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REVIEW

Precision oncology in biliary tract cancer: the emerging role of liquid biopsy

M. de Scordilli[1,2], M. Bortolot[1,2], S. Torresan[1,2], C. Noto[2,3], S. Rota[1,2], P. Di Nardo[1], A. Fumagalli[1], M. Guardascione[1], E. Ongaro[1], L. Foltran[1*] & F. Puglisi[1,2]

[1]Department of Medical Oncology, Centro di Riferimento Oncologico di Aviano (CRO), IRCCS, Aviano; [2]Department of Medicine, University of Udine, Udine; [3]Medical Oncology, ASUGI, Ospedale Maggiore, Trieste, Italy

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Liquid biopsy has already proven effective in aiding diagnosis, risk stratification and treatment personalization in several malignancies, and it could represent a practice-changing tool also in biliary tract cancer, even though clinical applications are currently still limited. It is promising for early diagnosis, especially in high-risk populations, and several studies on circulating free DNA (cfDNA), circulating tumour cells and differential microRNA (miRNA) profiles in this setting are ongoing. Circulating tumour DNA (ctDNA) also appears as a feasible noninvasive biomarker in the curative setting, in detecting minimal residual disease after resection and in monitoring disease recurrence. As of today, it can be particularly valuable in biliary tract cancer for genomic profiling, with a good concordance with tissue samples for most molecular alterations. CtDNA analysis may especially be considered in clinical practice when the tumour tissue is not sufficient for next-generation sequencing, or when urgent therapeutic decisions are needed. Moreover, it offers the possibility of providing a real-time picture to monitor treatment response and dynamically identify resistance mutations, potentially representing a way to optimize treatment strategies.

Key words: biliary tract cancer, cholangiocarcinoma, liquid biopsy, ctDNA, molecular profiling, minimal residual disease

INTRODUCTION

Biliary tract cancer (BTC) includes a variety of different malignancies: intrahepatic cholangiocarcinoma (iCCA), extrahepatic cholangiocarcinoma (eCCA) and gallbladder cancer (GBC). Cancers of the ampulla of Vater are rare and sometimes included in this classification. However, they have distinct clinical behaviours, with an intestinal or pancreatobiliary histology, and cannot be univocally considered as BTC; therefore, they are not usually included in clinical trials in the advanced setting.

BTC incidence varies greatly among different world regions based on distinct risk factors; however, it is considered a rare form of cancer in the Western world, with an incidence between 1 and 3 per 100 000.[1] It has been constantly increasing over the past few decades, however, especially iCCA.[2] Despite improvements in treatment options, such as the introduction of immune checkpoint inhibitors (ICIs) in the advanced setting, prognosis remains abysmal, with a diagnosis of advanced disease in most cases due to inadequate screening strategies, disease recurrence after surgery in ∼60% of patients, and a 5-year survival rate <20%.[3]

BTC diagnosis usually requires histological or cytological confirmation with samples obtained from transpapillary tumour biopsy, endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA), brush cytology via endoscopic retrograde cholangiopancreatography (ERCP), or biopsy of a metastatic lesion, with varying sensitivity and high possibility of nondiagnostic samples or low tumour cellularity tissue. Therefore, the risk of delayed diagnosis due to repeated procedures is high. Also, tumour tissue may prove insufficient for adequate molecular profiling, considering the several genomic alterations that may be clinically relevant in BTC patients.[4] Liquid biopsy has already proven effective in aiding diagnosis, risk stratification and treatment personalization in many other malignancies[5-8] and could also represent a powerful tool in BTC. In particular, it may also offer the opportunity to closely monitor disease response and to extensively study acquired alterations conferring resistance to systemic treatments.

LIQUID BIOPSY TECHNIQUES

Usually, the term liquid biopsy indicates the retrieval of biological fluids for the isolation of different molecular components. In oncology research, blood liquid biopsy has proven the most useful so far for the isolation of cancer-related molecules.

*Correspondence to: Dr Luisa Foltran, Department of Medical Oncology, Centro di Riferimento Oncologico di Aviano (CRO), IRCCS, Via Franco Gallini 2, 33081 Aviano (PN), Italy. Tel: +39 0434 659253
E-mail: luisa.foltran@cro.it (L. Foltran).

Circulating tumour cells

From blood samples, circulating tumour cells (CTCs) can be isolated through an enrichment process based on size and shape, or captured by tumour-specific antibodies such as EpCAM or mesenchymal proteins that help distinguish them from haematopoietic circulating cells (immunoselection).[9] CTCs in BTC are correlated with prognosis,[10] postsurgical recurrence and treatment response.[11,12] Moreover, genetic material can be retrieved from CTCs, allowing genomic profiling and molecular analyses.[13] However, the concentration of CTCs in blood can be very low, making CTC processing expensive and time consuming.[9]

Circulating free DNA

Circulating free DNA (cfDNA) consists of DNA fragments that can be retrieved from serum and analysed. In solid tumours, cfDNA can be used to study the genomic landscape of cancer cells, which are often heterogeneous in space and time, to a better extent in comparison with tumour biopsy. CfDNA levels correlate with tumour volume,[14] could aid early diagnosis[15] and are associated with prognosis.[16] The most interesting potential biomarker from cfDNA is methylation in its distinct patterns, which could be useful for BTC diagnosis[17] and in distinguishing malignancies from benign conditions.[18,19] Furthermore, cfDNA methylation levels seem to be prognostic and predictive of response to immunotherapy.[19,20] Correspondence between tissue biopsy and cfDNA in detecting targetable mutations has proven inconsistent, as have sensitivity and specificity. In particular, when identifying *FGFR2* fusions, the limitation is represented by the number of different fusion partners, for which partner-agnostic techniques might help.[21] CfDNA, nonetheless, could be a valuable tool in identifying resistance mutations, especially when polyclonal mechanisms must be addressed.[22] CfDNA specifically derived from cancer cells [circulating tumour DNA (ctDNA)] has proven effective in aiding diagnosis and prognostic and predictive evaluation in many solid tumours, including BTC.[23] CtDNA is used to test for targetable mutations when the tumour tissue is insufficient or not obtainable, having the advantage of being easily retrievable.[24]

Circulating RNA and extracellular vesicles

Moreover, all circulating RNA classes have been investigated as potential cancer biomarkers, including microRNAs (miRNAs), long noncoding RNAs and circular RNAs. Retrieval techniques are the same as used for ctDNA, but RNA usually derives from active secretion rather than cell apoptosis, and it is more unstable in plasma when not bound to proteins or included in exosomes.[25] RNA analysis provides both quantitative and qualitative information about the circulating transcriptome.[26] In BTC, plasma and exosomal miRNAs could play a role in diagnosis[27] and prognostic evaluation.[28,29]

More broadly, extracellular vesicles, including exosomes, represent new promising cancer biomarkers. They are small spheres with a bilayer lipid-membrane secreted by cells and containing several types of biomolecules, which can vary in pathological conditions. They can be isolated from body fluids by serial ultracentrifugation/sized-based assays and precipitation,[30] and their content can be analysed for diagnostic and prognostic purposes.[31] Interestingly, extracellular vesicles themselves can be used in cancer diagnosis, considering tumour-associated microparticles (taMPs) with specific proteins on the surface that can be quantified in the plasma and are quantitively higher in malignancies.[32]

Application on bile samples

The most interesting and peculiar perspective for liquid biopsy application in BTC regards bile samples, which can be retrieved both by ERCP and percutaneous transhepatic biliary drainage. The advantage of bile liquid biopsy is avoiding the interference of molecules and cells derived from other organs; the direct contact between cancer cells and bile could lead to a higher concentration of biomarkers and could provide more comprehensive information even in heterogeneous tumours. As for concordance with tissue biopsy, preliminary data seem to be acceptable[33] and more valuable than cytology,[34] but a balance between invasiveness and utility has yet to be defined.

All the above-mentioned biomarkers normally found in blood have also been studied in bile samples. Free RNA is quickly degraded; however, specific miRNA levels in bile exosomes could be useful to aid diagnosis[35] and even to explore new pathways of CCA progression.[36] Preliminary data on long noncoding RNAs and circular RNAs are also encouraging.[37] CfDNA concentration in bile is reported as low, but still higher than in plasma and with longer fragments, and similar data are reported for ctDNA.[33] A higher allele frequency for mutations in comparison with plasmatic frequency has also been reported, with notable concordance with tissue samples and a potential role in diagnostics.[38] As reported for blood samples, DNA methylation status could be analysed in bile samples also.[39] New possible bile biomarkers could be represented by proteins, both well-known blood biomarkers that seem to have higher biliary concentration in BTC patients, such as carcinoembryonic antigen (CEA), and others such as proteins in the mucin family, insulin-like growth factor-I (IGF-I), vascular endothelial growth factor (VEGF), heat shock proteins, neutrophil gelatinase-associated lipocalin (NGAL) and S100 family.[40]

DIAGNOSIS

Early diagnosis of CCA remains a major challenge, particularly in high-risk subjects such as patients with primary sclerosing cholangitis (PSC). Diagnostic procedures like fine-needle endoscopic biopsy or biliary brush cytology by ERCP may be technically difficult and sometimes result in non-diagnostic outcomes due to the paucity of the sample. The only serum biomarkers currently recommended for the management of BTC patients are carbohydrate antigen 19-9 (CA19-9) and CEA, the diagnostic utility of which is limited by the relative lack of sensitivity and specificity.

All these considerations highlight the unmet need for accurate and possibly noninvasive biomarkers to help in diagnostics and early tumour detection in high-risk populations.

cfDNA and ctDNA

Although the potential of cfDNA and ctDNA as surrogates for conventional tumour biopsy is attractive, few data are available about their use in localized disease. In a recent study, plasma cfDNA levels were quantified from 62 CCA patients, 33 benign biliary disease (BBD) patients and 30 healthy controls (HCs) by fluorescent assay: plasma cfDNA levels in CCA were significantly higher than those in the other two groups (24-fold higher than in the HC group and ∼3-fold higher than in the BBD group). Plasmatic cfDNA showed high sensitivity (88.7%) and specificity (96.67%) [area under the curve (AUC) 0.972 in the receiver operating characteristic curve analysis] to discriminate CCA patients from HCs, and the diagnostic efficacy of cfDNA level was superior to serum CA19-9 and CEA.[16] Similarly, a study of cfDNA detected by quantitative PCR in 34 patients with GBC and 39 controls (including 22 with cholecystitis) demonstrated significantly higher cfDNA levels in the malignant group.[41] This evidence suggests that cfDNA quantitative analysis could be used as a diagnostic biomarker for BTC and could also play an important role in distinguishing BTC from BBD. As seen in other tumours,[42,43] cfDNA concentration correlates with stage, tumour size and lymph node metastases. A positive correlation between the estimated tumour volume and cfDNA ($r = 0.9326$, $P < 0.0001$) was confirmed in a recent study.[14]

CfDNA gene methylation has also been explored as an adjunctive tool in differentiating malignancies from BBD. A study by Qiu et al. identified 3369 common differentially methylated regions in 105 patients with BTC.[20] Although several genes have been reported to be methylated in BTC,[17] data are not sufficient yet to determine potential biomarkers for cancer detection. Recently, methylation of *OPCML*, *HOXA9* and *HOXD9* was quantified in serum cfDNA of 80 patients (40 with CCA, 40 with other BBD-like cholecystitis, cholangitis and papillary adenoma), showing that methylation levels of *OPCML* and *HOXD9* were significantly higher in the malignant group, with an AUC of 0.850 and 0.789, respectively; no significant difference was evidenced for *HOXA9* methylation levels.[19]

CtDNA sensitivity in early stage BTC is modest due to the lower tumour burden and ctDNA shedding in comparison with the advanced setting. Therefore, its possible use as a diagnostic marker remains a challenge. Additionally, the high concordance between ctDNA and tumour tissue noted in the advanced disease has not been confirmed in this setting. The concordance has been described as low (56.3%) comparing molecular profiling on preoperative ctDNA and tumour samples; 35.2% of mutations were found in tumour but not in ctDNA.[16]

miRNAs. Several studies have reported the evolving role of miRNAs as novel biomarkers in cancer diagnosis. They are single-stranded noncoding RNAs acting as post-transcriptional regulators of gene expression and recent studies have highlighted their important role in the pathogenesis of BTC.[44] Various miRNAs, such as miR-21, miR-26a, miR-122 and miR-150, have been identified as possible blood-based biomarkers for noninvasive diagnosis of BTC.[29] When compared with HCs and patients with PSC, CCA patients exhibited a differential RNA profile, with up-regulation or reduced expression of specific miRNA expression.

Plasma samples of 94 patients who had undergone curative or noncurative resection for BTC and 50 HCs were tested, showing that miR-21 was significantly elevated in BTC compared with BBD (AUC 0.93 comparing BTC with HC, 0.83 when compared with BBD).[45] No differential significance in miR-21 expression between CCA and BBD was found by Cheng et al., although its up-regulation was confirmed in CCA compared with HC.[46] This suggests that miR-21 could be unsuitable to differentiate between CCA and benign diseases; in addition, miR-21 has been reported to have a diagnostic value for other tumours, such as pancreatic ductal adenocarcinoma, colorectal carcinoma and hepatocarcinoma, but without distinguishing between tumour types and thus reducing its potential role as a diagnostic instrument.

Recent studies have provided evidence for miR-26a dysregulation in blood samples from CCA patients, with contrasting results. Wang et al. evidenced elevated serum miR-26a in CCA compared with HC.[47] Conversely, in Cheng's study, miR-106a was significantly downregulated in CCA in comparison with BBD or HC and was an independent predictor for poor prognosis.[46] Moreover, Voigtländer et al. found reduced expression of miR-26a in serum in CCA compared with PSC.[35] MiR-122 is known for its high expression in liver-associated pathological conditions[48] and was found significantly up-regulated in serum in CCA compared with HC.[49] Remarkably, miR-122 was also significantly up-regulated when comparing PSC with CCA.[49]

Other miRNAs were investigated as possible blood-based biomarkers, including miR-150. Its expression levels were examined in tumour tissues, peritumoral noncancerous tissues and blood samples of 15 patients with iCCA, and they were significantly elevated in CCA compared with HC.[50] The discriminative ability (AUC 0.791) was better when compared with CA19-9 (AUC 0.747), with a sensitivity of 80.6% and a specificity of 58.1%. Notably, miR-150 was elevated in plasma but downregulated in matched tissue samples. More recently, reduction of expression of miR-150 was described in both serum and matched tissue samples of CCA; serum expression levels were significantly lower in both CCA and PSC compared with HC.[51] These findings are in line with previous results by Kojima et al., who provided evidence for reduced miR-150 expression in serum of BTC patients compared with HC.[52]

Assessing miRNAs not only in blood/serum but also in bile has emerged as a possible alternative. Wu et al. analysed miR-150 serum levels in CCA patients and matched the results with bile samples, showing a strong positive correlation in miR-150 expression.[51]

CTCs

Studies on CTCs in BTC are limited to EpCAM-enriched CTCs. Ustwani et al. were the first to describe the possibility of detecting CTCs in 25% of patients with BTC (3/13) or GBC (1/3).[53] Awasthi et al. focused on the diagnostic role of CTCs in 27 cases of treatment-naive GBC and 6 HCs. EpCAM-positive and CD45-negative CTCs were isolated in 25 of 27 GBC patients (5 stage I-II, 22 stage III-IV) and CTC count (cut-off point of $\geq$1) discriminated GBC from controls with a sensitivity, specificity and diagnostic accuracy of 92.6%, 91.7% and 92.3%, respectively.[54] Higher cut-off points for CTCs were able to distinguish between disease stages.

CTCs expression of other proteins linked to the epithelial—mesenchymal transition has been considered. In a prospective trial by Han et al., vimentin-positive CTCs (V-CTCs) were analysed alongside CTCs in 62 patients (52 with BTC and 10 with BBD). A CTC count >40/ml, VCTC count >15/ml and V-CTC/CTC ratio >40% were significantly different in BTC and BBD patients.[55]

Other approaches

A study by Lapitz et al. analysed protein biomarkers in serum extracellular vesicles (EVs) from patients with isolated PSC, patients with concomitant PSC-CCA, patients with PSC who developed CCA during follow-up, patients with CCA of non-PSC aetiology and healthy individuals, revealing distinct EV protein profiles for each subgroup.[31] Compared with PSC, the expression of 68 different proteins was altered in the PSC-CCA group (37 up-regulated, 31 downregulated). A total of 47 EV proteins showed significant AUC values for CCA diagnosis, with FRIL providing the highest score when compared with individuals with isolated PSC (AUC 0.909) or with the nonmalignant control group (AUC 0.931). The combination of these proteins and CA19-9 showed higher accuracy than CA19-9 alone. CRP/FIBRINOGEN/FRIL/PIGR levels showed a predictive capacity for CCA development in PSC before clinical evidence of malignancy. This differential proteomic signature suggests a possible diagnostic role in distinguishing patients with CCA from those with BBD or HC.

In another study, the transcriptomic profile of serum and urine EVs from patients with CCA, PSC, ulcerative colitis and HCs was characterized. Differential RNA profiles were found in serum and urine EVs in CCA patients and control groups (with benign diseases and HCs), showing high diagnostic capacity. Overall, patients with CCA present specific RNA profiles in EVs, mirroring tumour characteristics and constituting novel promising liquid biopsy biomarkers.[56]

MINIMAL RESIDUAL DISEASE ASSESSMENT

Given the high risk of relapse after BTC resection, a biomarker that aids the identification of patients at major risk of recurrence and who may benefit most from adjuvant therapies is an attractive prospect. This perspective is especially relevant for iCCA, leading to a better chance of receiving a second resection or other liver-directed therapies that may improve survival.

Currently, ctDNA appears as the most promising tool in detecting minimal residual disease (MRD) after curative surgery and in monitoring disease recurrence during follow-up. Although this field is rapidly evolving, very few studies have been published on CCA in this setting. The role of ctDNA analysis in detecting MRD was evaluated in a small cohort of 11 patients with resected pancreatobiliary malignancies (8 BTC). Although not statistically significant, a trend towards increased relapse risk in patients with ctDNA detected after surgery was evidenced (66.67% in ctDNA-positive versus 37.5% in ctDNA-negative patients).[57] In a retrospective study on 56 patients with early stage BTC after curative resection, ctDNA detection after surgery (MRD window period of 2-12 weeks) or during surveillance (>12 weeks after surgery) was associated with lower relapse-free survival (RFS).[58] Patients with positive ctDNA in the MRD window period had an RFS of 6.6 months versus not reached in patients with negative ctDNA [hazard ratio (HR) 26, 95% confidence interval (CI) 2.6-265, $P < 0.0001$], and an RFS of 19.3 months versus not reached during follow-up (HR 20, 95% CI 2.6-153, $P < 0.0001$). CtDNA identified recurrence in 93.8% of the recurred patients, with an average lead time of 3.7 months.

No data are available using approaches other than ctDNA, although most recent efforts in liquid biopsy application in BTC have focused on miRNAs. Expression levels of miR-21 were found to be significantly higher at baseline in patients with BTC and significantly decreased after surgery, representing a possible marker to assess MRD.[45]

METASTATIC DISEASE

In the past few years, the CCA clinical landscape has been significantly changing in terms of new treatment options, thus defining personalized therapeutic strategies.

For over a decade, cisplatin plus gemcitabine was the only standard first-line therapy for advanced CCA patients.[59] Recently, two phase III randomized trials demonstrated that the combination of standard chemotherapy with an ICI (durvalumab or pembrolizumab) provides greater efficacy when compared with chemotherapy alone.[60,61] Based on these results, chemo-immunotherapy has become the new standard of care in the first-line setting.

However, the majority of CCA patients experience disease progression and subsequent treatment lines are often needed. In this setting, precision oncology plays a fundamental role in offering innovative therapeutic options as an alternative to standard second-line chemotherapy.[62]

Molecular profiling

Using next-generation sequencing (NGS) technologies, recent translational studies have revealed a complex molecular landscape in CCA patients, identifying several potentially targetable genomic alterations. Up to 50% of patients harbour actionable genetic alterations: in eCCA the most prevalent are *KRAS* and *TP53* mutations and *ERBB2* amplifications, while in iCCA the most common are *FGFR* fusions and *BRAF*, *ARID1A* and *IDH* mutations.[63]

To date, several *IDH* and *FGFR* inhibitors have shown efficacy as second-line therapies in patients with these druggable alterations, leading to European Medicines Agency and/or Food and Drug Administration approvals; it is the case, for example, of ivosidenib for *IDH1*-mutant CCA patients and pemigatinib and futibatinib for patients with *FGFR2* rearrangements.[64-66] The testing of other driver alterations is suggested by international guidelines for their possible impact in clinical practice considering the availability of targeted therapies, such as *ERBB2* mutations/HER2 expression status, *BRAF* mutations, *NTRK* fusions, and microsatellite instability and mismatch repair (MSI/MMR) deficiency status.[4] Furthermore, several trials are still ongoing, evaluating targeted therapies also in the first-line setting, both alone [i.e. the FIGHT-302 trial (NCT03656536) with pemigatinib, while the FOENIX-CCA3 trial (NCT04093362) with futibatinib and the PROOF-301 trial (NCT03773302) with infigratinib have been closed due to poor recruitment] or in combination with chemotherapy (NCT04088188 with pemigatinib or ivosidenib) or immunotherapy (NCT05913661 with pemigatinib and anti-programmed cell death protein 1).

Due to the clinical relevance of novel treatment approaches, molecular profiling is essential to guide treatment decisions and offer biomarker-directed therapies.[4,63] Considering the several genetic alterations identified in BTC and their complexity (i.e. *FGFR2* fusions), NGS is currently the preferred approach and should be carried out upfront in all patients.[67,68] CtDNA analysis may be considered if the tumour tissue is not sufficient for NGS and it is recommended also by European Society of Medical Oncology (ESMO) guidelines,[69] especially since BTC sampling often yields low tumour cellularity tissue. CtDNA can be chosen also when urgent therapeutic decisions are needed. Moreover, liquid biopsy offers the possibility of providing a real-time picture to monitor treatment response and to dynamically identify driver alterations and resistance mutations.

A positive correlation ($r = 0.9326$, $P < 0.0001$) between estimated tumour volume (based on radiological imaging) and cfDNA shedding was described.[14] Furthermore, ctDNA variant allele frequency (VAF) strictly correlated with tumour load, as well as with progression-free survival (PFS).[23] A similar tumour variant burden (considering gene variants with a VAF >2%) was found comparing liquid biopsy and tissue results.[14]

In this scenario, several studies have tested ctDNA and cfDNA to detect molecular alterations, showing high concordance with tissue biopsies.[15,70,71] An American prospective experience compared NGS results from plasma cfDNA and tumour biopsies of 26 pancreatobiliary carcinomas reporting a concordance of 90%.[15] A study on 102 Korean patients showed a ctDNA sensitivity of 84.8% for mutations detected in tumour tissue.[71] Another study analysed 138 liquid biopsy samples of advanced BTC patients; excluding variants of uncertain significance, therapeutically relevant alterations were observed in 55% of cfDNA samples.[72] More recently, an extensive analysis of 1671 patients with advanced BTC identified genetic alterations in cfDNA in 84% of them (44% had targetable aberrations). In particular, concordance between liquid biopsy and tissue samples was the highest for *IDH1/2* and *BRAF* point mutations, ranging from 87% to 100%. A significant discordance has been described for *FGFR2* fusions, with the lower detection rate in cfDNA potentially due to the inability to correctly identify all fusion partners.[21] However, custom NGS panels have recently proven to be precise in identifying also *FGFR2* fusions from plasma samples, focusing on the regions most commonly involved in the genomic breakpoints of *FGFR2* fusions (intron 17 and exon 18).[73]

Of note, another study showed higher concordance of ctDNA genetic alterations with metastatic tumour tissue in comparison with primary tumour tissue.[74] These findings suggest that novel genetic alterations may be involved in metastatic dissemination and disease progression, and that ctDNA analysis may be an interesting option for a more precise and timely molecular characterization.[14]

Resistance mechanisms

As previously noted, monitoring treatment response is an important setting for potential ctDNA/cfDNA application. A German study evaluated ctDNA and tumour tissue samples in 24 patients with iCCA and eCCA before and during chemotherapy.[23] In therapy-naive patients, the mutational profile between tumour tissue and ctDNA was concordant in 74% of cases (92% in iCCA, 55% in eCCA). New detectable ctDNA mutations were observed at disease progression, with ∼63% of CT-naive patients who developed changes in their mutational profile during chemotherapy. The most frequently emerging mutated genes were *ERBB2*, *KMT2C*, *MUC1*, *ARID1A*, *CBLB*, *FOXE1*, *GATA6* and *MAP3K4*.

Another recent study evaluated a liquid biopsy-based model to predict clinical benefit from ICIs in the first-line setting.[75] CtDNA concentration changes after treatment start, *KRAS* mutational status and leukocyte transcriptome profile (analysed by whole transcriptome sequencing from RNA extracted from peripheral blood mononuclear cells) were used to predict 6-month PFS rate with an AUC of 0.909 (95% CI 0.795-1.024).

CtDNA analyses have been tested in patients with *FGFR2* rearrangements to identify monoclonal or polyclonal acquired alterations conferring resistance to pemigatinib and infigratinib.[22,76-78] In a recent study by Gonzalez-Medina et al., most patients progressing to *FGFR2* inhibitors presented increased fusion allele fraction and tumour fraction in cfDNA ∼3 months before radiological disease progression, but changes in cfDNA concentration were detected only when progression occurred.[73] Multiple point mutations in the *FGFR2* kinase domain have been evidenced at progression, with a marked inter- and intralesional heterogeneity and different mutations in individual resistant clones (up to 13 independent mutations per patient). The most frequently detected mutations involved the tyrosine kinase domain.[78,79] Interestingly, an increase in

VAF of resistance mutations has been evidenced several months before clinical progression.[73] Also, different numbers of *FGFR2* resistance mutations have been detected for each *FGFR2* inhibitor (higher with pemigatinib, lower with futibatinib, with a mean of nine and two mutations per patient, respectively).[73] In a cohort of 82 patients treated with *FGFR* inhibitors, 60% developed a secondary kinase domain alteration, with a total of 128 distinct *FGFR2* mutations detected; N550 molecular brake (N550D, N550H, N550K, N550T) and V565 gatekeeper (V565F, V565I, V565L) mutations were the most common (63% and 47% of all *FGFR2* kinase domain mutations, respectively).[79] The gatekeeper residue controls the accessibility of ATP competitive inhibitors to the hydrophobic pocket, while the molecular break residue forms hydrogen bonds that regulate kinase activation. In another cohort of 36 patients with tumours harbouring activating *FGFR2* alterations (27 with cholangiocarcinoma) and treated with reversible (i.e. pemigatinib and erdafitinib) or irreversible (i.e. futibatinib) *FGFR2* inhibitors, the same polyclonal kinase domain resistance mutations were evidenced in the majority of patients.[80] At least one mutation affecting either the molecular brake N550 or the gatekeeper V565 was found in 59% of the patients progressing to reversible inhibitors, and they were the only kinase domain mutations found at progression to futibatinib. Interestingly, polyclonality was rare in patients with tumours other than cholangiocarcinoma (one among nine patients). Other off-target resistance mechanisms have also been evidenced, even if less frequent, such as acquired mutations of *PTEN*, *PIK3CA*, *NRAS*, *CDKN2A/B* and *TP53.*[78,81] They can be evidenced often together with on-target mutations.[80] Notably, some of the off-target alterations can be evidenced also before *FGFR* inhibitor treatment (i.e. *PIK3CA* mutations concomitant with *FGFR2* alterations in 15%-20% of iCCAs) and may represent pre-existing subclonal alterations only emerging at drug resistance due to a different resistance mechanism.[79]

In this context, the efficacy of a new selective irreversible *FGFR2* inhibitor (RLY-4008 or lirafugratinib) was evaluated in a phase II study analysing serial cfDNA samples. This third-generation drug has been designed as a multitarget inhibitor against the most common mutations conferring resistance to other *FGFR2* inhibitors. Interestingly, though, the presence of acquired resistance mutations (i.e. V564F) was still described at the time of progression.[82]

Regarding patients with *IDH1/2* mutations, a lower VAF of mutated ctDNA determined by NGS before treatment start was described as associated with a longer median time to treatment failure (3.6 versus 1.5 months, $P = 0.008$).[83] Similarly, patients with a decrease in *IDH* mutations evidenced by droplet digital PCR on ctDNA had a trend towards a longer median survival in comparison with patients with no changes or increased VAF. Mutant *IDH* isoform switching (both from cytoplasmic mutant *IDH1* to mitochondrial mutant *IDH2* and vice versa) has been described as a mechanism of acquired resistance to *IDH* inhibition, even though data mainly derive from acute myeloid leukaemia patients.[84] A patient with *IDH1* R132C-mutant iCCA with a partial response to ivosidenib showed an *IDH2* R172V mutation at disease progression. Cleary et al. described two other iCCA patients with *IDH1* R132C mutation developing resistance mutations during treatment with ivosidenib.[85] One patient acquired an *IDH2* R172K mutation at ctDNA analysis, with the persistence of *IDH1* R132C and *PIK3CA* E545K mutations, even though with a lower MAF (2.1% versus 15% at baseline and 2.2% versus 9%, respectively); the other patient developed a second-site mutation in *IDH1* (D279N) co-existing with the basal *IDH1* R132C mutation, detected by a new biopsy of a liver metastatic lesion. Several mechanisms of both primary and secondary resistance have been described, including the activation of other pathways (i.e. PI3K/AKT/mTOR).[86]

Data on resistance mutations have been reported also with HER2-targeted treatment in HER2-positive BTC patients: isolated *TP53* mutations at baseline predicted a lower PFS compared with patients with other alterations (i.e. *TERT* promoter or *PIK3CA*) or no detected mutations (6.51 versus 12.02 versus 10.58 months, respectively; $P < 0.001$).[87]

Another promising noninvasive biomarker in advanced BTC is represented by miRNAs; altered miRNA profiles have been described in CCA as involved in resistance to CT.[44,88] For instance, miR-21 experimental inhibition sensitized CCA cells to gemcitabine through the inhibition of *PTEN*, resulting in decreased PI3K signalling.[89] Reduced expression of miR-200b/c has also been reported, and its enforced expression restores 5-fluorouracil (5-FU) sensitivity in CCA cells.[90] Similarly, expression of miR-29b, miR-205 and miR-221 are downregulated in gemcitabine-resistant CCA cells, but their experimental overexpression restores gemcitabine sensitivity.[91] The same happens with a restored expression of miR-106b, which leads to re-sensitization to 5-FU, mainly through the modulation of *ZBTB7A*, a proto-oncogenic transcription factor.[92] MiR-130a-3p levels mediate resistance to gemcitabine by targeting the expression of another transcription factor, peroxisome proliferator-activated receptor (*PPARG*).[93] Functional high-throughput approaches on CCA cell lines combined with analyses of BTC tissues have identified miR-1249 as a driver of the expansion of the CD133+ subpopulation that is responsible for primary and secondary resistance of CCA cells to cisplatin and gemcitabine.[88]

DISCUSSION

There are several settings in which liquid biopsy application could constitute a powerful tool to improve the clinical management of BTC (Figure 1). However, available data are still quite immature in comparison with other malignancies.

Few data are available regarding ctDNA/cfDNA application for BTC screening in high-risk subjects and MRD assessment after curative resection.

The analysis of methylation patterns appears as a promising noninvasive tool for early diagnosis, especially to distinguish BTC from other benign conditions of the biliary tract. The methylation of several genes has been reported

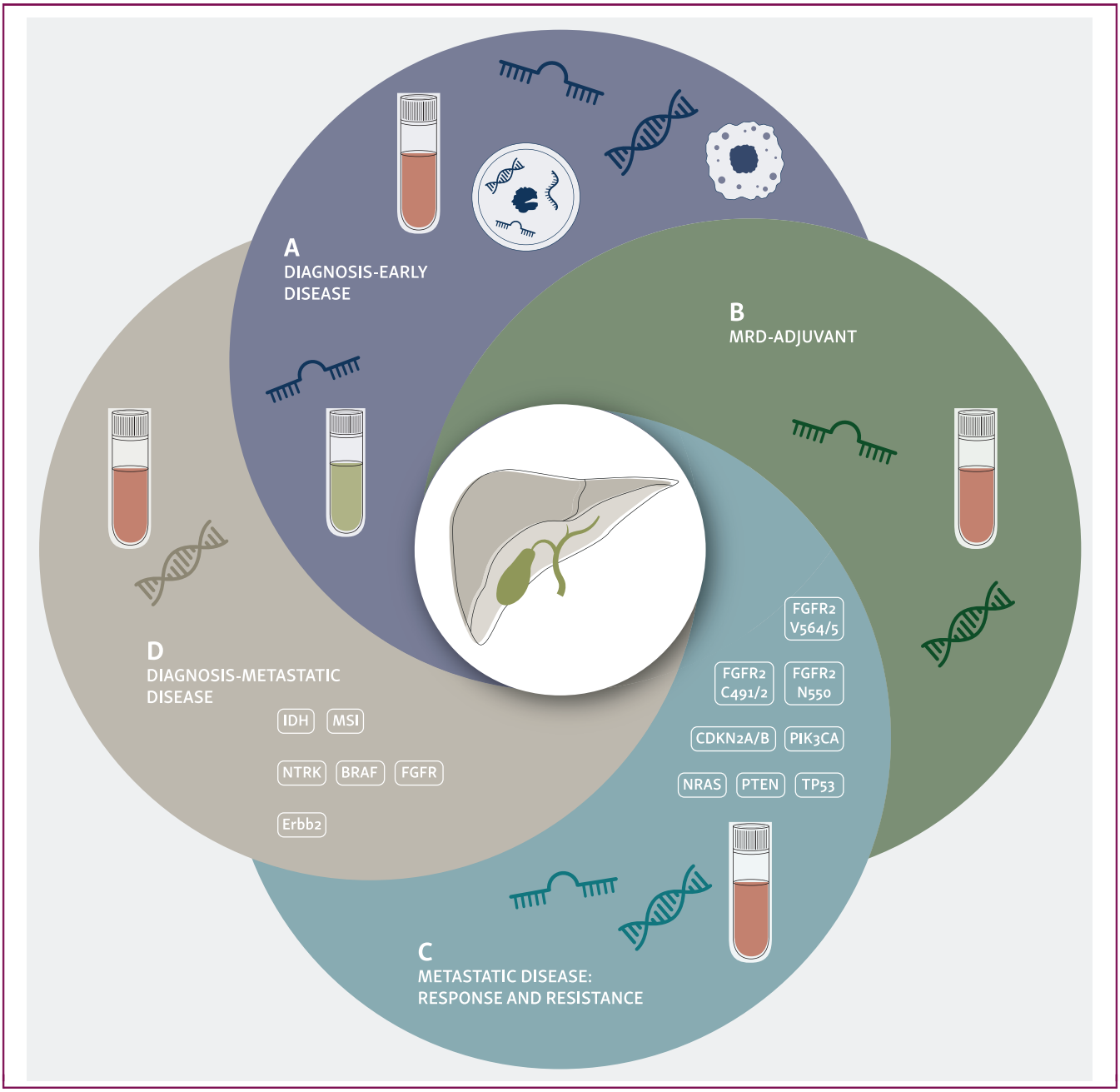

Figure 1. Liquid biopsy applications in BTC. (A) Blood and bile samples in diagnosis and early disease detection, exploiting circulating free/circulating tumour DNA (cfDNA/ctDNA), microRNAs (miRNAs), circulating tumour cells or extracellular vesicles. (B) Evaluation of minimal residual disease (MRD) by ctDNA or miRNAs. (C) Molecular profiling in the advanced setting by ctDNA analysis. (D) CtDNA and miRNAs as tools to study primary and secondary resistance to systemic therapies.

as a potential biomarker for cancer detection, but data are still limited and need to be confirmed prospectively. Also, ctDNA sensitivity remains low in early stage BTC due to the limited shedding.

Furthermore, miRNAs have been a central focus as an innovative instrument for BTC diagnosis. Several miRNAs have been studied and identified as possible biomarkers for early noninvasive diagnosis, considering the differential expression levels in BTC patients in comparison with healthy subjects. A good discriminative ability has been described for the up-regulation or reduced expression of several specific miRNAs.[35,45-47,51] Clinical application remains problematic, though, due to the possible alteration in the expression levels, also in patients with benign clinical conditions. In particular, decreasing levels of some miRNAs (i.e. miR-26a), which have been described as downregulated in CCA but up-regulated in PSC, would be difficult to relate to early CCA rather than to a recovery from PSC.[47]

The role of perioperative ctDNA testing for BTC is not known yet, especially regarding its potential impact in the neoadjuvant setting. Available data only derive from small experiences, using both ctDNA detection and miRNAs expression levels as possible biomarkers to assess MRD. No statistical significance for a higher risk of relapse has been shown in patients with ctDNA detection after surgery.[57] Another retrospective study has been recently presented,

considering 14 patients with iCCA who underwent curative resection.[94] Preoperative ctDNA was reliably detected in all patients except for one, independent of disease stage, but ctDNA concentration did not show a linear correlation with disease recurrence or survival, while a linear correlation was shown with pathological tumour size. Therefore, the prognostic value of ctDNA detection in the perioperative setting has yet to be proven.

CtDNA levels for specific mutations or as a quantitative marker have been proven to be prognostic.[14,95] Furthermore, longitudinal monitoring could help anticipate recurrence after definitive treatment,[57] evaluate disease response[83,96] and intercept resistant clones.[22,77-79,84,85] However, the variability in tumour shedding hinders technique replicability and makes it difficult to clearly establish concordance between ctDNA and tissue samples, both from intra-/extrahepatic primary and metastatic lesions.[74,97]

CtDNA can be effectively used to molecularly profile the disease when the tumour tissue is unavailable. A good concordance with tissue analysis has been shown, in particular with metastatic lesions.[15,70,74] However, testing for *FGFR* and *NTRK* fusions should be carried out on tissue, preferably with panel-based RNA methods able to identify fusion transcripts both with known and unknown fusion partners.[4] An RNA-based NGS approach should be preferred, especially when the available tissue is limited.[67]

In the expanding field of precision oncology, ctDNA analysis emerges as a valuable tool for providing a comprehensive molecular characterization of the disease and thus the most appropriate targeted therapy for BTC patients in the advanced setting. Moreover, it could become the pillar of future research by helping to understand molecular mechanisms of tumour response to systemic treatment and the escape pathways through which cancer cells acquire resistance, possibly offering to patients a sequence of therapies targeted to the emergence of resistance alterations. This is exemplified by new-generation *FGFR2* inhibitors designed to overcome common resistance mutations, or new drugs aimed at *IDH1*/*IDH2* dual inhibition to address *IDH* isoform switching as a resistance mechanism.

The clinical impact of miRNAs in BTC is also under investigation in the advanced setting, considering the alteration of RNA expression levels shown in CCA cells resistant to chemotherapy. The next step will be the evaluation of miRNAs *in vivo* in order to correlate expression levels with resistance to systemic treatments in BTC patients, possibly having an impact also as therapeutic targets to overcome acquired drug resistance.

CONCLUSIONS

Liquid biopsy represents a new and potentially invaluable tool for diagnosis, molecular analysis, patient selection and disease monitoring in the often-challenging setting of BTC.

Further studies are needed to evaluate the clinical impact of ctDNA analysis, particularly in the curative setting and for MRD detection, as well as to explore the potential impact of neoadjuvant systemic strategies.

As of today, liquid biopsy can be considered especially valuable for disease molecular profiling. Routine application in future clinical practice is desirable, given the sampling difficulties often encountered in BTC. Precision medicine in BTC could be significantly advanced through liquid biopsy by identifying primary and secondary resistance to targeted therapies, thus optimizing treatment strategies.

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