

Liquid biopsy in cholangiocarcinoma: Current status and future perspectives

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Abstract

Cholangiocarcinoma (CCA) are a heterogeneous group of tumors in terms of aetiology, natural history, morphological subtypes, molecular alterations and management, but all sharing complex diagnosis, management, and poor prognosis. Several mutated genes and epigenetic changes have been detected in CCA, with the potential to identify diagnostic and prognostic biomarkers and therapeutic targets. Accessing tumoral components and genetic material is therefore crucial for the diagnosis, management and selection of targeted therapies; but sampling tumor tissue, when possible, is often risky and difficult to be repeated at different time points. Liquid biopsy (LB) represents a way to overcome these issues and comprises a diverse group of methodologies centering around detection of tumor biomarkers from fluid samples. Compared to the traditional tissue sampling methods LB is less invasive and can be serially repeated, allowing a real-time monitoring of the tumor genetic profile or the response to therapy. In this review, we analysis the current evidence on the possible roles of LB (circulating DNA, circulating RNA, exosomes, cytokines) in the diagnosis and management of patients affected by CCA.

Key Words: Liquid biopsy; Cholangiocarcinoma; Circulating biomarkers; Biliary tumors; Circulating DNA; Circulating RNA; Exosomes; Cytokines

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Core Tip: Liquid biopsy allows to access tumoral components and genetic material from fluid samples. In patients affected by cholangiocarcinoma could play a major role as minimally invasive screening and diagnostic biomarker, prognostic tool and therapeutic monitoring target, but its role in the clinical practice is still marginal and further research is necessary.

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INTRODUCTION

Cholangiocarcinoma (CCA) still represents a challenging entity despite the efforts of researchers and clinicians that led to constant advances in the characterization, diagnosis and treatment of this malignancy. It can arise from the bile duct epithelium at any level and is characterized as an adenocarcinoma in the vast majority of cases, although some other rare histological subtypes have been described[1,2]. The term CCA groups together tumors with substantial heterogeneity in terms of aetiology, natural history, morphological subtypes, molecular alterations and management. CCA is generally classified according to the anatomical location as: Intrahepatic CCA (iCCA) when arises from the second-order bile ducts or proximally, perihilar CCA (pCCA) when arises distal to the second-order bile ducts but above the cystic duct and distal CCA (dCCA) when affects the bile duct between the insertion of the cystic duct and the ampulla of Vater. The two most common forms are pCCA and dCCA, accounting for approximately 50%-60% and 30%-40% of the cases respectively[3,4], with iCCA representing only 10%-20% of the total[5]. CCA presents three distinct growth patterns according to the macroscopic appearance: Mass-forming (the most common variant, represented in two-thirds of cases), periductal infiltrating and intraductal growing[6-8]. A rare and independent entity that comprises features of both iCCA and hepatocellular carcinoma (HCC) is represented by the mixed HCC-CCA[9-11].

CCA is the second most common liver malignancy after HCC[12], and its incidence is highly variable across the World, ranging from 0.3/100000 population to as high as 85/100000 in parts of North East Thailand[6,12,13]. The mortality is similarly variable, with rates between 0.7-6/100000[14,15]. This represents a manifestation of the significant role of the genetic predisposition and the environment as risk factors for the CCA carcinogenesis, which is likely to be a multifactorial process leading to variations in the three subtypes[16]. Despite knowledge of aetiological factors, the majority of cases are attributable to sporadic events[2,17]. Multiple risk factors have been identified and have been shown to markedly increase the risk of CCA development in specific groups of patients or geographic areas. A predisposition to CCA has been observed in patients with biliary pathology [bile duct cysts[18,19], primary sclerosing cholangitis (PSC)[20], choledocholithiasis[21-23], pre-malignant biliary lesions[24-27]], liver diseases (cirrhosis[28], viral hepatitis[29-31]), inflammatory bowel disease[18,32], pancreatitis[18], parasitic infections (from *Clonorchis sinensis* and *Opisthorchis viverrini*) [33], metabolic disorders (diabetes[34] and non-alcoholic fatty liver disease[35]), alcohol consumption[36,37], tobacco use[18,37,38] and genetic polymorphism[16].

Despite the apparent heterogeneity in risk factors associated with CCA, all result in damage to the biliary epithelium secondary to combinations of cholestasis and chronic inflammation, indicating that these pathological processes are key to disease biology. Chronic inflammation leads to activation of cytokines and growth factors and is characterized by an influx of immune cells and fibroblasts. This generates an oncogenic microenvironment typified by unregulated epithelial proliferation, increased mutational burden and genomic instability which in turn drive the inflammatory cascade[39,40]. This process also promotes the development of an aberrant vascular network (the angiogenic switch[41]), which promotes uneven distribution of oxygenated blood and ultimately hypoxia; a key driver of metastatic dissemination. Whilst CCA may develop de-novo in a chronically inflamed bile duct, CCA also

develops on the background of premalignant lesions including biliary epithelial neoplasia[25], intraductal papillary neoplasm of the bile duct[26,27,42], mucinous cystic neoplasm[43] and intraductal tubule papillary neoplasms[24,26]. Interestingly, only a proportion of these pre-malignant lesions will develop into malignancies and the role that chronic inflammation plays in driving their malignant progression remains unclear.

Mutation status has been extensively studied in CCA and intra- and extrahepatic forms can be defined as two distinct genetic entities characterized by substantial differences in their patterns of mutation[44-46]. Among the most commonly mutated genes are *IDH1/2*, *FGFR2* fusions, *BAP1*, *BRAF*, *ARID1A*, *KRAS*, *TP53* and *SMAD4* in iCCA; *KRAS*, *TP53*, *SMAD4*, *ERBB3*, *PRKACA-PRKACB* fusions and *ELF3* in pCCA and dCCA[15]. Some of these mutations are associated with prognosis, whilst others may indicate sensitivity to a particular pathway inhibitor or use as a predictive biomarker[46-48]. As well as mutational analysis, epigenetic changes have also been identified in CCA, including methylation-driven inactivation of several tumor suppressor genes, histone modification and aberrant expression of non-coding RNAs[49,50]. Dysregulation of non-coding RNAs has been linked to changes in gene expression that regulate cell survival, proliferation, and chemoresistance in CCA[15]. A better understanding of the epigenetic alterations in CCA therefore has the potential to identify new diagnostic and prognostic biomarkers and novel therapeutic targets[51].

pCCA or dCCA are often diagnosed following presentation with biliary obstruction, weight loss and non-specific abdominal pain, whereas iCCA less frequently cause jaundice and are detected at a later stage in their evolution[5,52], with 20%-25% of cases being an incidental finding[53]. In cases of suspected iCCA the diagnostic algorithm should include a contrast-enhanced computed tomography (CT) and/or a magnetic resonance imaging (MRI), in order to evaluate the relationship of the tumor with the adjacent structures, the extent of local invasion, calculate the future liver remnant volume, and can be associated to a chest CT for the detection of distant metastases[15,54,55]. One of the critical aspects is the difficulty in discriminating between iCCA and HCC, especially in case of small tumors < 2 cm of diameter[56,57]. This differentiation is of great importance considering that management, chemotherapy options and prognosis are different between these two forms. In case of pCCA or dCCA the majority of patients present with jaundice, and the initial investigation performed is an ultrasound in order to identify the site of biliary obstruction and exclude the presence of stones with a sensitivity of over 90%[58]. CT and MRI with magnetic resonance cholangiopancreatography are essential investigations to be performed in the diagnostic and staging process of pCCA and dCCA, where an important role is played by endoscopic procedures. Endoscopic ultrasonography (EUS) provides a high detection rate, especially in dCCA, and the possibility of sampling tumor tissue through fine-needle aspiration that comes at the price of risk of bleeding, infections and tumor seeding[59-61]. Endoscopic retrograde cholangiopancreatography can evaluate the biliary strictures and enables the placement of stents to relieve the biliary obstruction and the brushing sampling for cytological analysis. Percutaneous transhepatic cholangiography (PTC) is frequently performed in patients with CCA and can have both a diagnostic and a therapeutic role. PTC can achieve upstream biliary decompression of the future liver remnant in patients amenable of surgical resection or as a palliative procedure in case of unresectable malignancy, can provide accurate imaging of the biliary tree through cholangiography and allow endobiliary brush cytology[62]. Choledochoscopy is playing an increasing role in the diagnosis of extra-hepatic CCA, and with the implementation of the digital fiberoptic SpyGlass the sensitivity and specificity of this methodology are 90% and over[63,64]. Due to the difficult accessibility of CCA especially when located in the perihilar region and their highly desmoplastic and paucicellular nature, the biliary cytology sensitivity is as low as 20%-40%[65], but can be doubled when fluorescent *in situ* hybridization is used to analysis chromosomal instability[66,67]. This technique assumes a relevant role in discriminating malignant from benign strictures in patients affected by PSC, where the inflammatory changes can result in cellular alterations similar to neoplasia or by the multisystem fibroinflammatory disorder named immunoglobulin G4-related disease, where suspicious biliary strictures also can be present[68-70]. The difficulty in distinguishing a benign from a malignant stricture can result in a number of patients undergoing major surgical procedures burdened by a significant morbidity and mortality for a benign condition. Reports show that 3%-24% of patients undergoing surgery for suspected CCA are ultimately diagnosed with a benign condition[71-73]. The role of ¹⁸F-fluorodeoxyglucose positron emission tomography is marginal and controversial: Although can provide essential

information in the staging process, especially regarding potential lymph node and distant metastases, and in the identification of tumor recurrence; its incorporation in the diagnostic algorithm is still not justified given the low sensitivity[74]. The serum level of carbohydrate antigen 19-9 (CA19-9) has been extensively investigated as a potential biomarker of CCA and its main limitations are the sensitivity too low to be reliable at early stages of the disease, the non-specificity for CCA and the levels significantly altered in case of cholangitis or cholestasis[75,76]. Serum levels < 100 U/L in non-PSC patients and < 129 U/L in PSC patients have shown a negative predictive value of 92% and 99% respectively[54]. Since surgery is the treatment of choice for patients affected by all types of CCA, establishing a correct diagnosis is essential but represents a challenging and complex process that can result in delays of the surgical or medical treatments and have a negative impact on the outcome. Over half of the patients diagnosed with CCA present at an advanced stage, when surgery is not a suitable option and the prognosis is very poor[40,77-80]. Tumor stage, although with different classifications for iCCA, pCCA and dCCA[81], correlates with patient expected survival, with 5-year relative survival rates between 2 and 24% for iCCA and between 2% and 15% for extrahepatic CCA[82].

In this setting, there is a strong need for novel tools that can assist clinicians in diagnosing, managing and monitoring CCA.

LIQUID BIOPSY

Thanks to very recent breakthroughs such as the sequencing of the whole genomes of dozens of tumors[83], it became possible to identify and analysis the genetic changes that contribute to cancerogenesis. Tumors have been historically classified by anatomical site and histopathology, but different types of tumors have shown to carry similar recurrent mutations and apparently similar cancers very different sets of mutations, making every tumor a unique entity and giving way to a novel individualized oncologic approach[84-86]. In this scenario, accessing tumoral components and genetic material could play a crucial role in all stages of cancer management, from the diagnosis to the monitoring of the genetic profile that may be subject to a dynamic evolution, and to define targeted therapies for a tailor-made approach. Sampling tumor tissue is often performed with invasive procedures carrying significant risks for the patients and can result in insufficient amount of material for the analysis or be impossible to be done in case of tumors situated in inaccessible locations. Tumors are continually evolving from a mutational and tumor microenvironmental view-point and display immense intra-tumoral heterogeneity. Therefore, if precision medicine is going to become a realistic option, serial sampling from multiple sites within the tumor will be required to continually adjust the personalised therapeutic algorithm. Moreover, being tumors such dynamic and evolving entities, a systematic monitoring of the molecular alterations through serially repeated tissue biopsies, even when possible, would be not easily feasible and anyway provide only information of the biopsied site. A possible way around these technical issues is the liquid biopsy (LB).

The term "LB" comprises a diverse group of methodologies, centering around detection of tumor biomarkers from fluid samples. Blood, plasma, urine or other fluids are accessed in a minimally invasive approach that can be used in a serial manner to interpreted disease biology. A range of technologies can be applied to sample analysis, with next generation sequencing (NGS) providing a high level of sensitivity and a lower limit of detection[87-89], such that even small amounts of tumor-derived genetic material derived can be detected. Greater sequencing depth is obtainable through NGS compared to traditional Sanger sequencing[90], enabling detection of a wide variety of genetic alterations inclusive of single-nucleotide polymorphisms, mutations, insertions and deletions with high accuracy (sensitivity and specificity in the range of 95%-100%), increasing the ability to investigate mutations in cancer and other diseases[91]. Compared to the traditional tissue sampling methods LBs are less invasive and can be serially repeated, allowing a real-time monitoring of the tumor genetic profile or the response to therapy.

Types of LBs

Circulating tumor DNA: Cell-free DNA (cfDNA) may enter the circulation through apoptosis or necrosis of cells[92,93] and is present as small fragments of 140-200 base pairs[94,95]. Higher yields of cfDNA have been observed in patients affected by inflammatory processes, trauma and cancer, although the pathophysiological mechanisms are not completely understood and the cfDNA concentrations may vary

significantly[96]. The small fraction of cfDNA presenting tumor-specific alterations and originating from cancer cells during tumor progression and turnover, is named circulating tumor DNA (ctDNA)[93]. These fragments are generally characterized by the presence of somatic variants and can be difficult to isolate from cfDNA originating from normal cells[97]. The identification of cancer-specific mutations or methylation patterns that are indicative of the tumoral origin of the DNA can be obtained through the polymerase chain reaction (PCR) or NGS, the latter being more expensive but allowing a more comprehensive analysis of a larger set of mutations; an aspect valuable when considering the genetic heterogeneity of most tumors[98-100]. Detection is made difficult in tumors that display limited ctDNA shedding, stroma-rich cancers such as pancreatic adenocarcinoma or CCA[101-103] and from a technical perspective, most assays can only a small number of genes when compared to tissue-based panels[104]. This type of LB showed promising results as a potential screening and early diagnosis tool, as guidance for the selection of the most appropriate treatment strategy, for the detection of minimal residual disease and risk of relapse and prognosis[99,105,106].

Exosomes, cytokines and proteins: Exosomes are a subset of extracellular vesicles (EVs) originating from inward budding of the plasmatic membrane at the end of the endosomal pathway[107]. They can be found in all body fluids and are characterized by a small size (around 100 nm) and contain proteins, DNA and RNA[108], being involved in intercellular communication, cancer progression and metastasis[109,110]. Exosomes present both a set of protein regardless their tissue origin and other ones in common with their originating cell, giving them the possibility of being a potential diagnostic or prognostic tumor marker following isolation from body fluids[107,111-113]. An area of particular interest is the analysis of the genetic material that the exosomes carry contained within the phospholipid bilayer; a structure that effectively protects these materials from enzymatic degradation[114]. Upregulation of specific exosome micro-RNAs (miRNA) or the presence of mutations within exosome DNA has been associated with the presence of various cancers indicating that their assessment may have diagnostic value[108,115-117]. Cytokines are a broad and diverse group of proteins playing a key role in cellular communication but are also implicated in cancer development and progression[118,119]. Exosomes can deliver cytokines including interleukin (IL)-6, IL-8, IL-10, tumor necrosis factor- α and transforming growth factor- β , representing possible novel tumor biomarkers[120-122]. Moreover, it has been described how cancer cells-released EVs present a set of integral membrane proteins and membrane-anchored proteins that can be directly involved with tumor angiogenesis and anti-angiogenic therapies resistance[123].

Circulating tumor RNA: For over three decades extracellular RNA has been recognized as a tumor biomarker[124]. When in the bloodstream, is more unstable than cfDNA and with a half-life of only a few seconds when not incorporated within vesicles or bound to proteins[125]. Most circulating RNA does not originate from cell apoptosis or necrosis but from active secretion, enabling a real-time assessment of the cell population of origin[126]. The detection and analysis of circulating RNA is performed using techniques and technologies similar to the ones used for cfDNA, providing both quantitative and qualitative information about the circulating transcriptome[127,128]. All circulating RNA classes have been investigated as potential cancer biomarkers, including miRNA, long non-coding RNA and messenger RNA[129-133]. Having a key role in limiting the expression of tumor suppressors and increasing the activation of oncogenic pathways as well as mediating drug sensitivity and resistance mechanisms, circulating tumor RNA has a great potential of clinical significance in cancer early detection, treatment, monitoring and prognosis[134,135].

Circulating tumor cells: The ability of tumors to disseminate cells into the bloodstream was first described over 150 years ago[136]. They can originate from the primary or metastatic site and although could be a considerable amount, only a fraction of them will not be rapidly eliminated from the circulation where they can have entered even before the tumor is diagnosed[137-139]. Circulating tumor cells (CTC) can constitute an accessible source of tumor material which can be sampled repeatedly and not going through risky and invasive procedures such as biopsies. The CTC blood concentration is extremely low, in the range of 1-100 cells *per* mL, causing the counting process that usually requires the target cells to be enriched and then detected, isolated and released, to be challenging, time-consuming and expensive[140]. Despite the efforts in implementing new technologies that can ameliorate the CTC detection, the only Food and Drug Administration-approved technique is the CellSearch[®]. The discrimination of the CTC from circulating, mesenchymal-origin

hematopoietic cells is obtained by targeting epithelial markers such as EPCAM and cytokeratins. However, these surface markers are heterogeneous and can be lost during the epithelial-mesenchymal transition that cancer cells undergo as they leave the primary site[99,141-144]. The genetic material that is possible to recover from each isolated CTC is small (2-7 picograms), but after amplification can be sufficient to allow genomic and proteomic profiling and molecular screening analysis[145]. In addition to their established role in basic cancer research, CTC can represent a biomarker in patients affected by various tumors[146-151]. DNA methylation profiling in CTC has been shown to be useful in tumor diagnosis, staging, therapeutic monitoring and prognostication[152-154].

LB IN CCA

Surgical resection is the only potential curative treatment for CCA and the possibility of obtaining an earlier diagnosis would be of paramount importance, considering that the majority of patients diagnosed with CCA present with an advanced stage precluding any surgical treatment. In this setting, LB would have the fascinating potential to increase the chances of obtaining a diagnosis at less advanced stage, overcoming the difficulties and risks of tissue sampling and the diagnostic challenge when it comes to detecting iCCA over HCC. Additionally, it could offer the possibility to assess tumor heterogeneity, identify targeted therapeutic agents according to tumor biology and to evaluate treatment response and mechanisms of resistance to chemotherapy[155].

cfDNA/ctDNA

cfDNA and ctDNA are released into circulation from tumor cells undergoing metabolic secretion, apoptosis, or necrosis. The introduction of NGS assessments of cfDNA/ctDNA recently made possible the detection of genetic and epigenetic alterations associated with CCA, thus widening the potential diagnostic and therapeutic value of LB[156]. Mody *et al*[157] reported the largest profiling ctDNA series, based on 138 patients with biliary tract tumors. They found at least one genomic alteration in 89% of cases, including *TP53*, *BRAF*, *FGFR2* and *IDHA1* mutations, *ERBB2* amplifications, and *FGFR2* fusions. Nevertheless, the concordance between mutations observed in cfDNA/ctDNA with those seen in tumor tissue remains unknown. Zill *et al*[158] measured the concordance between tumor tissue biopsies and plasma-derived cfDNA in 26 pancreatobiliary malignancies (including 8 patients with CCA and 18 with pancreatic cancer). They reported a high concordance between mutations detected in tumor biopsies and cfDNA with the latter identifying the 90.3% of mutations also present in tissue biopsies. Similarly, Andersen and Jakobsen[159] screened 31 mutations in *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* in 11 CCA patients *vs* controls, finding a perfect agreement between mutations found in the tumor and in the plasma in all patients.

Importantly, some of the genetic changes seen in CCA are also demonstrated in benign or pre-malignant disease. For example, the identification of circulating BRCA-mutated DNA might imply a germline BRCA mutation without an underlying malignancy, a *KRAS* mutation as observed in patients with pancreatitis and circulating mutated *TP53* might be related to clonal hematopoietic cells of indeterminate potential[160]. Another role for ctDNA/cfDNA is represented by the possibility of monitoring the response to chemotherapy and targeted therapy, thus tracking emergence of chemotherapy resistance, guiding use of second or third line agents[161,162]. In a German study by Ettrich *et al*[163] ctDNA and tumor tissue samples were collected from 24 patients with CCA before and during chemotherapy, the two samples were subjected to deep sequencing of 15 frequently mutated genes in CCA, including *TP53*, *ARID1A*, *KRAS*, *IDH1*, *BAP1*, *PBRM1*, *SMAD4*, *PIK3CA*, *FBXW7*, *CDKN2A*, *ERBB2*, *NRAS*, *IDH2*, *BRAF* and *BLC2*. The overall blood/tissue concordance was 74% and 92% overall and for intrahepatic tumors respectively, and 63% of chemotherapy-naïve patients displayed a change in their mutational profile during chemotherapy. This mutation drift in circulating mutation status was associated with progression-free survival. Another example is represented by the role of genomic alterations in *FGFR2* which have recently been related to targeted therapies tested in a number of clinical trials, including specific anti-FGFR2 antibodies such as BGJ39[164]. Goyal *et al*[165] analyzed the cfDNA in 4 patients enrolled in a Phase II trial assessing the role of BGJ39. Three out of the four included patients experienced an

initial tumor regression followed by disease progression during the treatment, with cfDNA analysis demonstrating the presence of a V564F acquired mutation and multiple recurrent point mutations in the *FGFR2* kinase domain at progression. A high concordance was observed between tissue and plasma measurements, with post-progression tumor biopsy and autopsy confirming the different *FGFR2* mutations. Although based on a small group of patients, this study highlights the potential of cfDNA in monitoring CCA targeted therapy, where real-time detection of resistance mutations may provide useful information in guiding treatment selection.

Lastly, aside from using plasma as a source of ctDNA/cfDNA, assessment of bile has recently shown promising results as alternative DNA-containing body fluid in CCA[166,167]. A recent study by Shen *et al*[168] on 6 patients with CCA and 4 with gallbladder cancer, compared bile cfDNA and tumor DNA for mutational variants using a panel of 150 tumor-related genes. Their analysis demonstrated that bile cfDNA consisted of longer fragments, than those detected in plasma cfDNA. This was in agreement with the DNA lengths detected from the tumor tissue sample. Bile cfDNA and tumor DNA for single nucleotide variation, insertion and deletion revealed high sensitivity (94.7%) and specificity (99.9%). Therefore, the authors concluded that bile cfDNA could represent a promising source of tumor genetic material for LB in CCA patients, although having access to bile samples before or after surgery or in patients not undergoing an endobiliary procedures could be a difficult and demanding task.

EVs and miRNAs

EVs and miRNAs have received a special attention due to their stability and abundance in biofluids. Recent studies have demonstrated that bile EVs contain a significant amount of miRNAs[169]. These small, noncoding RNAs (18-25 nucleotides) play important roles in the regulation of a number of essential biological processes and are critical for the development of various cancer types[170]. A number of deregulated miRNAs have been recognized to function as oncogenic and/or tumor suppressors. One of the most extensively described miRNAs in CCA is miR-21, an oncogenic miRNA whose overexpression correlates with tumor stage and poor survival[171,172]. Correa-Gallego *et al*[173] compared miRNA-21 expression between 21 iCCA and 11 tumor-free liver samples. Significant overexpression of miR-21 and miR-221 was found in plasma from iCCA patients and circulating miR-21 demonstrated a high discriminatory ability between patients with iCCA and healthy controls [area under the curve (AUC): 0.94]. Similarly, Wang *et al*[174] analyzed the role of circulating miR-21 as a biomarker in patients with a variety of carcinomas in a recent meta-analysis. A total of 36 studies were included for the systemic review, with overall pooled results for sensitivity and specificity of 75.7% [95% confidence interval (CI): 67.1%-82.6%] and 79.3% (95%CI: 74.2%-83.5%), respectively. Interestingly, a higher circulating miR-21 expression was associated with poorer prognosis [odds ratio 2.37 (95%CI: 1.83-3.06, $P < 0.001$)], therefore the authors concluded that in addition to the role as a diagnostic biomarker, circulating miR-21 could have a prognostic value in patients with cancer. Nevertheless, similarly to ctDNA/cfDNA detection in plasma, the identification of miRNA should be interpreted with caution since this biomarker can also be identified in the serum of patients affected by HCC or other liver diseases[175]. Several other circulating miRNAs have shown potential as biomarkers with the ability to discriminate between CCA and other liver diseases[176-179]. Loosen *et al*[177] analyzed the concentrations of a 4 miRNAs panel (miR-122, miR-192, miR-29b and miR-155) in serum samples from 94 patients with CCA undergoing surgical resection and 40 healthy controls. The serum concentrations of all mi-RNAs were significantly elevated in patients with CCA compared to healthy controls or patients with PSC without malignant transformation. Furthermore, a strong postoperative decline of miR-122 serum levels was significantly associated with a more favorable prognosis and outcome. Similarly, Cheng *et al*[180] observed different expression levels of circulating miR-106a, sufficiently to discriminate patients affected by CCA from healthy controls and also from patients affected by benign bile duct diseases inclusive of biliary tract calculi and congenital biliary cysts. The presence and role of miRNA in bile samples has been recently investigated with some promising results[181,182]. Han *et al*[176] analyzed a panel of 1209 miRNAs in bile samples from 106 patients with obstructive biliary disease and found that miR-30d-5p and miR-92a-3p were significantly upregulated in patients with CCA. Similarly, Voigtländer *et al*[183] identified distinct circulating and bile miRNA profiles in patients affected by PSC with or without CCA, showing potential as a screening and diagnostic biomarker. CCA patients had a higher expression of miR-1281, miR-126, miR-26a, miR-30b and miR-122 in serum samples and different concentrations of miR-412, miR-640, miR-1537 and miR-3189 in bile samples compared to PSC patients. The diagnostic value of miRNAs

for CCA has been reviewed and confirmed by two meta-analyses, that found an AUC of 0.88-0.9 and pooled sensitivities of 0.76-0.85 and specificities of 0.79-0.91[184,185]. Notably, in these analyses bile was the biological fluid that yielded the highest diagnostic capacity followed by serum, tissue and urine, with AUCs of 0.95, 0.913, 0.846 and 0.745 respectively.

Several studies have highlighted the potential of miRNAs as diagnostic and prognostic biomarkers for CCA, however there is still lack of strong evidence regarding the source of mi-RNA and which subgroup would provide the most reliable information to be translated into clinical practice. The available evidence is usually limited by super selected study populations with an artificially high prevalence of CCA, with the risk of overestimating the diagnostic accuracy. A possible option to overcome this issue could be to plan to prospectively collect and analysis samples as part of a trial.

Proteins, cytokines and other serum metabolites

Proteins, cytokines and other serum metabolites have recently been evaluated as potential diagnostic and prognostic biomarkers for CCA. Elevated circulating levels of cytokeratin-19 (CYFRA 21-1), MMP-7, osteopontin, periostin and IL-6 have been found in the serum of patients with CCA, differently from healthy individuals and patients with benign biliary diseases[186-193]. Huang *et al*[192] assessed the serum levels of CYFRA 21-1 in comparison with CA19-9 and carcinoembryonic antigen preoperatively, postoperatively and during follow-up in 134 patients with biliary tract cancers and 52 controls without tumors. CYFRA 21-1 had the best diagnostic performance with levels presenting a high concordance with the clinical and oncological status of the patients and showing a decline after surgical resection and an increase when the tumor recurred. Additionally, it represented an independent predictor of 1-year recurrence-free survival and overall survival on multivariate analysis. Osteopontin has also been investigated by Loosen *et al*[193] in 107 patients undergoing surgical resection for CCA. Here, elevated osteopontin was significantly elevated in CCA patients when compared to healthy controls and PSC patients, whilst pre- and postoperative serum concentrations correlated with patient survival. Banales *et al*[194] investigated whether serum metabolomes represent a useful source of biomarkers in the diagnosis of iCCA, HCC and PSC. They found several metabolites that demonstrated high diagnostic value for iCCA and developed two algorithms combining four and six metabolites in order to achieve the best diagnostic value in differentiating iCCA, HCC and PSC. Glycine, aspartic acid, SM (42:3), and SM (43:2) accurately differentiated iCCA from HCC with an AUC of 0.89, 75% sensitivity, and 90% specificity. The addition of PC (34:3) and histidine accurately permitted to differentiate iCCA from PSC with an impressive AUC of 0.99, 100% sensitivity, and 70% specificity. These results were subsequently validated in an independent cohort of patients.

Since cancer cells present a variety of marked metabolic alterations, measuring metabolites in distinct biological samples is now regarded as an encouraging way to identify accurate diagnostic and prognostic biomarkers. However, as yet, none have been adopted into routine clinical practice.

CTC

Although only a few studies have addressed the potential role of CTC as diagnostic and prognostic biomarkers in CCA, some recent research has postulated the association between the number of CTCs and patient survival in patients with biliary tract cancer[195]. After analysis the presence of CTC in 88 patients with CCA, Yang *et al*[196] found that a CTC count ≥ 5 was associated with tumor extent and more aggressive tumor characteristics, as well as independently predicting shorter survival [CTC ≥ 2 hazard ratio (HR) of 2.5; 95%CI: 1.1-5.4; $P = 0.02$ and CTC ≥ 5 HR of 4.1; 95%CI: 1.4-10.8; $P = 0.01$].

CONCLUSION

Despite the constant advances, CCA still represents an unsolved challenge for clinicians and researchers, burdened by difficult diagnosis, management, treatment and with a dismal patient survival. The only curative option is surgical resection, but it can be offered only to a fraction of patients and is burdened by high recurrence rates. Therefore, maximum effort should be applied to increase the current knowledge of this genetically highly heterogeneous group of tumors in order to obtain an earlier

Table 1 Liquid biopsy biomarkers

	Alteration	Source	Potential application	Ref.
DNA				
ARID1A, BAP1, BLC2, BRAF, CDKN2A, FBXW7, FGFR2, IDHA1, IDH2, KRAS, NRAS, PBRM1, PIK3CA, SMAD4, TP53	Mutations	Serum	Diagnostic value	[157-159,163,197]
ERBB2	Amplifications		Diagnostic value	[157]
FGFR2	Fusions		Diagnostic and prognostic value	[157,198]
microRNA				
miR-9, miR-21, miR-29b, miR-106, miR-122, miR-150, miR-155, miR-192, miR-200 family	↑ Expression	Serum	Diagnostic and prognostic value	[171-173,177,179-181,183,199-203]
miR-26a, miR-26a-5p, miR-30b, miR-126, miR-141-3p, miR-551B, miR-604, miR-1281, miR-96-5p, miR-151a-5p, miR-191-5p, miR-4732-3p			Diagnostic value	[183,199,204,205]
miR-30d-5p, miR-92a-3p, miR-412, miR-640, miR-1537, miR-3189, miR-191, miR-486-3p, miR-1274b, miR-16 and miR-484		Bile	Diagnostic value	[169,176,183]
Proteins and cytokines				
Cytokeratin-19, MMP-1, MMP-7, MMP-9, MMP-10, Periostin, IL-6, CYFRA 21-1, Osteopontin	↑ Expression	Serum	Diagnostic and prognostic value	[186,187,190-193]
Serum metabolites				
Glycine, aspartic acid, SM (42:3), and SM (43:2)	↑ Expression	Serum	Diagnostic and prognostic value	[194]

diagnosis, be able to identify patients that will benefit from a specific treatment and develop better targeted therapies. In this scenario, liquid biopsies could play a major role as minimally invasive screening and diagnostic biomarkers, prognostic tools and therapeutic monitoring targets (Table 1). The role of LB in the clinical practice of patients affected by CCA is still marginal and further research is necessary to appreciate its potential and move towards a multimodal, precision medicine approach.

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