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REVIEW ARTICLE – TRANSLATIONAL RESEARCH

Optimizing Circulating Tumour DNA Use in the Perioperative Setting for Intrahepatic Cholangiocarcinoma: Diagnosis, Screening, Minimal Residual Disease Detection and Treatment Response Monitoring

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ABSTRACT In this review, we present the current evidence and future perspectives on the use of circulating tumour DNA (ctDNA) in the diagnosis, management and understanding the prognosis of patients with intrahepatic cholangiocarcinoma (iCCA) undergoing surgery. Liquid biopsies or ctDNA maybe utilized to: (1) determine the molecular profile of the tumour and therefore guide the selection of molecular targeted therapy in the neoadjuvant setting, (2) form a surveillance tool for the detection of minimal residual disease or cancer recurrence after surgery, and (3) diagnose and screen for early iCCA detection in high-risk populations. The potential for ctDNA can be tumour-informed or -uninformed depending on the goals of its use. Future studies will require ctDNA extraction technique validations, with standardizations of both the platforms and the timing of ctDNA collections.

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The incidence of intrahepatic cholangiocarcinoma (iCCA) is rising worldwide, and the 5-year overall survival (OS) remains as low as 10% for all stages of the disease.[1-5] Late diagnosis of iCCA is one of the main contributing factors to the poor OS. Patients are often asymptomatic at diagnosis and traditional tumour markers, like carbohydrate antigen 19-9 (CA19-9), greatly lack specificity in the screening of high-risk populations.[6] Consequently, only 20% of people with newly diagnosed iCCA have localized disease that is amenable for curative-intent surgery.[7] For those who undergo surgery, however, the probability of experiencing an iCCA recurrence is as high as 70%, underscoring the need to identify high-risk groups for potentially utilizing more aggressive or personalized adjuvant treatments.[5,7-9]

In search of new effective diagnostic tools and therapies for this lethal disease, many studies have documented the common genetic alterations found in iCCAs.[10,11] IDH1 mutations (15–20%) and FGFR2 fusions (10–20%) are the most prevalent alterations considered "actionable" with Food and Drug Administration (FDA) approved targeted therapies.[12,13] For example, the IDH1 inhibitor, ivosidenib, has shown to improve progression-free survival (PFS) based on the ClarIDHy trial, but is mainly a cytostatic agent with a response rate of 2%.[14] In contrast, FGFR inhibitors (FGFRi), such as pemigatinib and futibatinib, have reported response rates of up to 42% in patients with advanced disease harbouring FGFR2 fusions.[12] Three FGFR inhibitors, pemigatinib, infigratinib and futibatinib,

have received FDA approval, and pemigatinib has also gained European Medicines Agency (EMA) approval. Notably, targeted approaches in iCCA currently appear to be confined to small duct iCCAs.[15]

FGFR2 fusions have also been noted to be enriched in patients who benefit from liver transplant for iCCA.[16-18] This would suggest that downstaging or a neoadjuvant approach may be possible in this cohort in order to help improve surgical outcomes and disease-free survival (DFS). However, several challenges exist in incorporating the molecular targeted therapy into the current iCCA surgical treatment sequence. Before surgery, sufficient tumour tissue needs to be obtained through biopsy to test for gene alterations, including the aforementioned. However, biopsy of iCCA is frequently not feasible due to its anatomical location.[19,20] One potential solution to overcome these challenges may be to utilize cell-free DNA (cfDNA) genotyping technology, which provides real-time and non-invasive methods for measuring tumour genetics through blood tests or sampling of bile.[21-23]

After surgery, there are currently no good surveillance methods besides radiological imaging to detect iCCA recurrences, which delays the recurrence diagnosis.[24] For example, up to 63% of patients with early iCCA recurrences were reported to not have received the appropriate adjuvant therapies in time due to aggressive disease progression.[25] It is currently unknown whether intensification of adjuvant treatment may improve outcomes in iCCA, and the results of the ACTICCA-1 phase III trial of adjuvant cisplatin/gemcitabine are awaited.[26] In addition, FGFR2 inhibitors are now in trial in a first line metastatic setting, which could change the treatment paradigm in advanced disease (NCT03656536 and NCT03773302).[27] Conceivably, like the DYNAMIC trial in colorectal cancer (CRC), the detection of molecular or minimal residual disease (MRD) may pave a more personalized approach for those undergoing surgical resection.[28]

In this article, we review recent and relevant evidence concerning the potential uses of cell-free, circulating tumour DNA as a diagnostic, prognostic, and therapy-guiding tool for people with iCCA undergoing surgery.

CIRCULATING TUMOUR DNA

Liquid biopsies are revolutionizing the field of oncology and consist of tumour-derived fragments including circulating tumour cells, circulating tumour DNA (ctDNA) and tumour-derived extracellular vesicles.[22] Most commonly, ctDNA is being evaluated as a potential non-invasive biomarker in oncology.[29,30] CtDNA consists of DNA fragments from the tumour released into the blood from the tumour cells undergoing apoptosis or programmed cell death.[22] In contradistinction, cfDNA incorporates DNA fragments of the normal cells circulating in the blood.[22] The percentage of cfDNA attributed to ctDNA can vary but may be as low as 0.01%, and is usually identified by tumour-specific mutations or epigenetic signatures.[22] Harvesting ctDNA holds promise as a non-invasive "liquid biopsy" because the tumour DNA fragments are hypothesized to carry the same genetic information as their primary tumour, which they shed from.[22] In fact, ctDNA technology has been entering clinics through the United States FDA's approval of ctDNA use for selecting targeted molecular therapies. The first ctDNA assay approved was the cobas (registered trademark) EGFR Mutation Test v2 using real-time PCR to identify mutations within the EGFR gene.[31] Subsequently the Guardant360 CDx assay was approved as a companion diagnostic to identify EGFR mutations that predict benefits from osimertinib in the setting of non-small-cell lung cancers.[32] Since then, the FDA has approved Foundations Medicine's FoundationOne Liquid CDx and both platforms have expanding indications to cover a number of actionable alterations.

In the context of detecting MRD, ctDNA analyses have been increasingly divided into tumour-informed and tumour-uninformed (or tumour-agnostic) assays.[33] While the tumour-informed platform requires a tumour tissue biopsy to customize a panel of genes to sequence for an individual patient's plasma ctDNA analysis, tumour-uninformed approaches (plasma only) do not require a tissue biopsy, which results in a faster turnaround time for MRD ctDNA analysis and potentially quicker delivery of adjuvant therapies.[34] With the rapidly evolving next-generation sequencing (NGS) technologies, assays are now being developed to accurately assess a large set of gene panels from harvested ctDNA.[35] For the plasma only assays, the combination of genomic and epigenomic signatures may increase sensitivities for MRD detection comparable to that of tumour-informed assays.[36] However, there is limited evidence for the utility of ctDNA in the setting of operable iCCAs, as iCCA has often been categorized with other pancreatic or liver cancer types.[19,37]

Mutational Profiles of CCA

Cholangiocarcinomas (CCAs) are often classified by their anatomical subtypes: intrahepatic (iCCAs), perihilar (pCCAs) and distal cholangiocarcinomas (dCCAs).[38] It can be difficult to differentiate CCAs from other liver cancers or metastases based on the radiology imaging or histopathology.[6] In fact, recent studies suggest that tumour genetics may be better at classifying CCAs, and even reclassifying those often labelled as cancers of unknown primary.[39] Distinct genotypes have been documented in iCCA with a high prevalence of IDH1/2 (10–15%) and

BAP1 mutations (~13%) together with FGFR2 fusions (15–20%).[12,13,20] Additional fusions documented include NTRK, ALK/ROS1 and NRG1, all of which are considered actionable with therapeutic options.[20,40-43] Notably, these alterations tend to occur in small duct iCCA whereas large duct iCCA can often resemble extrahepatic CCA genotypically, with enrichment of TP53, KRAS, RNF43, PIK3CA and SMAD4 mutations.[6,20,38] Furthermore, within iCCAs, those tumours harbouring IDH1 mutations or FGFR2 fusions tend to have fewer co-occurring mutations in the mitogen-activated protein kinase (MAPK) pathway.[44]

IDH1 mutations and FGFR2 fusions are rarely identified in pCCA or dCCA.[45] Additional actionable mutations found in iCCA include the BRAF mutations (~5%).[46] Approximately 2–3% of iCCAs will harbour the class I $BRAF^{V600E}$ mutation for which dabrafenib and trametinib have gained approval.[47] The ROAR trial in biliary tract cancers (BTC) demonstrated response rates of 51% in advanced BTC.[47] HER2 amplification is less common in iCCAs compared with CCAs occurring extrahepatically, and can be expected in 3–5% of cases.[13,20] Several HER2 directed therapies are now available with zanidatamab, a bispecific antibody demonstrating objective response rates (ORR) of 47% in a phase 1 trial of advanced BTC (NCT02892123). How these matched approaches will translate to earlier stage disease remains unknown. Aside from single driver alterations, whole genomic sequencing and integrative omic approaches to CCA have further identified varying clusters that also associate with heterogeneous tumour immune microenvironments.[43,48,49] Delineating the common and exclusive genetic mutations of CCA subtypes could facilitate the correct diagnosis and classification of their genetic subtypes.[20]

ctDNA and the Detection of Actionable Alterations

The potential of ctDNA to serve as a surrogate for conventional tumour biopsy is attractive; however, it is reliant on high concordance between the ctDNA and tumour tissue.[13] The available evidence in the field of iCCA is promising (Table 1). Ettrich et al. reported 13 cases of isolated iCCA undergoing palliative chemotherapy and showed a 92% match between the mutations of the primary tumour and the ctDNA across 15 cancer genes (IDH1 included, but not FGFR2 fusions).[37] Lamarca et al. studied six metastatic iCCA patients and showed that those with an identified IDH1 mutation, FGFR2 mutation or FGFR2 fusion in pre-treatment ctDNA had a 100% match with the mutations of their respective tumours.[50] This was further supported by Csoma et al., who successfully detected the same FGFR2 point mutation together with IDH1/2, KRAS, and TP53 mutations in both the ctDNA and iCCA tissue specimens in metastatic settings.[51] In a comparative genomic analysis of 1632 advanced iCCAs (1048 primary tumour biopsies vs 364 liquid biopsies) reported by Israel et al., actionable alterations were found in 35% of the liquid biopsy cohort (4% FGFR2 rearrangements including fusion and 9% IDH1); however, the IDH1 and FGFR2 alterations were detected at a lower frequency in a liquid biopsy compared with a primary tumour biopsy.[52] Furthermore, a study by Mody et al. analyzing 85 ctDNAs for advanced iCCA reported about 10% IDH1 and 7% FGFR2 fusion rates, which is lower than the detection rate published in other studies.[53] It is critical to note that these two studies had no matched tumour comparison to validate the reported detection rate from ctDNA. Importantly, Berchuck et al. recently reported the largest series to date addressing concordance between tumour and ctDNA, analyzing 1671 patients.[54] Targetable alterations were detected in 44% of patients with concordance notably high for IDH1 and $BRAF^{V600E}$ mutations together with HER2 amplification.[54] Disappointingly, however, the Guradant360 platform used in this study showed a low sensitivity in detecting FGFR2 fusions from ctDNA. This was not due to low sensitivity in ctDNA detection but rather the ability to detect FGFR2 fusion partners, highlighting the challenges in fusion detection from ctDNA and the need for platforms to be optimized for certain disease subtypes. This will be critical if the field were to consider a liquid biopsy to detect actionable alteration in early stage iCCA (Fig. 1).

The aforementioned studies have only included samples from metastatic iCCAs. The question remains whether levels of preoperative ctDNA will be detectable with appropriate limits of detection for actionable alterations if considering targeted neoadjuvant approaches. Wintachai et al. compared the mutations between preoperative ctDNA and the resected tumours; however, this study was limited by its unclear breakdown of intra- vs extra-hepatic cholangiocarcinoma in the analysis.[55] When ten subjects with CCA underwent preoperative ctDNA molecular profiling, a 56% match was observed between detected somatic mutations and primary tumours compared with ctDNA.[55] This match rate was lower than in other studies conducted in metastatic iCCA settings.[55] It was also unclear what stages these cancers were in before resection (i.e., stage I–II vs. III–IV).[55] To fill in the knowledge gap, well-designed prospective studies will be needed to investigate whether ctDNA may serve as a surrogate for the iCCA tumour biopsy in a localized disease setting.[56] Variable tumour shedding may be a challenge in early stage disease, and if attempting to analyze ctDNA for therapeutics platforms, the technology will need to be optimized not only for the detection of mutations but also for potential partners like fusions.

TABLE 1 iCCA-ctDNA studies in the context of molecular profiling

Year	First/senior author	Type	Journal	Periop setting?	Population	ctDNA genes analyzed	Intervention/comparison	Outcomes	Platforms used
2022[54]	Berchuck/ Goyal	R	Annals of Oncology	No	1671 advanced BTCs (91% CCA, 9% GBC)	Top 20 most frequently altered genes in BTC categorized by alteration type (i.e., TP53, KRAS, FGFR2, IDH1, PIK3CA, ERBB2)	Large mix of targeted therapy and chemotherapy	Targetable alterations detected in 44% of patients Concordance between cfDNA and tissue for mutation detection was high for IDH1 (87%) and BRAF V600E (100%) Low concordance for FGFR2 fusions (18%)	Guardant360 cfDNA NGS assay
2022[51]	Csoma/ Mokanszki	P	Cancers	No	Metastatic BTC (15 iCCA, 5 eCCA, 5 GBC)	iCCA- APC, CSF1R, FGFR2, IDH2. KDR, ATM, KBXW7, IDH1, JAK3, RET, MET, CDH1, DRAS, PTEN, STK11, FOXL2, PK3CA, TP53	Gemcitabine-based chemotherapy, no resections	Positive correlation between the estimated tumour volume and cfDNA yield FGFR2, IDH1, IDH2, KRAS, and TP53 aberrations detected in matched liquid biopsy with similar tumour variant burden SNVs were shown in 84% of the cases	QIAamp Circulating Nucleic Acid Kit for cfDNA extraction. Libraries constructed using Archer VariantPlex Solid Tumor Kit
2021[52]	Israel/Ross	P	The Oncologist	No	1632 advanced iCCAs (1048 primary tumor biopsies, 364 liquid biopsies)	BRAF V600E, BRCA1/2, ERBB2, FGFR2, IDH1/2, KRAS, PIK3CA, TP53, CDKN2A/B, BAP1, MTAP	Primary tissue biopsy vs metastatic biopsy, liquid biopsy	Actionable alterations found in 35% in liquid biopsy cohort (4% FGFR2 and 9% IDH1) IDH1 and FGFR2 alterations were detected at a lower frequency in liquid biopsy	FoundationOne
2020[50]	Lamarca/ Valle	R	Journal of clinical medicine	No	Metastatic. pre-treatment BTCs (8 iCCA, 1 eCCA,1 GBC, 2 Amp)	IDH-1 mutation, FGFR2 fusion, FGFR-2 mutation in the tumour sample and confirmed on ctDNA analysis	Palliative chemotherapy initiated for 1 ctDNA patient only	Rate of targetable alterations 40%: IDH1 mutations (19%), FGFR2 alterations (10% and 5% had FGFR2 fusions and mutations, respectively) Concordance of findings for paired tissue and paired tissue-ctDNA was very high ctDNAs prior to palliative treatment showed no significant association with PFS or OS	FoundationOne CDx and/or Oncomine used for tumor tissue and FoundationOne Liquid platform for ctDNA
2019[37]	Ettrich/ Berger	P	Nature research scientific reports	No	Mixed 13 iCCA & 11 eCCA (locally advanced or metastatic only)	TP53, ARID1A, KRAS, IDH1, BAP1, PBRM1, SMAD4, PIK3CA, FBXW7, CDKN2A, ERBB2, NRAS, IDH2, BRAF, BCL2	ctDNA measured prior to and during chemotherapy	Variant allele frequency in ctDNA correlated with tumor load and PFS. -63% of therapy naive patients had their mutational profile changed during chemotherapy Trend towards shorter PFS in patients with a mutation in either of BAP1, PBRM1, KRAS and TP53	QIAamp Circulating Nucleic Acid Kit for ctDNA extraction NGS of 15 gene panel, selected frequently mutated genes

Table 1 (continued)

Year	First/senior author	Type	Journal	Periop setting?	Population	ctDNA genes analyzed	Intervention/comparison	Outcomes	Platforms used
2019[53]	Mody/Borad	R	JCO Precision Oncology	No	BTC (85 iCCA all locally advanced or metastatic)	FGFR2, PIK3CA, IDH1, TP53, KRAS. MYC, ARID1A, BRAF	None, one sided ctDNA analysis	Analysis of iCCA ctDNA without comparative tissue showed 5% IDH1 and 7% FGFR2 alteration rates Only locally advanced or metastatic, lumped with gallbladder cancer and eCCA	Guardant Health. Gene panel of 70-73 genes, from National Comprehensive Cancer Network somatic genomic targets
2015[102]	Zill/ Collisson	P	Cancer discovery	No	6 CCA (does not specify if iCCA), total 26 advanced pancreatobiliary cancers	BRAF	n/a	cfDNA accurately detects tumor-derived mutations in advanced cancer without a priori knowledge of tumor genotype or cfDNA burden Of the 31 mutations detected by tumor biopsy NGS, 28 were also detected by the cfDNA test	QIAamp circulating nucleic acid kit for cfDNA, sequencing performed at Guardant health. The exons of 54 cancer genes captured
2016[103]	Andersen/ Jakobsen	P	Clinica Chimica Acta	No	11 CCA (does not specify if iCCA)	KRAS, NRAS, BRAF, PIK3CA	vs controls	Perfect agreement as to wild type status in tumor and plasma Multiplex dPCR is a suitable method of screening plasma for RAS/RAF muations	Multiplex digital PCR, Maxwell 16 Blood DNA purification kit Mutations selected based on previous publications

Amp ampullary cancer, BTC: biliary tract cancer, *CCA* cholangiocarcinoma, *cfDNA* cell-free DNA, ctDNA: circulating tumor DNA, *CNV* copy number variation, *dPCR* digital polymerase chain reaction, *eCCA* extrahepatic cholangiocarcinoma, *GBC* gallbladder cancer, *iCCA* intrahepatic cholangiocarcinoma, *OS* overall survival, *PFS* progression free survival, *periop* perioperative, *SNV*: single-nucleotide variant, Type = Study type, *P* = prospective, *C* = case report, *R* = retrospective

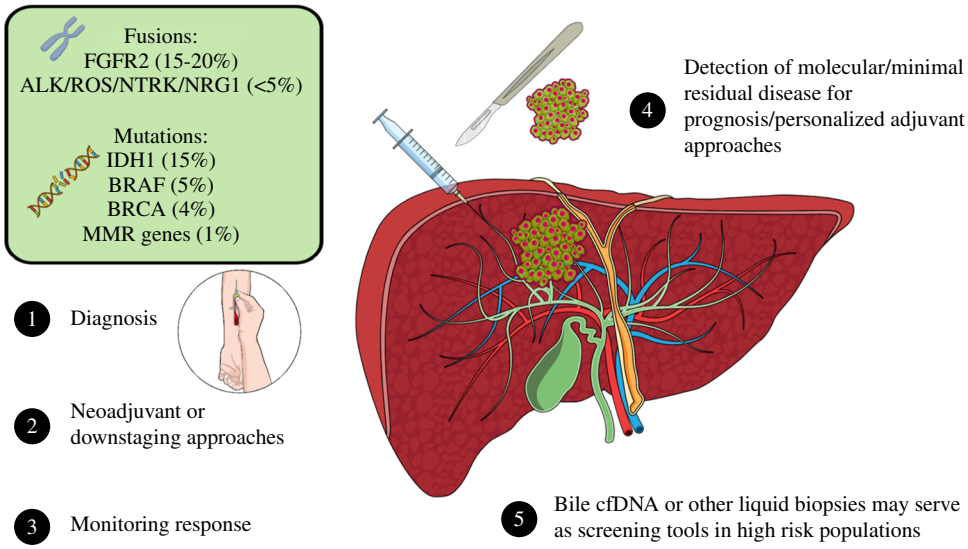

FIG. 1 Potential ctDNA usage in the perioperative setting

Minimal Residual Disease—Tumour-Informed and -Uninformed Approaches

After curative-intent surgery of CCAs, the assessment of MRD may help in determining prognosis and in pursuing personalized adjuvant approaches. One example of this application is the Signatera™ MRD technology, which generates 16 tumour-specific clonal somatic variants unique to the individual's primary tumour, and uses this "tumour signature" to target the presence of ctDNA in the plasma.[57] If at least two mutations are detected it can be considered a positive result. This assay has been shown to detect ctDNA levels as low as 0.01% with high sensitivity. Although this field is rapidly evolving, few studies have been published on CCA. Kasi et al. have presented their work using the Signatera assay from 62 patients (151 samples) with CCA.[58] Of these, 26.2% were documented to have positive MRD and, notably, ctDNA detection was associated with stage.[58] Tumour burden may be the lowest at this clinical stage as the tumour has been completely removed and potentially cured, and further studies will be needed to validate the assay use for MRD in iCCA.[22]

Currently, postoperative therapy capecitabine remains the standard adjuvant treatment option for patients with iCCA.[59] However, it should be noted that in the setting of perihilar and extrahepatic CCAs, with at least one lymph node metastasis after complete macroscopic resection, adjuvant cisplatin/gemcitabine did not improve OS compared with capecitabine alone in a multicenter phase 2 randomized controlled trial (STAMP).[60] As adjuvant treatment strategies evolve, it may be important to determine who is at high risk of recurrence through detection of residual disease after surgery. Studies from other cancer fields such as melanoma, colorectal, lung and pancreatic cancers have highlighted the prognostic role of ctDNA level measured in the immediate postoperative period in predicting cancer recurrence after surgery.[61-65] In fact, tumour-informed ctDNA was able to detect pancreatic cancer recurrences earlier than conventional radiologic diagnosis (3.1 vs. 9.6 months, $p = 0.0004$).[62] The DYNAMIC study is another example, evaluating stage II CRC patients in a randomized controlled trial to make decisions on adjuvant treatment based on ctDNA vs standard clinicopathological features after surgery.[28] This trial utilized a tumour-informed approach measuring ctDNA at 4 and 7 weeks postoperatively, and demonstrated that the ctDNA guided adjuvant therapy decision reduced adjuvant chemotherapy use without compromising recurrence-free survival (RFS).[28]

In the context of hepatocellular carcinoma (HCC), serial ctDNA was demonstrated to predict early recurrence after resection.[66] In this study, 41 patients with resectable HCC underwent serial ctDNA measurements before and after surgery, and the detection of postoperative ctDNA was associated with RFS ($p = 0.03$). After adjusting for HCC cancer stages, even baseline or preoperative ctDNA was associated with a higher risk of early recurrence after surgery.[66] A similar conclusion was arrived at in another study that analyzed 46 patients with HCC who underwent hepatectomy or liver transplant, in which detection of preoperative ctDNA was significantly associated with a higher incidence of postoperative recurrence and extrahepatic metastasis.[67] Another study finding was reported in the setting of CRC liver metastasis in which detectable postoperative or post-adjuvant chemotherapy ctDNA was associated with shorter RFS.[64] The role of ctDNA in predicting cancer recurrence is especially relevant for iCCA, as most postoperative iCCA recurrences occur relatively early, about 25% within 6 months and 50% within 2 years after surgery.[7,8,68] Earlier detection of iCCA

recurrence may lead to a better chance of receiving repeat resection or other liver-directed therapies that may improve survival.[69] There are limitations to MRD, and patients require counselling on the potential for false negatives and false positives. The former may be overcome with serial monitoring of ctDNA, which could also allow for the outgrowth of subclones that may have been below threshold levels for detection.[22] In the context of plasma-only or tumour-uninformed ctDNA analyses, clonal haematopoiesis of indeterminate potential (CHIP) can account for false positives.[22]

TREATMENT—MONITORING OF SYSTEMIC TREATMENT RESPONSE

When administering systemic or locoregional therapies for cancers, ctDNA may provide information on prognosis and an opportunity for real-time monitoring of tumour dynamics and evolution.[22] For patients who are receiving platinum-based chemotherapy for metastatic biliary tract cancers (80.6% were iCCAs), the pre-treatment dominant clone allele frequency (detected gene with the highest variant allele frequency) detected in ctDNA was associated with worse OS (median 10.8 vs. 18.8 months, $p = 0.03$) and PFS.[70] Interestingly, in this study there was no difference in the treatment response rate between high or low dominant clone allele frequency groups, implying that it is a measure of prognosis but not treatment response.[70] There are, however, other examples of ctDNA associating with treatment response. Winter et al. measured ctDNA at multiple time points for four patients receiving selective internal radiation therapy (SIRT) for metastatic iCCA (post palliative chemotherapy, Table 2).[71] Throughout these serial ctDNA measurements, a reduction in the burden of copy number variants (CNV) was observed corresponding to treatment response.[71] Similar findings were observed in breast cancer, CRC, and CRC liver metastasis studies using serial ctDNA measures to monitor treatment response.[22,64,72] The application of ctDNA has also been extended to detecting methylation markers in HCC, highlighting how epigenetics may come into play in measuring treatment response through ctDNA.[73]

In the setting of neoadjuvant systemic therapy, one rectal cancer study showed a correlation between ctDNA detection rate and treatment response from neoadjuvant chemotherapy.[74,75] A similar observation was reported in the setting of pancreatic cancer, in which administration of neoadjuvant chemotherapy resulted in a drop in ctDNA detection rate.[76] Such findings are relevant for the treatment of iCCA as the use of neoadjuvant chemotherapy is increasing (though not yet representing standard of care) because of potential advantages compared with using adjuvant chemotherapy alone.[77] As an example, neoadjuvant chemotherapy can have a "downstaging" effect on unresectable iCCA tumours by shrinking the disease and making them resectable.[78,79] It is also theorized that neoadjuvant chemotherapy treats micro-metastatic systemic cancer, potentially resulting in improved OS after iCCA resections.[80-82] As neoadjuvant chemotherapy is slowly integrating into the iCCA treatment sequence, ctDNA could play a critical role in assessing treatment response in such a preoperative setting. Moreover, targeted treatments such as pemigatinib may be used in the setting of locally advanced iCCAs, and a trial is underway (NCT05565794).[83]

Treatment—Identification of Resistance Mechanisms

Molecular profiling of the cancer during treatment allows us to detect new genomic alterations that arise either from acquired resistance or clonal evolution.[84] Clonal dynamics describe different genetic subclones that develop within a tumour and get passed down the evolving tumour cells.[22] These clonal variants carry unique signatures of the individual's original tumour. Acquired resistance may arise by a clonal outgrowth of resistant subclones to a certain treatment.[22] CtDNA has the advantage of capturing information on these various subclones and perhaps clarify multiple resistance mechanisms at once.[22] This is especially relevant in iCCA disease as both the IDH1 (10–15%) and FGFR2 (15–20%) alterations, which are almost exclusively seen in iCCAs, have targeted treatments (i.e., pemigatinib, infigratinib and futibatinib for FGFR2; and ivosidenib for IDH1) that can be monitored for resistance.[13,84] For instance, Varghese et al. studied eight patients with locally advanced or metastatic iCCA who were on pan-FGFR treatment for confirmed FGFR2 alterations (Table 3).[85] This study showed up to 31 acquired FGFR2 mutations detected through ctDNA during the treatment period and captured drug resistance mechanisms.[85] Furthermore, Goyal et al. conducted two studies investigating the mechanism of acquired resistance from FGFR inhibitor therapies using serial ctDNA measurements in metastatic iCCA patients.[86,87] In the context of IDH1 treatments, Cleary et al. identified secondary IDH1 mutations and acquired IDH2 mutations as resistance mechanisms when patients were treated with ivosidenib.[88] In general, the resistance profiles differed across subjects and within the serial measurements in each subject under the same kind of targeted treatment. A few more actionable gene mutations (i.e., ERBB2) are under investigation to delineate resistance mechanisms to their respective molecular targeted therapies.[89] Standardizing serial ctDNA measurements in molecular targeted treatment trial protocols may help with earlier detection of acquired resistance,

TABLE 2 iCCA-ctDNA studies in the context of early iCCA detection and treatment responses monitoring

Year (study reference)	First/senior author	Type	Journal	Periop setting?	Population	ctDNA genes analyzed	Intervention/comparison	Outcomes	Platforms used
2022[70]	Uson Junior/ Borad	R	Precision medicine	No	67 iCCA, 7 eCCA, 6 gall bladder cancers	TP53, KRAS, FGFR2, ARID1A, STK11, and IDH1	Platinum-based chemotherapy, high vs low dominant clone allele frequency detected in pre-treatment ctDNA	Worse OS (median 10.8 vs 18.8 months, p = 0.03) and PFS with higher dominant clone allele frequency No difference in the treatment response rate between groups	Guardant360 and their database
2021[55]	Wintachai/ Jusakul	P	Diagnostics	Yes	62 CCA (31 iCCA, 27 eCCA, 4 iCCA + eCCA), 10 CCA (4 stage I-II, 6 III-IV) mutational profiling with matched samples collected from surgery	ARID1A, PBRM1, MTOR, FGFR3, IDH1/2, ERBB2, FGFR2	CCA vs 33 benign biliary disease (BBD) and 30 controls,	Preop cfDNA levels discriminated CCA from healthy controls cfDNA level increased with higher TNM staging or lymph node metastasis Mutation-level concordance of 56% between ctDNA and tumour DNA	QIAamp MinElute ccfDNA Kit (Qiagen). NGS panel customization: Capture-based probes designed for 60 exon genes based on previously reported high frequency mutations and genomics-driven therapy database
2019[71]	Winter/ McCullagh	P	Cancers	No	4 metastatic iCCA	NRAS and IDH1 mutations	None. iCCA vs healthy control	Recurrent somatic SNVs and CNVs identified in ctDNA from patients with both NRAS and IDH1 mutations Reduction in the number of CNVs was observed with SIRT treatment	QIAamp Circulating Nucleic Acid kit (Qiagen). Targeted sequencing conducted using 50 cancer gene panel, from Ion AmpliSeq™ Cancer Hotspot Panel

CCA cholangiocarcinoma, *cfDNA* cell-free DNA, *ctDNA* circulating tumour DNA, *CNV* copy number variation, *eCCA*: extrahepatic cholangiocarcinoma, *iCCA* intrahepatic cholangiocarcinoma, *preop* preoperative, *periop* perioperative, *SIRT* selective internal radiation therapy, *SNV* single-nucleotide variant. Type = Study type, *P* = prospective, *C* = case report, *R* = retrospective

TABLE 3 iCCA-ctDNA studies in the context of identifying drug resistance mechanism or clonal dynamics

Year	First/senior author	Type	Journal	Periop setting?	Population	ctDNA genes analyzed	Intervention/ comparison	Outcomes	Platforms used
2022[88]	Cleary/ Losman	P	Nature Npj precision oncology	No	2 IDH1 mutated metastatic iCCAs	IDH1 and IDH2	Ivosidenib (one arm)	Secondary IDH1 mutations and acquired IDH2 mutations identified as resistance mechanisms	Guardant 360
2022[90]	Lapin/Janku	P	JCO Precision Oncology	No	Advanced (stage IV) CCA with known IDH1/2 mutations	IDH1 and IDH2	IDH1 or IDH2 inhibitor treatment for clinical trial (one arm)	IDH mutations in ctDNA concordant with tumour tissue Lower variant allele frequency of ctDNA detected by NGS was associated with longer time to treatment failure Emerging alterations with predicted oncogenic potential were detected in ctDNA at the time of progression	QIAamp Circulating Nucleic Acid kit to isolate cfDNA ddPCR in cfDNA: mutation-specific assays were used Targeted digital NGS of cfDNA using the 74 genes assay Guardant360
2021[85]	Varghese/ Berger	P	JCO Precision Oncology	No	8 locally advanced or metastatic iCCA	FGFR2	Infigratinib pan-FGFR inhibitor for 7 patients	31 acquired mutations in FGFR2 were identified at resistance in 6/8 patients with detectable ctDNA. Genomic concordance among resistant subclones ctDNA effective means to longitudinally monitor for acquired resistance in FGFR2- altered iCCA	QIAsymphony DSP Circulating DNA Kit used for cfDNA Sequenced with custom, ultra-deep coverage NGS panel, MSK-ACCESS
2019[86]	Goyal/ Bardeesy	P	Cancer discovery	No	4 metastatic iCCA	FGFR2	FGFR inhibitor RAS-120	ATP-competitive FGFR inhibitors show efficacy in FGFR2-altered iCCA Irreversible FGFR inhibitor benefits patients with resistance to ATP-competitive FGFR inhibitors and overcomes several FGFR2 mutations	Targeted sequencing of ctDNA: panel of 70 genes from Guardant Health. Targeted sequencing for tumour tissue: SNaPshot platform, the FoundationOne platform, or MSK-IMPACT,
2019[89]	Yarlagadda/ Kasi	C	nph precision oncology	No	1 metastatic CCA (case report)	ERBB2 (HER2)	HER2 directed systemic therapy (trastazumab/ pertuzumab)	mCCA with ERBB2 amplification identified on ctDNA testing Response to now over 12 months of dual-anti-HER2 therapy	Gaurdant360 for ctDNA Tempus platform for tumour tissue for confirmation.

Table 3 (continued)

Year	First/senior author	Type	Journal	Periop setting?	Population	ctDNA genes analyzed	Intervention/ comparison	Outcomes	Platforms used
2017[87]	Goyal/Zhu	P	Cancer discovery	No	3 metastatic iCCA	FGFR2 fusion	FGFR inhibitor BGJ398, pre-treatment and post progression cfDNA	9/32 (28%) had FGFR2 fusions detected Post-progression sequencing of the FGFR2 gene demonstrated de novo point mutations that conferred resistance to BGJ298	Targeted sequencing of ctDNA: panel of 70 genes from Guardant Health Tumour tissue: targeted sequencing via the FoundationOne platform

CCA cholangiocarcinoma, *ctDNA* circulating tumor DNA, *iCCA* intrahepatic cholangiocarcinoma, *mCCA* metastatic cholangiocarcinoma, *NGS* next-generation sequencing, *periop* perioperative. Type = Study type, *P* = prospective, *C* = case report, *R* = retrospective

which would help guide how and when to start alternative treatments.

Furthermore, Lapin et al. studied people with metastatic iCCAs undergoing IDH1 or IDH2 inhibitor treatments for known IDH1 or IDH2 mutations (Table 3).[90] In this study, the treatment group with lower variant allele frequency in ctDNA experienced a longer time to treatment failure than those with higher variant allele frequency in ctDNA (3.6 vs. 1.5 months; $P = 0.008$). And through serial ctDNA measurements during disease progression, emerging alterations with oncogenic properties were detected in ctDNA. The most common ones were ARID1A and TP53 mutations.[90] Both ARID1A and TP53 mutations were from the cancer clones and not from the original cancer.[90] Similarly, Varghese et al. studied eight patients with metastatic iCCAs who were on pan-FGFR treatment for confirmed FGFR2 alterations, and reported up to 13 independent FGFR2 mutations detected per patient.[85] Such a polyclonal mechanism or evolution implies that a single site biopsy may not suffice to capture the complete picture of cancer clonal dynamics. As different clones of cancer simultaneously shed their ctDNAs into the blood, ctDNA has the unique advantage of providing a cross-sectional snapshot of multiple clone genetics evolving at different stages.[22]

Diagnosis and Screening—Early Intrahepatic Cholangiocarcinoma Detection Before Surgery, and Screening High-Risk Populations

Wintachai et al. studied the diagnostic accuracy of preoperative cfDNA levels using 62 resected CCA samples, of which 31 were iCCAs (Table 2).[55] Compared with their healthy controls, patients with CCA had up to 24-fold higher mean cfDNA levels.[55] Plasma cfDNA level showed 89% sensitivity and 97% specificity in diagnosing a heterogeneous group of patients with CCA (including a mix of intra- and extrahepatic CCA), outperforming conventional tumour markers such as the carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9).[55] Furthermore, this study showed how a higher cfDNA level was correlated with a higher TNM staging (American Joint Committee on Cancer 7th edition).[55,91]

Screening for iCCA is controversial, primarily as identifying high-risk groups has been a challenge. One candidate cohort would be those with underlying primary sclerosing cholangitis (PSC), where CCA is the most common cause of death if transplantation has not occurred.[92] In the PSC population, the cumulative incidence of CCA is 20–25% at 20 years.[93] CCA tends to occur in those with dominant strictures in the perihilar region; however, many cases of PSC-associated CCA present within a 6-month period of PSC diagnosis, precluding effective screening.[94] Nevertheless, given the evolving role of

transplantation in CCA, particularly in PSC patients, early identification of malignancy provides an opportunity.[95-97] Unfortunately, in the presence of PSC, CA19-9 is poorly sensitive to detecting malignancy.[98] CtDNA in this regard may provide an opportunity, in conjunction with imaging and pathology, to increase the diagnostic yield of the underlying malignancy. However, challenges exist, given that ctDNA might be more difficult to detect in the earlier stage iCCA settings due to smaller cancer cell volume or burden.[32] For instance, in a study by Csoma et al., a positive correlation between tumour volume (in the setting of metastatic biliary tract cancers including 15 iCCAs) and cfDNA harvest yield ($r = 0.93$, $p < 0.0001$) was noted. Similarly, Wintachai et al. reported merely a 50% match between the gene mutations of the ctDNA and primary tumour in the stage I–II iCCA group, which was lower than the match reported from the more advanced stage III–IV iCCA group.[55] Notably, PSC-CCA rarely occurs in small ducts, and the genomic profiles from a limited series reveal a high prevalence of TP53 and KRAS mutations.[99] However, what is appealing is the potential utility of bile cfDNA.[100] In a study reported by Arechederra et al. evaluating strictures, bile cfDNA at the time of first Endoscopic retrograde cholangiopancreatography (ERCP) was processed using the Oncomine pan-cancer cell-free assay.[100] In patients with a stricture initially identified as benign or indeterminate, the sensitivity of the cfDNA assay was 100% in identifying malignancy.[100] Additional innovative technologies seek to use methylation markers from blood to provide early detection assays and predict cancer origin. One example is the Circulating Cell-free Genome Atlas (CCGA) study (NCT02889978), which developed and validated a multi-cancer early detection (MCED) test using whole genome bisulfite sequencing.[101] The sensitivity of this test was high (93.5%) in the 46 bile duct cancers included.[101] In the future, these tests may complement evolving screening strategies.

SUMMARY AND FUTURE DIRECTION

In this review, we summarized some of the evidence for the potential application of ctDNA in the perioperative setting for a target-rich cancer like iCCA. Circulating tumour DNA in the perioperative setting may become a biomarker for guiding neoadjuvant treatment decisions, postoperative follow-up intensity and selection of patients with detected MRD who might benefit from personalized adjuvant therapy approaches.[23,81]To overcome current limitations, further ctDNA sequencing assay validations and standardization of ctDNA collection time relative to the time of surgery are required. Furthermore, given that not all genetic mutations discovered from ctDNA will be "actionable" or have targeted treatments available,[32] some genetic mutations detected might be irrelevant to the oncogenesis process, and an understanding of tumour-informed and -uninformed platforms is required. Finally, not all patients with early iCCA will have enough ctDNA to be harvested for meaningful genetic analysis; however, it is expected that newer NGS-based assays and technology will be able to detect lower levels of ctDNA, potentially enabling screening and diagnostic tools for early iCCA detection.[32,35,94] The integrative approach of cell-free methylation or epigenomic signatures, together with mutational analysis, is likely to move the needle with regard to MRD and early detection.

CONCLUSION

Components of liquid biopsies including ctDNA may be utilized to: (1) determine the molecular profile of the tumour to integrate targeted molecular therapies into the surgical treatment sequence, (2) form a surveillance tool for the detection of MRD or cancer recurrence after surgery, and (3) diagnose and screen for early iCCA detection in high-risk populations. In the future, ctDNA could become the standard biomarker in iCCA care for guiding neoadjuvant treatment decisions, for postoperative follow-up intensity, and for the selection of people who show signs of residual disease after surgery and who could benefit from adjuvant therapies. Future prospective clinical trials studying neoadjuvant or adjuvant systemic therapies for iCCA should incorporate serial ctDNA measurements in their protocols to fully elucidate its potential.

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