed by CAFs in GBC. TSP-4 can bind to its receptor on cancer cells to induce the phosphorylation of heat shock factor 1 (HSF1), which in turn upregulate the expression of TGF- $\beta$ 1 to promote the transdifferentiation of Peritumoral fibroblasts (PTFs) into CAFs, thus forming a positive feedback loop. This discovery provides a potential therapeutic target for GBC patients.<sup>179</sup>

Via single-cell transcriptomes analysis, Lewinska et al discovered that iCAF are responsible for upregulated Lysyl oxidase (LOX) expression in CCA. LOX is copper-dependent ECM protein which promote collagen and elastin cross-linking, leading to increased ECM stiffness.<sup>180</sup> CCA cells exposed to LOX show a significantly decreased expression of apoptotic genes and an increased cell migration. The interaction between LOX and mitochondrial transcription factor TFAM contributes to the increase in mitochondrial fitness, which in turn promote the metastasis of CCA. Accordingly, LOX is correlated with poor survival in CCA patients. Moreover, LOX overexpression is also found in CCA early lesions and livers of patients with primary sclerosis cholangitis (PSC), indicating that LOX is involved in CCA initiation.<sup>181</sup> Although both cancer-promoting CAFs (pCAFs) and cancer-restraining CAFs (rCAFs) have been reported in several

cancers so far,<sup>182</sup> only pCAFs have been found in CCA. Hu et al measure the CAFs' effects on the tumor progression by transwell assays and demonstrate the existence of both subtypes of CAFs in CCA. Intriguingly, pCAFs and rCAFs regulate the protein stability of polycomb group ring finger 4 (PCGF4) in a reversed way to affect the migration of the CCA. pCAFs activate the IL-6/phosphorylated STAT3 pathway to increase the stability of PCGF4, while rCAFs trigger the proteasome-dependent degradation of PCGF4.<sup>183</sup>

## ICI Combination Trials

In the Phase II, multi-cohort, non-randomized KEYNOTE-158 study, the efficacy of pembrolizumab, a PD-1 inhibitor, is assessed in 104 patients with previously treated BTC. The mOS is 7.4 months, and the mPFS is 2 months, with a poor objective response rate (ORR) of 5.8%. This study reported a patient with grade 5 renal failure and no patient with grade 4 treatment-related adverse events (TRAE).<sup>184</sup> The similar outcomes are shown in several other studies applying ICI monotherapy.<sup>34,185</sup> Moreover, ICI monotherapy and immune-based combinations may lead to increased risk of all-grade and grade 3–4 hypertransaminasemia.<sup>186</sup> These disappointing results prompt researchers to explore combination therapies involving ICIs and other drugs with lower TRAE incidence. Trials of ICI use in treatment to biliary tract cancers are shown in Table 1.

**Table 1** Trials of Immune Checkpoint Inhibitor (ICI) Use in Treatment to Biliary Tract Cancers

Study Name	Regimen	Mechanism	Phase	Patient Number	PFS (m)	OS (m)	Report Year	Citation	Line of Therapy
Immune checkpoint inhibitors monotherapy									
KEYNOTE-158	Pembrolizumab	PD-1	2	104	2	7.4	2020	[33]	2nd line
KEYNOTE-028	Pembrolizumab	PD-1	1b	24	1.8	5.7	2020	[33]	2nd line
NCT02829918	Nivolumab	PD-1	2	54	3.68	14.24	2020	[34]	2nd line
PD-1/PD-L1 plus CTLA-4									
NCT03101566	Nivolumab Ipilimumab	PD-1/CTLA-4	2	33	3.9	8.2	2022	[187]	1st line
NCT01938612	Durvalumab Tremelimumab	PD-L1/CTLA-4	2	65	/	10.1	2022	[188]	2nd line
NCT03482102	Durvalumab Tremelimumab Radiotherapy	PD-L1/CTLA-4	1	Ongoing	/	/	/	/	2nd line
PD-1/PD-L1 plus TGFβR									
NCT02699514	Bintrafusp alfa	PD-L1/TGFβRII	1	30	2.5	12.7	2020	[189]	2nd line
NCT03833661	Bintrafusp alfa	PD-L1/TGFβRII	2	159	1.8	7.6	2023	[190]	2nd line
NCT04066491	Bintrafusp alfa GemCis	PD-L1/TGFβRII	2	Terminated	/	/	2021	/	1st line
PD-1/PD-L1 plus VEGFR									
LEAP-005	Lenvatinib Pembrolizumab	PD-L1/VEGFR	2	31	6.1	8.6	2021	[191]	2nd line
NCT03895970	Lenvatinib Pembrolizumab	PD-L1/VEGFR	2b	32	4.9	11	2020	[192]	2nd line
IMbrave-151	Atezolizumab Bevacizumab GemCis	PD-L1/VEGFR	2b	79	8.3	/	2023	[193]	1st line
NCT03892577	Toripalimab Lenvatinib Radiotherapy	PD-1/VEGFR	2	20	13.7	10.8	2023	[194]	1st line

(Continued)

Table 1 (Continued).

Study Name	Regimen	Mechanism	Phase	Patient Number	PFS (m)	OS (m)	Report Year	Citation	Line of Therapy
ICI plus chemotherapy									
NCT03046862	Durvalumab Tremelimumab GemCis (BMC)	PD-L1	2	30	13	15	2020	[195]	1st line
NCT03046862	Durvalumab GemCis (3C)	PD-L1	2	45	11	18.1	2020	[195]	1st line
NCT03046862	Durvalumab Tremelimumab GemCis (4C)	PD-L1	2	46	11.9	20.7	2020	[195]	1st line
TOPAZ-1	Durvalumab GemCis	PD-L1	3	341	7.2	12.8	2022	[32]	1st line
KEYNOTE-966	Pembrolizumab GemCis	PD-1	3	533	6.5	12.7	2023	[196]	1st line
JS001-ZS-BC001	Toripalimab Gem S-1	PD-1	2	48	7	16	2021	[197]	1st line
ChiCTR2000036652	Sintilimab GemCis	PD-1	2	30	5.1	15.9	2023	[198]	1st line
IMMUCHEC	Durvalumab Tremelimumab Gem (arm A)	PD-1/CTLA-4	2	22	2.75	7.38	2022	[199]	1st line
IMMUCHEC	Durvalumab Tremelimumab GemCis (arm B)	PD-1/CTLA-4	2	22	5.98	12.32	2022	[199]	1st line
IMMUCHEC	GemCis (arm C)	/	2	35	8.7	16.93	2022	[199]	1st line
IMMUCHEC	Durvalumab Tremelimumab GemCis (arm D)	PD-1/CTLA-4	2	30	8.13	22.73	2022	[199]	1st line
IMMUCHEC	Durvalumab GemCis (arm E)	PD-1	2	29	6.37	15.3	2022	[199]	1st line
Other Strategies									
NCT02375880	DKN-01	DKK-1	1	47	8.7	/	2020	[200]	1st line
NCT04057365	Nivolumab DKN-01	PD-1/DKK-1	2	Ongoing	/	/	/	/	2nd line
NCT03937895	Allogeneic NK Cell Pembrolizumab	PD-1/NK Cell	1/2a	40	4.1	/	2022	[201]	2nd line
NCT05023109	Tislelizumab Ociperlimab GemCis	PD-1/TIGIT	2	Ongoing	/	/	/	/	/

**Abbreviations:** PD-1, programmed cell death protein-1; PD-L1, programmed cell death-ligand 1; CTLA-4, cytotoxic T lymphocyte-associated antigen-4; VEGFR, vascular endothelial growth factor; TGF $\beta$ -RII, transforming growth factor  $\beta$  receptor II; DKK-1, dickkopf-related protein 1; TIGIT, T cell immunoglobulin and ITIM domains; GemCis, cisplatin-gemcitabine; NK Cell, natural killer cell; ICI, immune checkpoint inhibitor; OS, overall survival; PFS, progression-free survival.

## Combination of PD-1/PD-L1 and CTLA-4 Blockade

Due to the joint effects PD-1/PD-L1 and CTLA-4 have on Effector T cells, Tregs, NK cells and macrophages, combination of inhibitors of these two immune checkpoints is expected to improve the poor prognosis of BTC patients. The phase II study (NCT03101566) of nivolumab and ipilimumab combination demonstrates improved mOS and mPFS in patients with advanced BTC. Of interest, the likelihood of survival at 2 years in this trial is significantly higher than in patients receiving chemotherapy alone. Treatment is feasible with no grade 3 or higher hematologic TRAE reported and the most common nonhematologic TRAE are Elevated aspartate aminotransferase and alanine aminotransferase.<sup>187</sup> The low

TRAE incidence of this combination is also confirmed in the Phase I trial (NCT01938612).<sup>188</sup> Recently, a phase II study investigating the combination of Tremelimumab plus Durvalumab with Radiation (NCT03482102) is ongoing.

## Combination of ICI and Transforming Growth Factor Beta Receptor (TGF $\beta$ R)

Bintrafusp alfa, the first bifunctional fusion protein comprising the extracellular domain of TGF- $\beta$ R2 and a PD-L1 blocker, is expected to revolutionize BTC treatment. A phase I study (NCT02699514) reports its promising antitumor activity in 30 previously treated patients. Importantly, five patients have responses lasting over 12.5 months, and responses are observed even in patients with immune-desert and immune-excluded TME, highlighting the significant value of bintrafusp alfa.<sup>189</sup> These results are further verified in the following phase II study (NCT03833661) including 159 patients.<sup>190</sup> Unfortunately, the phase II/III study (NCT04066491) evaluating bintrafusp alfa in combination with GemCis as a first-line treatment is terminated.

## Combination of ICI and Vascular endothelial growth factor receptor (VEGFR) Blockade

The combination of ICI and VEGFR inhibitors is highly anticipated in the treatment of advanced BTC due to their potential to reprogram the immunosuppressive TME into an immunostimulatory one.<sup>202</sup> In the ongoing phase II LEAP-005 study, the durable efficacy of lenvatinib plus pembrolizumab is observed in 31 patients previously treated for BTC. Encouraged by the promising results, the study enrollment is expanded to include 100 patients.<sup>191</sup> Patients receiving the same therapy report a much higher ORR in another phase IIb study (NCT03895970), probably due to a high percentage of patients who receive two or more anticancer treatments previously in this study.<sup>192</sup> To investigate the combination of PD-L1 and VEGF inhibition with chemotherapy as a first-line treatment for BTC, the phase II double-blind, placebo-controlled IMbrave-151 trial randomly assigns 162 participants to receive bevacizumab plus atezolizumab and GemCis or placebo plus atezolizumab and GemCis. The bevacizumab group shows a superior mPFS.<sup>193</sup> The feasibility of combining toripalimab and lenvatinib with radiotherapy (RT) is evaluated in the NCT03892577 clinical trials. This study randomizes BTC patients into the RT group or non-RT (NRT) group, demonstrating significant benefits for patients receiving RT. However, patients treated with RT have a higher incidence of Grade  $\geq 3$  treatment-related adverse events (TRAEs).<sup>194</sup>

## Combination of ICI and Chemotherapy

Through immunogenic cell death (ICD) and directly reduction of immunosuppressive cells,<sup>203,204</sup> chemotherapy is proven to be able to reach a better result in treatment to many malignancies, including lung cancer,<sup>205</sup> breast cancer,<sup>206</sup> nasopharyngeal cancer,<sup>207</sup> gastric cancer<sup>208</sup> and urothelial cancer.<sup>209</sup> Via the application of GemCis combined with anti-PD-1 and anti-CTLA-4 in orthotopic murine models of iCCA, Chen et al discover that GemCis induce effector CD8<sup>+</sup> T cell recruitment by the normalization of iCCA vasculature, which is caused by proangiogenic factors from cancer cells killed by GemCis, thus enhancing the antitumor effect of ICI.<sup>210</sup>

In a phase II study conducted in South Korea, 121 advanced BTC patients are randomly divided into three cohorts: in the biomarker cohort (BMC) to receive GemCis, followed by GemCis, durvalumab and tremelimumab; in the 3 combo cohort (3C) to receive GemCis and durvalumab; in the 4 combo cohort (4C) to receive GemCis, durvalumab, and tremelimumab. Amazing benefits are observed in all three cohorts. The most common Grade  $\geq 3$  TRAEs are neutropenia and anemia.<sup>195</sup> This study lays the groundwork for subsequent phase III trials.

KEYNOTE-966, the second phase III study investigating ICI plus chemotherapy treatment for BTC following TOPAZ-1, enrolls 1069 patients at 175 medical centers worldwide. Among all the participants, 533 receive pembrolizumab plus GemCis and 536 receive placebo plus GemCis between 2019 and 2021. The efficacy boundary for a statistically significant overall survival benefit for the pembrolizumab group is met in this study. The most common TRAEs are decreased neutrophil count and anemia. Of note, an absence of the relationship between PD-L1 expression and outcomes is observed in both KEYNOTE-966 and TOPAZ-1,<sup>196</sup> which is also reported in other studies.<sup>195,211</sup>

The safety and efficacy of toripalimab in combination with gemcitabine plus S-1 are assessed in the single-arm, phase II JS001-ZS-BC001 study in patients with advanced BTCs. Of the 48 treatment naive patients with BTCs, ORR is 27.1%, the mPFS is 7.0 months and the mOS is 16.0 months. The activation of PI3K pathway may associate with shorter PFS in this research.<sup>197</sup>

In a single-arm, phase II trial (ChiCTR2000036652), 30 BTC patients receive sintilimab plus GemCis as a frontline treatment. The presence of gene alteration in homologous recombination repair (HRR) pathway might bring additional benefits to patients in this study, compared to the wild-type group. Differential expression gene enrichment between responders and non-responders is mainly concentrated in cytokine–cytokine receptor interaction and chemokine signaling pathways. High levels of TAMs are associated with significantly shorter survival outcomes,<sup>198</sup> consistent with other studies.<sup>212</sup>

To determine the efficacy of the combination of durvalumab (D) and tremelimumab (T) in addition with Gem or GemCis, the IMMUCHEC study, which includes five arms, is conducted. The trial interventions: arm A (n=22): D, T and Gem; Arm B (n=22): D, T and GemCis (n=35); Arm C (n=35): GemCis; Arm D (n=30): D, T and GemCis; Arm E (n=29): D and GemCis. The omission of Cis results in inferior outcomes: the ORR is only 4.6% in arm A, while it is 18.2% and 26.7% in arm B and D. Regimen B in arm D seems to have some benefits to patients, with higher mPFS and mOS compared with arm B. The incidence of Grade  $\geq 3$  TRAE in both arms is almost unanimous.<sup>199</sup>

## Other Strategies

Dickkopf-1 (DKK1), a secreted WNT signaling modulator, can promote tumor growth by disabling antitumor cells. It is found that DKN-01, an inhibitor of DKK1, reduces tumor growth in mice with CCA.<sup>213</sup> The feasibility and efficacy of DKN-01 is also demonstrated in several other tumors.<sup>214,215</sup> In a phase I study, 47 BTC patients receive DKN-01 plus GemCis. This therapy is well tolerated by participants and is consistent with the toxicity profile of GemCis. It is noteworthy that improved outcomes are associated with biomarkers of angiogenesis inhibition and reduced inflammation. However, this combination fails to show an additional activity beyond historically reported efficacy of GemCis.<sup>200</sup> To further explore the antiangiogenic and immunomodulatory activity of DKN-01, the phase II study (NCT04057365) investigating Nivolumab plus DKN-01 is currently ongoing.

Based on the previous finding that allogeneic NK cells (SMT-NK) show cytolytic activity against CCA in mice<sup>216</sup> and clinical trial investigating SMT-NK in patients with lung cancer,<sup>217</sup> The phase I and IIa study (NCT03937895) is carried out. In this study, SMT-NK plus pembrolizumab are given to 40 patients. Data from the 23 patients who can be obtained efficacy evaluation for highlight its therapeutic potential.<sup>201</sup> Meanwhile, ociperlimab, a TIGIT inhibitor, in combination with tislelizumab and GemCis, are being tested in an ongoing phase II trial (NCT05023109).

## Biomarkers for ICI Therapy

Based on the KEYNOTE-028 clinical trial, patients with high tumor mutation burden (TMB) and high PD-L1 expression may benefit more from ICI therapy.<sup>218</sup> The first study exploring the correlation between genomic characteristics and immunotherapy response in BTC is carried out by Li et al. In their study, genetic mutations of LRP1B show higher TMB and predict for better immunotherapy response, which is in line with another multicenter analysis involving multiple-tumor types.<sup>219</sup> Better PFS is also discovered in patients with ERBB2 and PKHD1 alterations. However, the functional significance of ERBB2 missense mutations remained unclear, and ERBB2 mutations are higher in patients with worse survival in another study.<sup>220</sup> Furthermore, Chromosome arm-level somatic copy-number alterations (SCNAs) can also affect the response to immunotherapy. 19q amplification and 9p deletion are associated with poor prognosis in this research.<sup>221</sup> KRAS, correlated with poor prognosis in patients treated with ICI, is one of the most common mutations in BTC.<sup>220</sup> It is also found that patients with both KRAS mutation and PD-L1 positivity have longer PFS than patients with KRAS mutation and PD-L1 negativity. Interestingly, this finding is not discovered in patients without KRAS mutation.<sup>222</sup> Fatty acid-binding protein 1 (FABP1) is reported to induce the uptake of fatty acids to mediate lipid metabolism of cancer cells during metastasis.<sup>223</sup> The expression of FABP1 is significantly higher in direct liver invasion tumor than in primary tumor of GBC and is associated with lymph node metastasis and OS.<sup>224</sup> The stem cell marker octamer-binding transcription factor 4 (OCT4) maintains the pluripotent properties of embryonic stem cells and is demonstrated to induce

chemoresistance in CCA.<sup>225</sup> Compared with patients with no circulating tumor cell (CTC) or patients with OCT4-CTC, patients with OCT4+CTC have a higher chance of developing lymph node metastases and a worse survival.<sup>226</sup> Moreover, CD8+ T cell infiltration, NLR, cutaneous TRAEs, expression of immune checkpoint and DPT can also be used to predict the efficacy of ICI in BTC.<sup>159,220,227–230</sup>

## Future Directions and Conclusion

BTCs are rare and devastating solid tumors characterized by a highly heterogeneous TME comprising adaptive and innate immune cells. Due to the typically asymptomatic nature of BTC, there is significant variability in patient responses to immunotherapy, highlighting the challenge of developing personalized treatment strategies. Researchers can use molecular biology, genetics, bioinformatics, and other technologies to explore tailored treatment approaches based on specific gene expressions or mutations. Although the roles of immune cells in tumor progression were previously believed to have fixed positive or negative impacts, it is increasingly evident that their exact functions within the tumor microenvironment are influenced by various factors, such as TME crosstalk and tumor stage. It is feasible to employ single-cell sequencing to explore the phenotype and functional characteristics of different immune cell types within BTC tissues. We can also collect and analyze clinical samples from BTC patients, such as tissue biopsies or blood samples. Analyzing immune cell types, functional states, and levels of immune suppression factors in these samples provides insights into the immune status of BTC patients and its relationship with disease progression and treatment responses. While BTC often exhibit resistance to immune ICI monotherapy, combination therapies, which combine immunotherapy with emerging treatment modalities such as targeted therapy or gene therapy to form comprehensive treatment strategies, could offer BTC patients more treatment options and opportunities. To find the optimal combination, we should conduct preclinical studies to elucidate tumor microenvironment changes and potential resistance mechanisms when combining therapies. Clinical trials are needed to help determine treatment sequences, dosing schedules, and potential synergistic effects in the following research. Furthermore, identifying biomarkers for prognosis and treatment response in BTC patients is a critical research direction. Biomarker discovery can help identify patient populations best suited for immunotherapy, thereby advancing personalized treatment strategies. In order to find powerful biomarkers, it is crucial to collect and analyze biological samples from clinical patients, such as tissue biopsies or serum samples. By comparing immune therapy treatment responses and biomarker expressions in different patient populations, potential biomarkers associated with treatment response can be identified. Meanwhile, combining big data and bioinformatics analysis will help us integrate information from different sources, discover new biomarker candidates, and validate their potential value in immunotherapy. However, immunotherapy can induce immune-related adverse reactions such as immune-related hepatitis. As a result, focusing on optimizing treatment protocols to minimize adverse effects and identifying more effective management strategies is important. Currently, immunotherapy for BTC remains in the exploratory stage. Over the next five years, more clinical trials animal model studies will be necessary to verify its effectiveness and safety across different patient groups, particularly exploring its potential in advanced-stage BTC.

Overall, the future development of immunotherapy for BTC will depend on deeper understanding of immune mechanisms, which may inspire researchers to invent new combination therapies with minimized adverse effects. Patients can be treated by personalized treatment strategies targeting at specific gene expressions or mutations. Their response to immunotherapy will be predicted by measuring the expression of biomarkers. With advancements in technology and theory, we anticipate immunotherapy to play an increasingly important role in the treatment of the exquisitely heterogeneous BTC, providing new hope and choices for patients.

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