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REVIEW

Best practices in sample management and molecular profiling of cholangiocarcinoma: a practical guide

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ABSTRACT

Introduction: Cholangiocarcinoma (CCA) is an uncommon yet aggressive malignancy often diagnosed at advanced stages. Its management is challenged by significant molecular heterogeneity and limited treatment options. Advances in next-generation sequencing (NGS) have identified actionable alterations, such as FGFR2 fusions, thereby facilitating a precision oncology approach for CCA management.

Areas Covered: This review consolidates current evidence and expert insights on molecular profiling in CCA. It examines the histopathological subtypes and addresses diagnostic challenges associated with their diagnosis. Critical pre-analytical factors, including biopsy techniques, tissue handling, and tumor heterogeneity, are discussed in relation to their impact on molecular testing. The review also evaluates DNA-based versus RNA-based NGS methodologies, highlighting their strengths and limitations in detecting complex genomic alterations. The role of liquid biopsy as a minimally invasive tool for dynamic tumor monitoring is also explored.

Expert Opinion: The routine integration of molecular profiling for CCA requires the best histopathological diagnosis and pre-analytical preparation practices. Diagnostic workflows should prioritize meticulous tissue handling to ensure robust molecular analyses to avoid tissue exhaustion and preserve the integrity of nucleic acids. Employing DNA plus RNA sequencing platforms, supported by molecular tumor boards, is recommended to enhance patient stratification and guide therapeutic decision-making in CCA.

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Cholangiocarcinoma; precision medicine; molecular profiling; tissue analysis; next generation sequencing; targeted therapy

1. Introduction

Cholangiocarcinoma (CCA), a malignancy originating from the biliary epithelium, is a rare (incidence rate < 2 cases per 100,000 individuals) yet highly aggressive cancer, accounting for approximately 15% of primary liver cancers and 3% of all gastrointestinal malignancies [1]. The global incidence of CCA varies widely, with the highest rates reported in Southeast Asia due to endemic liver fluke infections and hepatolithiasis [2]. Several risk factors are implicated in the development of CCA, including primary sclerosing cholangitis, hepatolithiasis, parasitic infections, metabolic dysfunction-associated steatohepatitis (MASH), metabolic dysfunction-associated steatotic liver disease (MASLD), obesity, and type 2 diabetes, alongside genetic alterations [3]. Histopathologically, CCA is classified into intrahepatic (iCCA), perihilar (pCCA), and distal (dCCA) subtypes based on the anatomical location of the tumor [4].

Although surgical resection is potentially curative, approximately 70% of CCA cases are diagnosed at advanced, unresectable or metastatic stages, which is partly attributed to the insidious onset of symptoms [1]. Advanced CCA has been associated with a severe prognosis, with a 5-year overall survival (OS) rate of < 10%, median survival of <11 months in patients treated with chemotherapy, and approximately 4 months in those receiving the best supportive care [5–7]. The integration of molecular testing, particularly with the advent of next-generation sequencing (NGS), has significantly improved the treatment landscape and prognosis of CCA by identifying actionable genetic alterations with therapeutic implications. Several genetic alterations were identified for iCCA, including *IDH1* and *FGFR2*, and extrahepatic CCA (eCCA), including *KRAS*, *TP53*, and *SMAD4*, which impact tumor prognosis [8,9]. Among the most clinically relevant alterations are *FGFR2* fusions and *IDH1* mutations, predominantly observed in iCCA. FGFR inhibitors, such as pemigatinib and futibatinib, and the *IDH1* inhibitor, ivosidenib, are approved for patients with previously treated unresectable CCA and targeted alterations [10,11]. In addition, further agents – including zanidatamab and trastuzumab-deruxtecan (for *HER2* overexpression and/or *ERBB2* gene amplification), dabrafenib-trametinib (for $BRAF^{V600E}$-mutated tumors), pembrolizumab for microsatellite instability-high (MSI-high) CCA, as well as tumor-agnostic approvals of entrectinib/larotrectinib (targeting *NTRK* fusions), selpercatinib (targeting *RET* alterations), and PARP inhibitors (in patients with *BRCA1*/2 and *PALB2* mutations responding to platinum-based therapy) – expanded the approved

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Article highlights

- Rigorous histopathological diagnosis is essential to prevent tissue exhaustion and ensure sufficient material for molecular analysis.
- Optimal pre-analytical preparation – including careful biopsy technique, proper tissue handling, and judicious use of immunohistochemistry – helps improve nucleic acid yield and integrity.
- Standardized protocols for sample processing are critical for accurately detecting actionable genomic alterations in CCA.
- Combined DNA and RNA sequencing approaches are recommended for comprehensive molecular profiling, enabling reliable detection of point mutations, copy number alterations and gene fusion events.
- Targeted NGS panels should be selected and executed with an understanding of the specific limitations of amplicon-based versus hybrid capture methods to optimize the detection of actionable alterations.
- RNA-based NGS is particularly recommended for *FGFR2* fusion detection, given its superior sensitivity in identifying diverse fusion partners.
- Liquid biopsy offers a minimally invasive alternative for molecular profiling, although challenges related to sensitivity, specificity, and clonal hematopoiesis must be carefully addressed.
- Integrating molecular tumor boards is essential for interpreting NGS findings, enhancing patient stratification and guiding personalized therapeutic strategies.
- These foundational practices and NGS recommendations are prerequisites for the routine integration of advanced molecular profiling into clinical protocols for CCA management.

therapeutic options for CCA [12]. Several other targeted therapies are being tested for advanced CCA patients [13].

Consequently, molecular profiling in CCA has grown substantially in Europe. The European Society for Medical Oncology (ESMO) has emphasized the importance of routine molecular profiling in patients with advanced biliary tract cancer (BTC), including CCA [12]. The ESMO guideline recommends targeted multigene NGS panels covering level I actionable alterations in CCA patients [14]. Nonetheless, implementing NGS-based molecular profiling for CCA in routine clinical practice faces several clinical, logistical, and economic challenges [15–17]. From a pathological standpoint, the limited availability of high-quality tumor samples with sufficient DNA and RNA quantity is one of the key challenges in NGS profiling for CCA. Tissue exhaustion, low number of neoplastic cells, DNA degradation, or sample cross-contamination during handling and storage can also increase the risk of sample failure [15]. The heterogeneity of CCA also poses challenges, as small biopsies may not capture the full genetic landscape of the tumor [18]. Pathological workflows often lack integration with molecular diagnostics, leading to delays and inefficiencies in testing and reporting [15].

This expert opinion review integrated real-world insights with the latest evidence to outline the challenges and best practices for sample management and molecular profiling of CCA. The present review provides an overview of current NGS molecular profiling techniques and their limitations, focusing on *FGFR2* fusion detection. We also present our experience integrating the molecular tumor board (MTB) into clinical practice for CCA management.

2. Methods

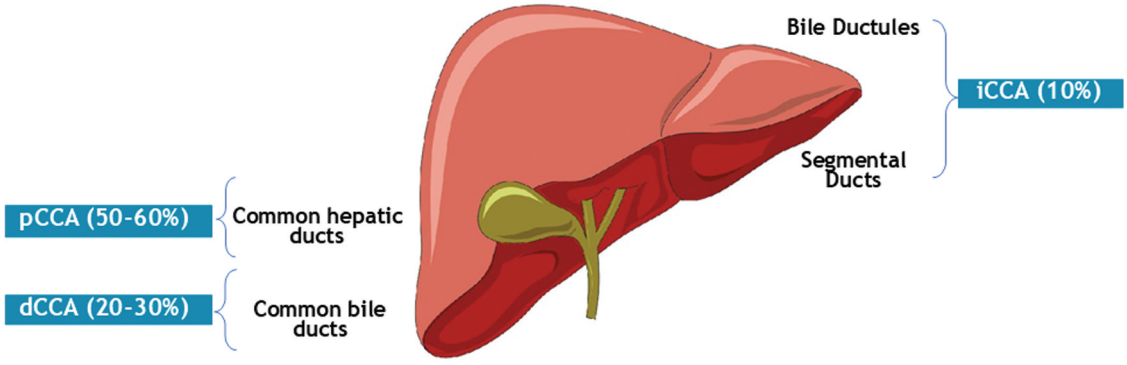
This expert opinion review was developed by integrating insights from a multidisciplinary panel of experts and a comprehensive bibliographic review of relevant literature. The expert insights were obtained during a preceptorship held in Barcelona on 7 November 2024. This in-person meeting brought together leading oncology, pathology, and molecular diagnostics experts with extensive experience in the management of CCA. The preceptorship was attended by European pathologists, and focused on sample management and molecular profiling of CCA to provide practical recommendations. To supplement expert insights, an online bibliographic search was conducted across multiple databases, including Medline via PubMed, Embase, Scopus, and Web of Science. The search strategy employed a combination of keywords and Medical Subject Headings (MeSH) terms such as 'cholangiocarcinoma,' 'molecular profiling,' 'sample management,' 'biopsy techniques,' 'diagnostic biomarkers,' 'tissue quality control,' 'next-generation sequencing,' 'liquid biopsy,' and 'precision oncology.' Boolean operators (AND, OR) were used to refine the search, and truncation was applied to include variations of the terms.

3. Precision medicine for cholangiocarcinoma: clinical value and survival benefits

The emergence of actionable genetic alterations in CCA (Figure 1) has provided the foundation for significant advancements in precision medicine. These alterations have led to multiple pivotal clinical trials evaluating the efficacy of molecularly targeted agents in patients with advanced CCA or BTCs [19], Table 1.

FGFR2 fusions are present in approximately 8–10% of patients with iCCA [32,33]. These fusions disrupt normal *FGFR2* signaling, driving oncogenesis and tumor proliferation [34,35]. This has led to developing of FGFR inhibitors based on clinical trials demonstrating meaningful efficacy in patients harboring these alterations. For instance, pemigatinib, a FGFR1–3 inhibitor, was evaluated in the phase II FIGHT-202 trial, involving patients with advanced or metastatic iCCA harboring *FGFR2* fusions or rearrangements. The trial reported an overall response rate (ORR) of 35.5%, with a median progression-free survival (PFS) of 6.9 months and a median OS of 21.1 months [20]. Infigratinib, another FGFR1–3 inhibitor, has also demonstrated significant efficacy in this population, with an ORR of 23.1%, a median PFS of 7.8 months, and a median OS of 23.1 months [36]. The phase II FOENIX-CCA2 trial evaluated futibatinib, an irreversible FGFR1–4 inhibitor. Futibatinib achieved an ORR of 42%, with a median PFS of 8.9 months and a median OS of 21.7 months [21]. Derazantinib, a multi-kinase inhibitor targeting FGFR1–3, is being investigated as an alternative FGFR2-targeted therapy, with an ORR of 21.4% and a disease control rate (DCR) of 74.8% [37]. Erdafitinib, another pan FGFR inhibitor, showed an ORR of 60% and a DCR of 100% [38]. Currently, pemigatinib and futibatinib are approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for patients with *FGFR2* fusion-positive CCA [39].

Although FGFR inhibitors have demonstrated significant efficacy, acquired resistance remains a challenge. Secondary mutations in the FGFR kinase domains, mainly N550 and V565 mutations, have been identified as a common resistance mechanism, prompting the development of next-generation



Actionable alterations	% iCCA	% eCCA
ERBB2/ HER2 amplification/overexpression	5.8%	1.3 -20%
FGFR2 fusion	10-16%	----
IDH1 mutations	13-30%	4.7%
NTRK fusion	3-9%	----
BRAF^{V600E} *mutations*	1.5%	----
BAP1 mutations and deletions	15-20%	----
BRCA1/BRCA2 mutations	3.1%	4.7%
MSI-H/dMMR	4.7-18.2%	4%
PI3K mutations	5%	7%
KRAS mutations	8-54%	36.7-46%
TP53	18-27%	18-68%
MTAP Loss	3%	1.1%

Figure 1. Frequency of actionable alterations in cholangiocarcinoma. BAP1: BRCA1 associated protein 1, BRAF: v-raf murine sarcoma viral oncogene homolog B, dCCA: distal cholangiocarcinoma, eCCA: extrahepatic cholangiocarcinoma, HER2 protein: erb-B2 receptor tyrosine kinase 2 (*ERBB2* gene), FGFR2: fibroblast growth factor receptor 2, iCCA: intrahepatic cholangiocarcinoma, IDH1: isocitrate dehydrogenase 1, KRAS: Kirsten Rat sarcoma viral oncogene homolog, MSI-H: microsatellite instability-high, NTRK: neurotrophic tyrosine receptor kinase, pCCA: Perihilar cholangiocarcinoma, PI3K: phosphoinositide 3-kinase, TP53: tumor protein P53.

Table 1. An overview of clinical trials evaluating targeted therapies in CCA.

Target (Gene)	Drug	Trials	Phase	No	ORR, % (95 CI%)	OS, months (95% CI)	PFS, months (95% CI)	TRAEs (Grade ≥3), %	References
FGFR2 fusion or rearrangements	Pemigatinib	FIGHT-202	II	107	35.5 (26.5, 45.4)	21.1 (14.8, NE)	6.9 (62, 9.6)	64%	[20]
	Futibatinib	FOENIX-CCA2	II	103	42 (32, 52)	21.7 (14.5, NE)	8.9 (6.9, 13.1)	48%	[21]
IDH1	Ivosidenib	ClarIDHy	III	185	2 (0.5, 6.9)	10.8 (7.7 to 17.6)	2.7 (1.6 to 4.2)	6%	[22]
BRAF^{V600E}	Dabrafenib plus trametinib	ROAR	II	43	51 (36, 67)	14 (10, 33)	9 (5, 10)	NR	[23]
HER2 overexpression or *ERBB2* amplification	Pertuzumab plus trastuzumab	Javle et al., 2021	II	39	23 (11, 39)	10.9 (5.2, 15.6)	4 (1.8, 5.7)	8%	[24]
	Zanidatamab	HERIZON-BTC -01	II	87	41 (NR)	15.5 (10.4, 18.5)	NR	21%	[25]
	Trastuzumab-deruxtecan	DESTINY-PanTumor02	II	41	26.8 (14.2, 42.9)	7 (4.6–10.2)	4.6 (3.1, 6)	39%	[26]
NTRK gene fusion	Entrectinib	Doebele et al., 2020	I/II	54	80 (67, 90)	NR	NE	NR	[27]
	Larotrectinib	Drilon et al., 2018	I/II	55	50	NR	NR	7%	[28]
	Repotrectinib	TRIDENT-1	I/II	88	Naïve: 62 (38, 82) Pre-treated: 42 (18, 71)	NR	NR	51%	[29]
MSI-high/dMMR	Pembrolizumab	KEYNOTE-158	III	351	30.8 (25.8, 36.2)	20.1 (14.1, 27.1)	3.5 (2.3, 4.2)	64.7%	[30]
RET fusion	Selpercatinib	LIBRETTO-001	I/II	45	43.9 (28.5, 60.3).	18 (10.7, NE)	13.2 (7.4, 26.2)	22%	[31]

CCA: Cholangiocarcinoma; iCCA: Intrahepatic cholangiocarcinoma; NE: Not estimable; NR: Not reported; OS: Overall survival; ORR: Objective response rate; PFS: Progression-free survival; TREAs: Treatment-emergent adverse events.

inhibitors [40,41]. Combination strategies involving third-generation irreversible FGFR inhibitors [42] and other therapies, such as immunotherapy or chemotherapy, are being investigated to enhance treatment durability and overcome resistance [43]. For instance, RLY-4008, a highly selective oral FGFR2 inhibitor, showed promising anti-tumor activity in patients with prior FGFR2 inhibitors, with a duration of response of 5.6 months [44]. Tinengotinib, a potent FGFR2 kinase domain inhibitor, was associated with an ORR and DCR of 34% and 89.7%, respectively, in patients with prior FGFR inhibitors [45].

Isocitrate dehydrogenase 1 (*IDH1*) mutations, identified in 14.3% of iCCA and 4.7% of eCCA [22,46–48], represent another actionable alteration. These mutations produce an

oncometabolite, 2-hydroxyglutarate, which disrupts cellular metabolism and promotes tumorigenesis [49]. The development of IDH1 inhibitors, such as ivosidenib, was fueled by the results of the ClarIDHy trial, which demonstrated improved PFS in advanced CCA patients with *IDH1* mutations compared to placebo (median PFS: 2.7 vs. 1.4 months, respectively; hazard ratio (HR) = 0.37, $p < 0.001$) [22]. Ivosidenib was approved by the FDA and EMA for patients with unresectable locally advanced or metastatic *IDH1*-mutated CCA.

HER2 overexpression (protein) and *ERBB2* amplification (gene), both involving the *ERBB2* gene that encodes the HER2 receptor – a member of the epidermal growth factor receptor (EGFR) family – are actionable molecular targets in iCCA and eCCA, with a prevalence of 5.8% and 13–20%, respectively [50,51]. *ERBB2* amplifications and HER2 overexpression contribute to tumorigenesis by activating downstream signaling pathways, including MAPK and PI3K-AKT [52]. Clinical trials evaluating HER2-directed therapies in CCA have shown promising results. In a phase II study, the combination of the HER2-directed monoclonal antibodies, trastuzumab plus pertuzumab, was associated with an ORR of 23% and a median PFS of 4 months in patients with *HER2*-positive BTCs [24]. Zanidatamab, a bispecific *HER2*-directed antibody, was recently approved by the FDA based on the results of the open-label single-arm HERIZON-BTC-01 (NCT04466891) trial. In patients with unresectable or metastatic *HER2*-positive BTC, zanidatamab led to an ORR of 41% and a median duration of response (DOR) of 14.9 months [53] and recently received positive advice from the European CHMP. Similarly, HER2-targeted antibody-drug conjugates (ADCs) such as trastuzumab deruxtecan (T-DXd) are being investigated in this population. Early-phase trials have demonstrated an ORR of 36.7% in patients with HER2-positive BTCs [54].

BRAF mutations, a key component of the MAPK signaling pathway, are in approximately 5% of iCCA, with 1.5% of the cases showing $BRAF^{V600E}$ mutation [55]. The combination of BRAF inhibitors (dabrafenib) and MEK inhibitors (trametinib) has shown promising efficacy in CCA patients with $BRAF^{V600E}$ mutations. In a pivotal phase II study, this combination achieved an ORR of 51% in patients with advanced BTCs harboring $BRAF^{V600E}$. Median PFS and OS were 9 and 14 months, respectively [23]. Other emerging targeted therapies include tyrosine kinase (TRK) inhibitors for patients with *NTRK* fusions [27,28] and therapies targeting alterations in the mismatch repair (MMR) pathway or high tumor mutational burden (TMB) [56], *RET* rearrangements and mutations [57], novel *KRAS* mutations (such as $KRAS^{G12C}$ mutation) [58], murine double minute 2 (*MDM2*) amplification [59], and *MTAP* loss [60].

4. Pre-analytical preparation: best practices for sample management

4.1. CCA histopathology: classification and challenges in the histopathological diagnosis

CCA is histopathologically classified into iCCA, pCCA, and dCCA subtypes based on the anatomical location of the tumor within the biliary tract [4]. iCCA, accounting for 10–50% of the CCA cases [61], arises from intrahepatic bile ducts and can be further subclassified into small-duct and large-duct subtypes with distinct anatomical origins, histological features, and molecular characteristics (Figure 2).

The small-duct subtype originates in the peripheral hepatic parenchyma and involves bile ducts and ductules within this region. Its growth pattern is typically mass-forming (MF) and is not closely associated with chronic biliary inflammation. Instead, its development is often linked to systemic or non-biliary conditions such as chronic viral hepatitis or non-biliary cirrhosis. The precursor lesions for small-duct iCCA remain poorly defined, though it was proposed that the tumor arises from the progenitor cells or mature hepatocytes of small intrahepatic bile ducts [62,63]. The small-duct iCCA is characterized by a tubular growth pattern with low-columnar or cuboidal epithelial cells surrounded by a desmoplastic reaction. Some components may also exhibit ductular or cord-like arrangements with slit-like lumina. Unlike large-duct iCCA, small-duct iCCA typically does not produce mucin, resulting in non-mucin-secreting glands. Perineural and lymphovascular invasion is less commonly observed, contributing to a potentially better prognosis than large-duct iCCA [63]. Immunohistochemistry (IHC) markers, such as epithelial membrane antigen (EMA/MUC1), CK7, and CK19, are typically expressed. Additionally, characteristic features such as CD56, NCAM, and C-reactive protein (CRP) are observed [64]. Small duct iCCAs are more commonly associated with *FGFR2* fusions, *IDH1* mutations, and *BAP1* loss [65].

In contrast, large-duct iCCA arises from the proximal intrahepatic bile ducts located near the hepatic hilum. Its growth pattern can be periductal infiltrating (PI) or combined PI and MF patterns. This subtype is strongly associated with chronic biliary inflammation and related conditions. Precursors to large-duct iCCA include biliary intraepithelial neoplasia (BiN) and intraductal papillary neoplasms, contributing to the tumor's progression [65]. Histologically, large-duct iCCA exhibits a ductal or tubular pattern with columnar to cuboidal epithelium embedded within a prominent desmoplastic stroma. Mucin production is a hallmark feature, with mucin-secreting glands observed in most cases. Perineural and lymphovascular invasion are more frequent in large-duct iCCA, contributing to its aggressive clinical behavior and worse prognosis compared to small-duct iCCA [66]. IHC markers such as EMA/MUC1, CK7, and CK19 are also expressed in this subtype, along with additional markers such as S100, TFF1, and AGR2, highlighting its distinct molecular profile [67]. Large duct iCCAs exhibit molecular alterations similar to eCCA, such as *KRAS*, *TP53*, and *SMAD4* mutations [65].

On the other hand, pCCA arises at the bifurcation of the right and left hepatic ducts. It accounts for 50–60% of all CCA cases [4]. Histologically, pCCA typically displays a dense desmoplastic reaction and well-formed glands infiltrating the periductal tissue [68]. dCCA arises from the bile ducts below the cystic duct and often presents with symptoms of obstructive jaundice due to its anatomical location. It accounts for 20–30% of CCA cases and shares histological features with pCCA, including glandular architecture and mucin production [69].

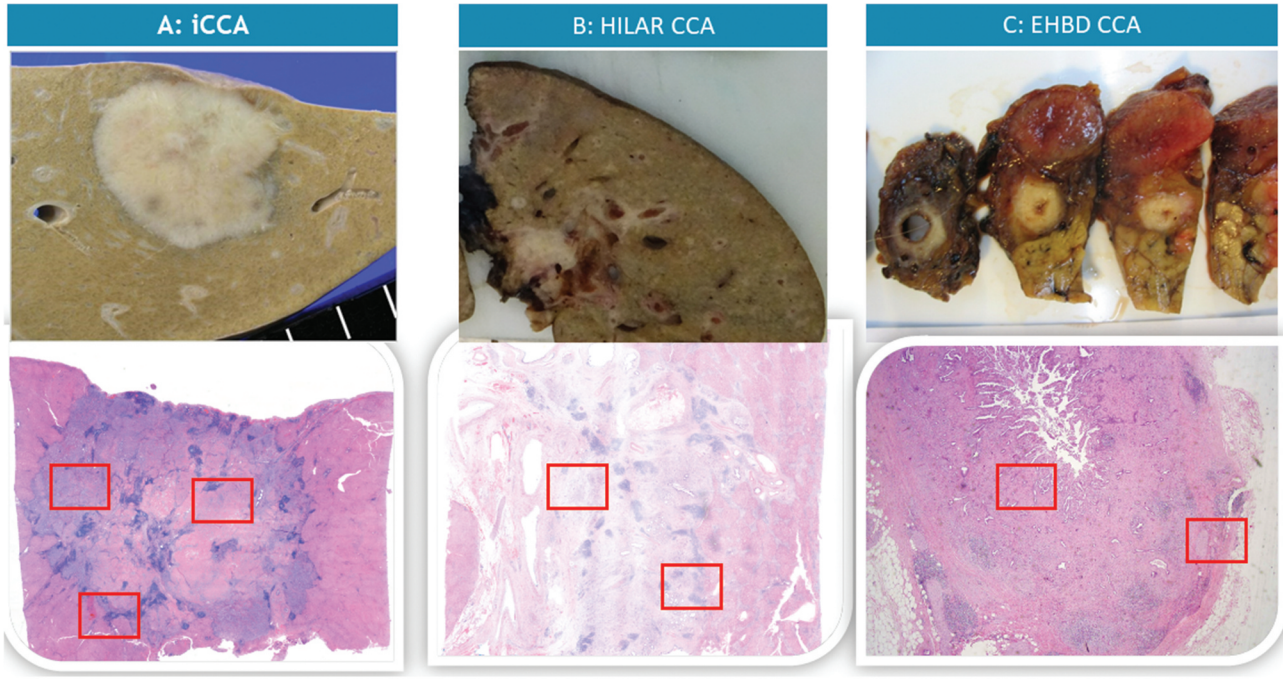


Figure 2. Intra-tumor heterogeneity in different subtypes of cholangiocarcinoma (CCA). Panel a depicts intrahepatic cholangiocarcinoma (iCCA), showing a well-circumscribed mass within the liver parenchyma with areas of necrosis and fibrosis. The histopathology slide highlights regions of cellular variation (red boxes), demonstrating tumor architectural diversity. Panel B represents hilar cholangiocarcinoma (hilar CCA), characterized by an infiltrative growth pattern with bile duct involvement. The corresponding histological section reveals stromal desmoplasia and tumor heterogeneity, as marked by red boxes. Panel C presents extrahepatic bile duct cholangiocarcinoma (EHBD CCA), exhibiting a more complex and fibrotic pattern with luminal obstruction. The histopathological image highlights distinct tumor subpopulations, emphasizing the variability in morphology within the same tumor.

The diagnosis of CCA from biopsy samples presents significant challenges. A primary challenge lies in distinguishing CCA from conditions such as primary sclerosing cholangitis (PSC), which shares overlapping clinical and radiological features. Histologically, PSC is characterized by inflammation and fibrosis, which can mimic the desmoplastic reaction observed in CCA [70]. Another diagnostic challenge arises in differentiating CCA from IgG4-related cholangitis. Both conditions may exhibit elevated serum IgG4 levels, adding complexity to the differentiation [71]. Distinguishing primary iCCA from metastatic adenocarcinoma remains a significant challenge. Both iCCA and metastatic pancreatic ductal adenocarcinoma exhibit immunopositivity for CK7 and CK19, limiting their diagnostic specificity. However, combining these markers with others, such as CRP, N-cadherin, BerEP4, and polyclonal CEA, has shown promise in distinguishing iCCA from metastatic lesions [72,73].

iCCA and hepatocellular carcinoma (HCC) are also challenging to tell apart since they have overlapping risk factors and imaging characteristics [74]. Combined hepatocellular-cholangiocellular carcinoma (cHCC-CC) is a rare and highly heterogeneous malignancy that exhibits features of both HCC and CCA [75]. The diagnosis of cHCC-CC poses significant challenges due to overlapping clinical, radiological, and histopathological characteristics with both primary liver malignancies. Histologically, cHCC-CC contains hepatocellular and biliary differentiation components within the same tumor. These tumors may exhibit a broad spectrum of morphologies, ranging from distinct hepatocytic and cholangiocytic areas to poorly demarcated regions where the two components are intermixed. The dual phenotype of cHCC-CC often requires an IHC panel to identify specific markers for each lineage. Emerging imaging techniques such as radiomics models using dynamic contrast-enhanced MRI (DCE-MRI) have shown promise in differentiating cHCC-CC from HCC and iCCA [75]. Additionally, cHCC-CCA exhibits a complex molecular landscape, often showing overlap with both HCC- and CCA-associated mutations, reflecting its biphenotypic nature [65].

Diagnosing CCA often requires multiple diagnostic tests, which can lead to tissue exhaustion in small biopsy samples. Tissue exhaustion and insufficient tissue may limit the ability to conduct comprehensive molecular analyses, potentially depriving patients of access to precision medicine approaches.

4.2. Sample biopsy and pre-analytical preparation-related Challenges:

Several pre-analytical challenges can increase the risk of sample failure for genomic profiling. The inherently low content of nucleated cells in CCA samples can lead to insufficient DNA or RNA yields and sample failure [76], particularly when dealing with small biopsy specimens. Surgical resection is not feasible for many patients with CCA, and biopsy specimens are the primary diagnostic and molecular testing source. Collecting adequate biopsy material from the biliary tree, especially for pCCA and dCCA subtypes, is technically challenging. These tumors are located in anatomically complex regions, and sampling is further complicated by the small caliber of bile ducts and the dense desmoplastic stroma characteristic of these malignancies. Percutaneous core needle biopsy (CNB) is

generally preferred over fine needle aspiration (FNA) or endoscopic brush cytology due to its higher tumor content and ability to provide tissue architecture [77]. However, CNB has limitations, such as small biopsy sizes, low tumor cellularity, and significant desmoplastic fibrosis, frequently resulting in insufficient material for molecular profiling [76]. Re-biopsy is often impractical for patients with advanced CCA or poor performance status and may entail some risks [78]. Tissue biopsy carries inherent risks, particularly in anatomically challenging or surgically inaccessible tumors. Complications such as bleeding, infection, and tumor seeding, although rare, can occur and may limit repeated sampling, especially in patients with comorbidities or fragile liver function [79]. While FNA or brush cytology can be considered, they are also limited by insufficient materials for NGS and low sensitivity for detecting BTCs [80,81].

Intratumoral heterogeneity is a prominent feature in CCA, whereby many tumor subclones coexist (Figure 2). These subclones may be intermixed within the tumor mass or spatially segregated across different primary tumor regions [18,82]. Metastases derived from the primary tumor also frequently exhibit additional heterogeneity, referred to as intrametastatic heterogeneity [82]. Intratumoral heterogeneity can lead to sampling bias during the biopsy, as small specimens may not fully represent the genetic diversity of the tumor. This may affect especially subclonal driver alterations, such as acquired resistance mutations in *FGFR2*-gene fusion carriers [83].

Low tumor cell content is one of the most common causes of sample failure during NGS profiling for CCA [76]. The desmoplastic stroma and fibrotic tissues in CCA samples dilute the tumor cell population, reducing the quantity and quality of tumor-derived DNA and RNA available for molecular testing and inducing false-negative results [15,81]. Many NGS-based platforms require a minimum tumor cell content threshold of 10–20% to identify variants and correctly remove sequencing artifacts [84]. In a previous analysis of 149 advanced BTC samples, the sample failure rate was 27% for tissue and 15% for liquid biopsy; the most frequent cause for sample failure was low tumor content < 20% [76]. Other reports have demonstrated low tumor cellularity in 21% of the BTC tissue samples [84,85].

Tumor ischemia and necrotic changes are common in CCA samples (Supplementary Figure S1), which may lead to degraded nucleic acids and compromised DNA quality [15]. Formalin fixation can introduce cross-links between proteins and nucleic acids, DNA fragmentation, and oxidative damage. These effects may lead to false-positive variant calls, most commonly involving thymine artifacts resulting from cytosine deamination [86]. Over-fixation may also reduce the efficiency of NGS sequencing, particularly for RNA-based analyses, due to nucleic acid damage [87].

4.3. Best practices for sample management

Accurate molecular profiling of CCA depends on effective sample management to preserve tissue integrity and ensure successful downstream analyses. Interventional radiologists and gastroenterologists should consider collecting adequate tissue material during biopsy procedures [81]. Where feasible, multiple biopsies from the same lesion may be considered to increase the likelihood of obtaining sufficient material for molecular testing [13]. Pathologists need to balance the requirements of histopathological diagnosis with the need to reserve sufficient tissue for NGS and other molecular tests. When tissue is insufficient or inaccessible, complementary diagnostic methods such as liquid biopsy may provide non-invasive alternatives for ctDNA molecular testing [15].

The clinical and radiological context should guide the diagnostic process to mitigate unnecessary tissue use, minimizing reliance on exhaustive tissue manipulation. Tissue can be divided into multiple paraffin blocks to ensure that material remains available for molecular testing even if one block is exhausted. Pathologists should consider minimizing the number of rounds of tissue sectioning and prioritizing the rational use of IHC to select the most relevant markers based on clinical and radiological findings [15]. When multiple tissue blocks are available, selecting the most suitable block for molecular profiling is critical. Blocks that have not been decalcified need to be prioritized since nucleic acids are critically damaged during this step. The ideal block should maximize the proportion of neoplastic cells relative to stromal or inflammatory components to improve nucleic acid yield and increase the likelihood of detecting genomic alterations [15].

Macrodissection can be a valuable tool for maximizing the neoplastic cell content of samples. Pathologists can identify and mark regions of high neoplastic cell content, ensuring that only tumor-rich areas are used for molecular testing. Non-neoplastic regions can be removed to preserve the quality and accuracy of the analysis. This approach is particularly useful when tumor cell content is low, but specific regions exhibit higher cellularity [88,89]. However, macrodissection should be performed carefully to avoid excessive complexity or overly small regions hindering analysis.

5. NGS-based molecular profiling for CCA

5.1. NGS techniques: advantages and limitations

In the clinical arena, targeted sequencing approaches play distinct roles in advancing precision medicine for CCA. Compared to genome-wide applications, such as whole-genome sequencing (WGS), whole-exome sequencing (WES), and whole-transcriptome sequencing (WTS), targeted NGS is associated with lower cost, shorter turnaround time, fewer infrastructure requirements, and greater throughput [81]. In this section, we focus on the design and execution of targeted NGS panels, which are recommended in clinical practice and adopted widely for CCA molecular biomarker determination [14]. This includes the selection of source materials (DNA vs RNA) and targeted NGS chemistry (hybrid capture vs amplicon-based).

Biomarker profiling needs to be considered first in the context of the nucleic acid type -DNA or RNA. DNA is characterized by its stability and suitability for detecting a broad spectrum of genomic alterations and can be extracted from a variety of sample types, including fresh tissue, formalin-fixed paraffin-embedded (FFPE) blocks, and even plasma in liquid

biopsy applications [90]. DNA-based NGS enables reliable identification of single-nucleotide variants (SNVs), insertions and deletions (indels), MSI status and copy number alterations (CNAs) [91]. In CCA, it is used to profile mutations of actionable genes such as *IDH1*, *BRAF*, or *ERBB2*. As such, gene panels can enable highly accurate detection of MSI status by sequencing mononucleotide microsatellite loci that lie within gene panels. For each microsatellite locus, the number of differently-sized repeats in experimental samples is compared to a population of normal controls [92]. Several hotspot microsatellite loci have been described in endometrial, colon, and stomach cancers [93]. This might become a challenge for MSI profiling in other tumor types, such as CCA. However, DNA-based gene-panel NGS approaches are limited in their ability to profile structural rearrangements, leading to gene fusions. To this end, RNA is particularly valuable for identifying fusions that may not be detected at the DNA level, such as *FGFR2* in CCA (discussed later). RNA sequencing also provides an understanding of alternative splicing events and differential gene expression [94]. However, RNA is less stable than DNA and is more susceptible to degradation, particularly in FFPE samples. Acceptable-quality RNA extraction is critical, which can be challenging in routine clinical workflows [95,96].

The two main chemistries for targeted NGS are hybrid capture and multiplex PCR/amplicon-based approaches (Figure 3). Amplicon-based approaches imply generating multiple polymerase chain reaction (PCR) products around specific genomic regions of interest. This option offers several advantages, including low cost, rapid turnover, and minimal input material, making it well-suited for small biopsy specimens [91]. The approach is highly sensitive to identifying SNVs and indels.

Despite its advantages, the amplicon-based enrichment chemistry also has some limitations. Generally speaking, the scalability of amplicon-based enrichment is typically limited to a few thousand products [97] and primer interactions, particularly in complex panels, can compromise sequencing uniformity [98]. Concerning point mutations and indel detection, allelic dropout, caused by mutations within primer-binding regions, can reduce amplification efficiency and inaccurate variant quantification [90]. In addition, end-point PCR amplification limits the ability of the amplicon-based approach to identify CNAs, such as *ERBB2* [98]. The accuracy of amplicon-based enrichment can be influenced by the bioinformatic thresholds for defining CNAs gain. High-level amplifications are generally more reliably detected than low-level gains, which may be prone to misclassification [99].

Furthermore, the amplicon-based approach is restricted to predefined genomic regions, requiring oligonucleotide primers that precisely define the target regions at their 5′and 3′ ends. This limits the ability to detect events without conserved 5′and 3′ genomic locations, such as genomic rearrangements. For RNA-based approaches, anchored multiplex PCR (AMP) is available, using an innovative approach that is only limited by one of its ends. Compared to other amplicon-based assays, AMP is highly sensitive to identifying gene fusions, particularly the multiple FGFR2 fusion partners in CCA [100].

Hybrid capture-based enrichment uses hybridization of probes to complementary sequences to selectively capture genomic regions. Unlike amplicon-based methods, which rely on predefined primers, hybrid capture offers greater flexibility and breadth, making it particularly suited for comprehensive molecular profiling. The homogeneity of target region coverage achieved in libraries based on hybrid capture is much higher than in amplicon-based chemistries [101]. Another significant advantage of hybrid capture is its ability to accommodate large and scalable sequencing panels [90], hence being highly effective in detecting a large number of genomic loci.

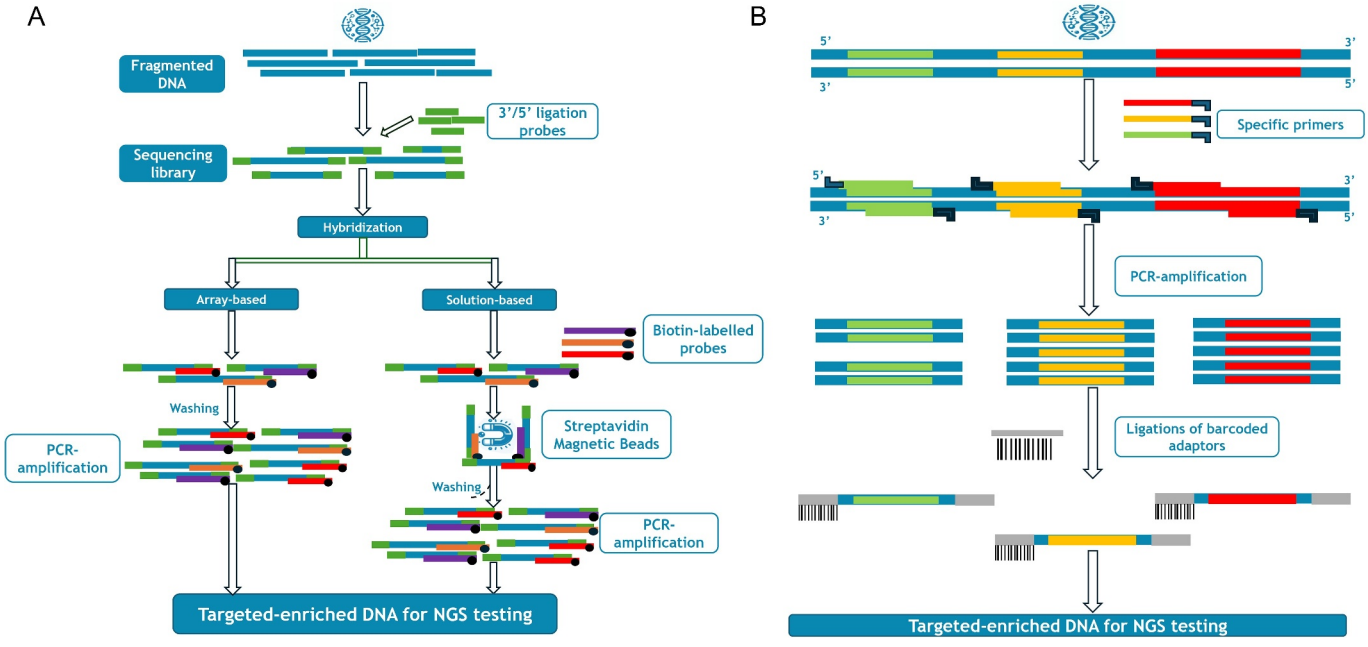


Figure 3. Approaches for targeted-enrichment DNA sequencing for next-generation sequencing (NGS) testing. Panel A represents hybridization-based capture techniques. Panel B depicts the amplicon-based enrichment method.

A clear advantage of hybrid capture is that flanking sequences to the targeted regions are not limited to the hybrid capture event, allowing the capture of regions close to rearrangement points [90]. Altogether, this allows profiling a wide range of genomic alterations, including SNVs, indels, CNAs, and rearrangements, as well as complex biomarkers such as MSI status and tumor mutational burden (TMB) quantification [46,81].

Despite its strengths, hybrid capture has limitations that must be considered. For FFPE-derived applications, hybrid capture typically requires higher input DNA or RNA amounts than amplicon-based methods (10ng vs > 100ng). The process is also more time-consuming and complex, resulting in longer turnaround times and higher costs [102]. Tolerance of hybrid capture to mismatches is limited to around 5, impacting the performance of capturing indels, especially when these fall in the central region of the probe. Another limitation lies in detecting rearrangement points in large DNA intronic regions. These regions can be highly repetitive and have reduced efficiency of probe binding. Repetitive sequences within introns may also increase the likelihood of off-target hybridization. This issue is particularly relevant in detecting specific *FGFR2* fusions or other structural variants with complex genomic architectures [81].

Thus, ideally, initial molecular profiling in CCA should be designed as a combined approach (DNA + RNA sequencing) to profile all relevant biomarkers to date. Table 2 shows the advantages and limitations of targeted NGS techniques.

5.2. NGS testing of FGFR2 fusions

Detecting *FGFR2* fusions in CCA presents unique challenges, requiring careful consideration of the sequencing approach. The architecture of *FGFR2* pathogenic fusions consists of a 5′ *FGFR2* exon1–17 portion (losing the most C-terminal part of the FGFR2 protein) fused to a 3′ partner (in general, contributing a dimerization domain) [103]. Although some are recurrent, the 3′ fusion partners are diverse and variable [13]. While DNA- and RNA-based NGS tests can be used for detection, RNA-based sequencing is often preferred [77]. DNA sequencing identifies gene fusions by detecting chromosomal breakpoints; chemistries that allow capturing these genomic fragments have been discussed in the previous section. However, this approach has notable limitations. RNA-based NGS, by contrast, sequences the fusion transcript product, allowing for direct identification of functional in-frame fusions and their partner genes. This approach also enables the detection of alternative splicing events, which cannot be identified by DNA-based methods [103]. RNA-based enrichment techniques such as hybrid capture and AMP are preferred to address these challenges. These methods are recommended by the ESMO for profiling *FGFR2* and *NTRK* fusions at the transcriptomic level, ensuring comprehensive and accurate fusion detection [12, 14]. However, RNA-based methods are not without challenges. RNA is inherently less stable than DNA and is prone to degradation, particularly in FFPE samples [15]. If RNA-based NGS is unavailable, optimized DNA-based panels with advanced bioinformatic pipelines can also be used to investigate gene fusions.

5.3. NGS testing of ERBB2 amplification and the "HER2 overexpression

NGS-based detection of *ERBB2* amplification can be performed through either low-pass WGS (LP-WGS) or targeted hybrid capture panels. Each approach has its specific strengths and limitations. LP-WGS is suitable for identifying broad copy number changes across the genome; however, its sensitivity depends heavily on the tumor fraction (typically requiring ≥ 3%) and may struggle to detect focal amplifications smaller than 1 Mb due to binning resolution limits [104]. In contrast,

Table 2. Advantages and disadvantages of NGS enrichment technologies. Table developed based on references [81,90].

Enrichment Method	Advantages	Disadvantages
Amplicon-Based Enrichment	– Low input requirement; suitable for small biopsy or degraded samples. – Cost-effective and fast turnaround time. – Customizable for focused panels targeting specific genes. – Works well for SNVs and indels	– Limited to predefined genomic regions (1000–2000 PCR products). – The DNA-based technique is not suitable to detect structural variants and CNAs. – RNA-based approaches require prior knowledge of both exon partners in gene fusions of interest for primer development. – Cannot detect novel fusion partners. – Prone to primer – primer interactions. – Allelic dropout can reduce efficiency. – Amplification biases in regions with high GC content.
Hybrid Capture Enrichment	– Broader and scalable, supports large panels for comprehensive profiling. – Effective for detecting complex structural variants, including gene fusions. – Fusion partner agnostic. – Concurrent analysis of distinct gene variants. – Provides uniform sequencing coverage across target regions.	– Requires higher input material (DNA/RNA). – More expensive compared to amplicon-based methods. – Longer processing time due to hybridization and washing steps. – Intronic regions may be included as enriched regions but may not be captured with the desired efficiency. – Capture probes allow mismatches but may have lower efficiency when > 5 mismatches are present between target and probe.
Anchored Multiplex PCR	– Effective for detecting fusions with 5′OR 3′ unknown partners. One of the exons involved in the gene fusion has to be predefined. – Lower probability of primer – primer interactions.	– RNA-based; prone to degradation and fragmentation. – Reliance on precise primer design is limited by the performance of primers in the PCR reaction.

CNV, copy number variation; NGS, next-generation sequencing; PCR, polymerase chain reaction; SNV, single nucleotide variation.

targeted gene panels, such as those using hybrid capture methods, benefit from flexible and higher-resolution binning, allowing for more reliable detection of small, high-level focal amplifications – such as those commonly seen in *ERBB2*—even at lower tumor fractions [105]. However, it is important to note that NGS-based quantification of amplification is semi-quantitative and can be influenced by factors such as coverage depth, normalization algorithms, and tumor purity. For instance, low coverage may obscure true gains, especially in samples with low-level amplifications, while normalization algorithms used to infer relative copy number can vary between platforms and pipelines, impacting the consistency and accuracy of CNA calls. Additionally, low tumor purity may dilute the amplification signal, making it more difficult to distinguish from background noise [105]. As such, NGS results for *ERBB2* amplification are often complemented by protein-level confirmation via IHC and/or FISH. Orthogonal validation by IHC and/or FISH remains essential for confirming HER2 status and guiding HER2-targeted therapy decisions.

6. Liquid biopsy: potential and pitfalls

Liquid biopsy is a minimally invasive technique that enables the detection of molecular biomarkers in bodily fluids, providing valuable insights into tumor dynamics and genetic alterations. Liquid biopsy offers the advantages of reduced procedural risks, ease of performance, and frequent sampling, allowing for longitudinal monitoring of tumor evolution, tumor burden metastasis, and treatment response [106,107]. In cancer clinical applications, this approach primarily focuses on profiling circulating tumor DNA (ctDNA) released by tumor cells through apoptosis, necrosis, or active secretion [108]. ctDNA carries tumor-specific genomic alterations, including point mutations, structural rearrangements, methylation changes, and copy number variations, differentiating it from cfDNA derived from normal cells. Plasma ctDNA is a part of the total circulating cell-free DNA pool (cfDNA), mainly derived from white blood cells [109]. ctDNA also exhibits unique characteristics, such as specific fragmentation patterns [110].

Recent advancements in sequencing applications have enhanced the sensitivity and specificity of liquid biopsy. Assays have been developed to detect a wide range of tumor-derived biomarkers, including SNVs, structural chromosomal alterations, and methylation changes, such as targeted sequencing, WGS, and whole-genome bisulfite sequencing (WGBS) [111]. These biomarkers have demonstrated utility in identifying actionable alterations, monitoring minimal residual disease, and predicting therapeutic response [111]. The ESMO Precision Medicine Working Group recommended ctDNA as an alternative when obtaining adequate tissue biopsy is not feasible [112]. Although plasma is the most widely utilized sample for liquid biopsy, ctDNA can be detected in various biological fluids, depending on the tumor's anatomical site. In CCA, bile-derived ctDNA/cfDNA was found to have a higher concordance rate with tissue biopsy than plasma ctDNA/cfDNA [113,114] and, upon additional validation, could become an alternative liquid biopsy source [81].

Despite all its undeniable advantages, liquid biopsy faces its own challenges. Limitations include false positive and negative results, affecting concordance rates between liquid biopsy and tissue analysis.

Detectable ctDNA levels in plasma depend on several biological and pathological factors, including tumor type, tumor size, vascularization, proliferative activity, and stage [115]. Within CCA, iCCA demonstrates higher ctDNA levels in plasma than eCCA, with studies reporting concordance rates of 92% between ctDNA and tissue-based mutational profiles in iCCA [116]. False-negative results in liquid biopsy are associated with low ctDNA levels that lead to, on many occasions, very low mutant allele fractions [117].

Sequencing depth plays a pivotal role in the sensitivity and accuracy of ctDNA analysis and in detecting low variant allele frequencies (VAF). In a study comparing ctDNA and tumor tissue samples, the mean sequencing depth for ctDNA was significantly higher than for tumor tissue samples [116]. However, increasing sequencing depth also presents challenges. Higher depths can amplify sequencing errors, necessitating robust error correction methods to distinguish true mutations from artifacts. The limit of detection (LoD) in NGS has been optimized for liquid biopsy management by the use of unique molecular identifiers (UMI)-based approaches and improved bioinformatic analysis, reaching the 0.01% VAF [118,119].

Similar to the situation in tissue, another challenge is encountered in gene fusion detection. In an NGS analysis of 1,671 BTCs, the concordance between ctDNA and tissue sample DNA was overall good for biomarkers such as *IDH1* mutations (87%) or *BRAF V600E* (100%), but was low for *FGFR2* fusions; however, it is worth noting that the ctDNA was compared to tissue DNA in only a small subset of cases [120]. The latter is most likely due to the poor testing performance of hybrid capture in intronic regions, rich in repetitive and homopolymeric regions, in addition to the frequent low tumor fraction [121]. In a recent report from our center, liquid biopsy detected 88.9% of the *FGFR2* fusion in patients with iCCA and known *FGFR2* fusion upon careful design and tiling in the *FGFR2* intron 17 region [121]. As previously discussed, RNA-based assays are often preferred for identifying active fusion transcripts, but plasma samples lack sufficient circulating RNA for widespread clinical testing.

Clonal hematopoiesis (CH) describes the presence of somatic mutations in the bone marrow or peripheral blood resulting from clonal hematopoietic stem and progenitor cell (HSPC) expansion [122]. CH or clonal hematopoiesis of indeterminate potential (CHIP) related variants are frequently detected in liquid biopsy [123], complicating the differentiation between tumor-derived mutations and other hematopoietic-borne genomic alterations. Studies have shown that up to 15% of *TP53* mutations identified in plasma correspond to CH rather than tumor-derived origins [123]. Additionally, ctDNA fragments harboring cancer-associated mutations may be shed from predisposing nonmalignant conditions such as PSC and cirrhosis, increasing the risk of false-positive results [124]. This reinforces the necessity for further well-designed prospective studies to validate specificity and predictive value across diverse clinical scenarios.

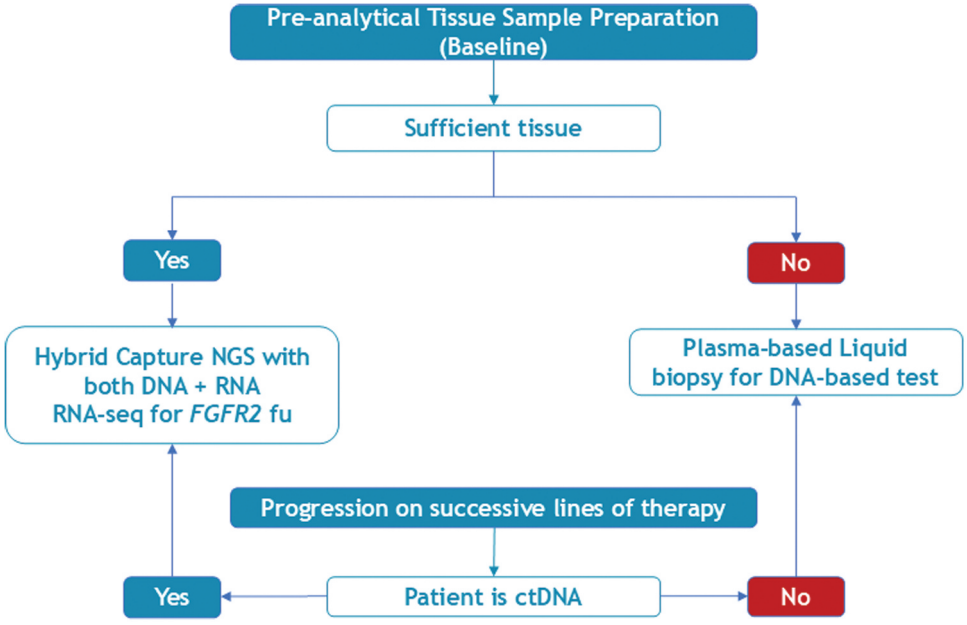


Figure 4. A practical workflow for molecular profiling of cholangiocarcinoma. The decision-making process is based on tissue availability. If sufficient tissue is available, hybrid capture next-generation sequencing (NGS) is performed using both DNA and RNA sequencing. In cases of insufficient tissue, a plasma-based liquid biopsy is conducted for DNA-based testing. Upon disease progression, ctDNA analysis may guide further therapeutic decisions.

In our opinion, liquid biopsy has promising applications in CCA despite its limitations (Figure 4). CtDNA analysis is generally a good alternative to tissue analysis when a short turnaround time is needed. Additionally, the quantification of ctDNA tumor fraction (TF) has emerged as a robust prognostic marker, positively correlated with OS in all common cancers [109]. In BTC patients, cfDNA VAF was associated with worse OS and shorter response to treatment [120]. A recent report also indicated that higher ctDNA levels correlated with worse OS in patients with FGFR2 fusion-positive CCA [121]. Liquid biopsy offers dynamic monitoring of therapeutic efficacy by assessing changes in specific biomarkers during treatment. For instance, alterations in fusion allele fraction (FAF) levels were shown to correlate with treatment responses to FGFR inhibitors. A reduction in FAF indicates partial or complete tumor response, while a lack of clearance signals potential resistance or progression [121].

A notable advantage of ctDNA is its ability to capture a broader genomic landscape of the disease by reflecting alterations from multiple tumor lesions, thereby potentially overcoming the spatial heterogeneity limitation of single-site biopsies [125]. This characteristic may be particularly valuable in CCA, where multifocality and intertumoral heterogeneity are common. Lastly, liquid biopsy may allow the identification of *FGFR2*-related genetic alterations in ctDNA, including mutations and fusions that are associated with acquired resistance [120]. These alterations are detected in plasma even when not found in tumor tissue biopsies, demonstrating the sensitivity of liquid biopsy for molecular profiling [83,120].

7. Value of molecular tumor boards

MTBs have emerged as a pivotal component in integrating precision oncology into clinical practice, particularly in complex malignancies such as CCA. These boards typically consist of clinical oncologists, treating physicians, pathologists, molecular biologists, and geneticists who collaborate to interpret molecular profiling results and develop individualized treatment strategies. This collaborative framework ensures that complex genomic data are not viewed in isolation but are contextualized within the patient's overall clinical picture, thereby enhancing the ability to assign personalized therapies [126].

One of the primary roles of MTBs is to provide a structured platform for discussing challenging cases. In CCA, where histopathological heterogeneity and complex molecular alterations often complicate treatment decisions, MTBs facilitate the integration of genomic insights into clinical decision-making [127]. By convening experts from various disciplines, MTBs can critically assess the quality and relevance of molecular profiling data. This process not only supports the identification of actionable alterations but also helps in matching patients with appropriate targeted therapies, clinical trials, or off-label treatment options (Figure 5) [128]. Studies have demonstrated that patients whose cases are reviewed by MTBs often experience improved outcomes, attributable to more informed therapeutic choices and timely intervention [129].

The efficacy of MTBs depends heavily on robust collaboration between pathology and genomics laboratories and clinical teams. Close cooperation is essential to ensure that tissue samples are processed optimally to avoid issues such as tissue exhaustion and maintain nucleic acid integrity for downstream NGS analyses [130]. As discussed in earlier sections, standardized pre-analytical protocols facilitate the generation of reliable molecular data, which forms the backbone of MTB deliberations. This collaborative environment streamlines the process of identifying patients eligible for targeted therapies, thereby optimizing the use of genomic testing in treatment planning [131].

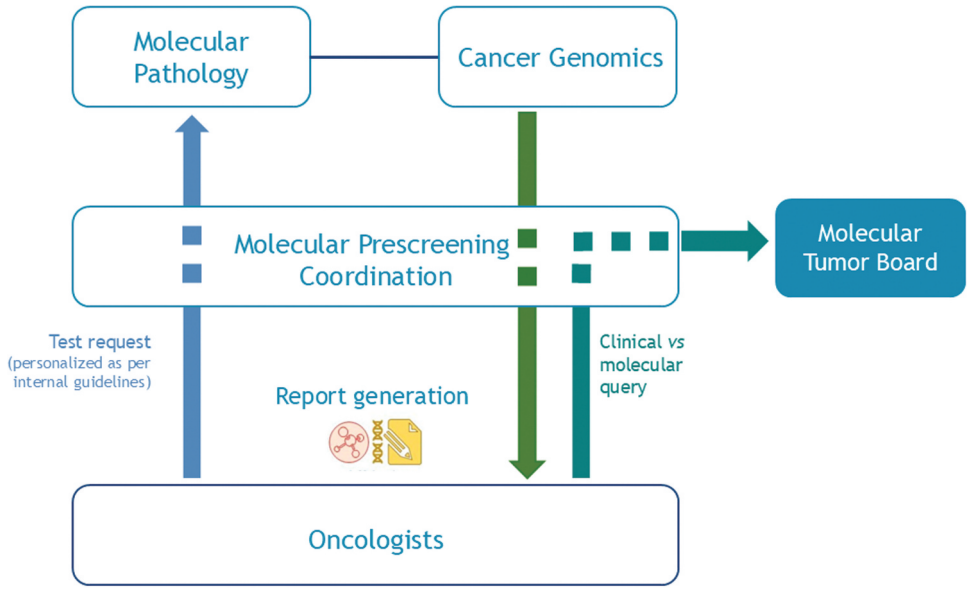


Figure 5. Workflow of molecular prescreening coordination for precision oncology. The diagram illustrates the interaction between molecular pathology, cancer Genomics, and oncologists through a centralized molecular prescreening coordination process. Test requests are initiated based on internal guidelines, and molecular data is integrated with clinical queries. Findings are discussed at the molecular tumor board to guide patient-specific treatment decisions. The generated reports support oncologists in selecting targeted therapies, ensuring a streamlined approach to precision oncology.

From an operational standpoint, MTBs can potentially improve the overall efficiency of cancer care. The structured discussion format of MTBs helps bridge the gap between rapidly evolving genomic technologies and their practical application in clinical settings, thus fostering a learning environment where successes and challenges are openly discussed and addressed [132].

Despite these advantages, several challenges still face the widespread adoption of MTBs. Variability in institutional resources, differences in the availability of genomic technologies, and a lack of standardized protocols across centers can limit the uniformity of MTB practices [132].

Looking forward, the future of MTBs lies in developing efficient network systems that harmonize patient selection criteria, molecular profiling technologies, and therapeutic recommendations across various institutions. In parallel, robust training programs and accreditation processes for laboratories performing NGS-based assays will be essential to ensure the quality and consistency of molecular data. As the number of actionable biomarkers continues to grow, MTBs will be instrumental in facilitating the transition from traditional histologic classification to a more genomically driven approach.

8. Expert opinion

The integration of advanced molecular profiling into the management of CCA holds transformative potential for real-world outcomes. Recent advances in NGS technology – particularly combining DNA and RNA-based approaches – can significantly enhance diagnostic accuracy and inform treatment guidelines. In clinical practice, a more precise understanding of the genetic landscape of CCA can lead to earlier and more accurate diagnoses, thereby enabling the selection of targeted therapies such as *FGFR*2 and *IDH1* inhibitors. Furthermore, incorporating liquid biopsy platforms offers a minimally invasive means of longitudinal tumor monitoring, which is critical for assessing therapeutic response and detecting emergent resistance mechanisms. Despite these promising advances, adopting molecular profiling for CCA in clinical practice faces several challenges, including tissue exhaustion during histopathological evaluation, variability in pre-analytical processing protocols, and limitations of some NGS techniques.

Improving the integration of molecular profiling into routine care necessitates a focus on best practices in both histopathological diagnosis and pre-analytical preparation. Ensuring optimal tissue handling begins at the biopsy stage, where techniques must be refined to maximize tumor cellularity and minimize sample degradation. Standardizing tissue processing protocols and judiciously selecting IHC markers are essential to prevent tissue exhaustion and preserve nucleic acid integrity. Additionally, a tailored approach incorporating DNA and RNA sequencing is recommended to capture the full spectrum of genomic alterations. While DNA-based methods are robust for detecting point mutations and copy number variations, RNA-based NGS is superior for identifying gene fusions, especially *FGFR2* fusions, which are known to have various fusion partners and complex rearrangements. In cases of tissue limitation, one should consider and decide upon a sequential approach: DNA testing (including liquid biopsy as an option); if positive, RNA testing is unnecessary. Additionally, due to certain NGS chemistry-specific technical limitations and/or tissue constraints for NGS, one might consider conducting further IHC for MMR and *HER2* status.

Tumoral heterogeneity and the tumor microenvironment further complicate the genetic characterization of CCA. Intratumoral clonal heterogeneity often challenges genetic profiling – particularly in cases of mixed iCCA – and intermetastatic diversity [82]. In addition, developing a reactive microenvironment in response to tumor growth is

a functional hallmark of CCA. The complex interactions between malignant cholangiocytes and the surrounding stromal and immune cells influence tumor behavior and represent key targets for novel therapeutic interventions. These dynamic spatial and temporal changes in tumor subclonal composition may lead to an underestimation or bias in the identified mutational landscape if genetic characterization is performed on a single tumor biopsy [133–135]. Recognizing these complexities is critical. Therefore, a thorough understanding of the histological characteristics and microenvironmental context of each CCA subtype is essential for optimal sampling, handling, and subsequent interpretation of molecular profiling results.

The current research landscape in CCA molecular profiling is rich with opportunities for further advancement. Although significant progress has been made, there remains no definitive endpoint in achieving comprehensive tumor characterization. Continued refinement of NGS methodologies, including improved hybrid capture techniques and advanced bioinformatics pipelines, is critical to overcoming the inherent challenges of low tumor content, sample heterogeneity, and the detection of complex structural rearrangements. In parallel, further validation of liquid biopsy platforms is warranted. Although liquid biopsies have some challenges, such as low ctDNA levels, clonal hematopoiesis, and limited sensitivity and specificity in certain settings, their minimally invasive nature and ability for dynamic monitoring emphasize their potential as a complementary diagnostic tool. Future research should also explore integrating multi-omic data, including proteomics and epigenomics, to provide a more holistic view of tumor biology, thereby enhancing patient stratification and informing personalized therapeutic strategies.

Looking toward the future, the field of CCA molecular profiling is poised for significant evolution. In the next ten years, we anticipate standard procedures incorporating more automated and standardized protocols for tissue processing and genomic analysis. Advances in sequencing technologies are likely to reduce costs further while increasing sensitivity and specificity, thereby enabling more widespread adoption of these techniques in both academic and community settings. Moreover, as our understanding of tumor heterogeneity and resistance mechanisms deepens, integrating serial liquid biopsy monitoring into routine practice may become standard, allowing for real-time adaptation of therapeutic strategies. While immuno-oncology and other emerging fields also hold promise, the precision provided by molecular profiling will remain a cornerstone of personalized cancer care, particularly for malignancies such as CCA that have historically been challenging to treat.

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