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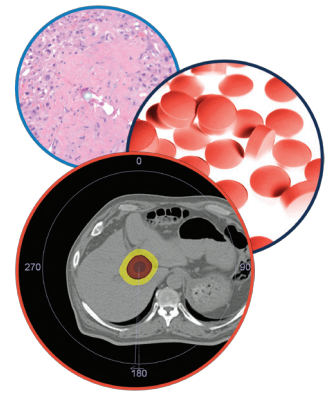

Hepatic Oncology

The clinical role of 'liquid biopsy' in hepatocellular carcinoma

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Practice points

- Circulating-free tumor DNA (ctDNA) and circulating-free tumor cells (CTCs) is released by tumor cells and can be detected in blood.
- Gene mutations and epigenetic changes in ctDNA can be detected and used for diagnostic and prognostic purposes in hepatocellular carcinoma (HCC).
- ctDNA therefore provides a 'liquid biopsy' of cancer which is minimally invasive and readily repeated, making this an attractive potential biomarker in HCC diagnosis and management.
- Evidence suggests ctDNA is a useful diagnostic marker for HCC, including for small or early lesions.
- ctDNA also provides prognostic information, such as tumor behavior, response to treatment, overall survival and recurrence postsurgery.
- Circulating tumor cells, or CTCs, are another source of ctDNA, allowing correlation between genetic and epigenetic alterations and individual cell phenotype.
- However, to date many studies in this field include small numbers of patients and further larger studies are warranted.

Circulating free tumor DNA (ctDNA) is DNA released from necrotic or apoptotic tumor cells into the bloodstream. Absolute levels of ctDNA, as well as genetic mutations and epigenetic changes detected in ctDNA are useful biomarkers of tumor biology, progression and response to therapy in many tumor types and recent evidence suggests they may be useful in hepatocellular carcinoma (HCC). ctDNA detected in blood, therefore, offers a minimally invasive, easily repeated 'liquid biopsy' of cancer, providing real-time dynamic analysis of tumor behavior and treatment response that could revolutionize both clinical and research practice in HCC. In this review, we provide a critical summary of the evidence for the utility of ctDNA as a diagnostic and prognostic biomarker in HCC.

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Future Medicine part of fsg

Liver cancer (hepatocellular carcinoma [HCC]) is the sixth most common malignancy worldwide (749,000 new cases per year) and the third most common cause of cancer-related death (692,000 cases per year) [1–3]. Despite major advances in the diagnosis and therapy of HCC, incidence of HCC is increasing worldwide and mortality remains very high despite the global trend of falling cancer death rates over the last decade [2,4,5]. Importantly, there are currently few validated biomarkers for diagnosis and prognosis in HCC to guide clinical management [3].

Circulating free DNA (cfDNA) released by cancer cells into the bloodstream has been recognized as an exciting potential biomarker in cancer for decades, though only more recently has this technology been investigated in liver cancer. Genetic mutations and epigenetic changes present in tumor cells can be detected in tumor cfDNA in blood, therefore providing a 'liquid biopsy' of cancer. cfDNA detection and analysis offers dynamic, personalized information about tumor biology and behavior in an individual patient [6,7]. When coupled with the ease of obtaining a blood sample in comparison to a tumor tissue biopsy, the potential benefits of cfDNA analysis could revolutionize both clinical management and research in hepatocellular carcinoma.

In this review, we explore the potential role of tumor cfDNA as a diagnostic and prognostic tool in hepatocellular carcinoma.

Circulating cell-free DNA from malignant & benign sources

Circulating free DNA (cfDNA) is DNA released from necrotic or apoptotic cells into the bloodstream. Both nonmalignant and malignant cells release cfDNA; in patients with cancer cfDNA reflects an admixture of wild-type nonmalignant cell cfDNA and cfDNA released from tumor cells, known as ctDNA (or circulating tumor DNA). ctDNA is therefore a subset of total cfDNA within blood. cfDNA is highly fragmented, consisting of short segments (185–200 bp) [8] generated by cellular apoptosis and long fragments generated by necrosis. ctDNA is rapidly cleared from serum and plasma and is therefore a dynamic marker of tumor biology and behavior [9–11]. However, the amount of tumor ctDNA in blood is usually lower than wild-type cfDNA, which limits sensitivity for detecting specific mutations in cancer [12]. cfDNA is likened to a 'liquid biopsy' of cancer because it offers dynamic, personalized information about tumor biology to guide clinical management [13]. cfDNA quantification has potential utility for HCC screening and diagnosis, prognosis and tumor recurrence post definitive therapy [14–16].

While evidence suggests that cfDNA levels are also elevated in inflammation [7,17] and trauma [18], several studies have determined significant differences between cfDNA levels in patients with cancer compared with patients with benign inflammatory diseases [19,20]. The presence of specific mutations in cfDNA can be markers of disease severity and prognosis, or predict responsiveness to chemotherapy agents and other treatment regimens [6,21].

In comparison to tumor tissue biopsy, cfDNA measurement in peripheral blood is minimally invasive and easily repeated and therefore more acceptable to patients for both clinical and research purposes. This lends itself well to monitoring tumor progression and recurrence postsurgery. cfDNA is also easier to process and store than tissue specimens, with less degradation than fixed tissue specimens [14]. This technique therefore has potential uses for oncology management in resource-limited settings, where histopathology resources are scarce or difficult to access. cfDNA may also prove a useful research tool for large-scale cancer genetic epidemiology studies, where biopsy of tumor specimens are not often feasible for reasons of cost, logistics and patient acceptance.

Methodology of cfDNA level quantification

There have been significant advancements made in cfDNA detection and quantification methods in recent years and there are several excellent reviews on technical aspects of cfDNA quantification and mutation detection to which we direct the reader [6,7,22]. Methodology will not be discussed in detail in this review.

To summarize the basic principles of cfDNA quantification methodology, cfDNA is isolated from plasma or serum using either standard DNA isolation procedures or specialized circulating free DNA extraction protocols. cfDNA is then quantified using methods such as UV spectrometry, mass spectrometry, sequencing methods such as polymerase chain reaction (PCR), Sanger sequencing or massive parallel sequencing and chip binding microarrays [7]. Allele-specific PCR methods allow detection of prespecified gene mutations [23], while massive parallel sequencing

techniques coupled with next-generation sequencing allow sequencing of the entire genome [24]. It is possible to detect down to 0.01% of mutation allele fractions using new methods [25].

Epigenetic changes such as DNA methylation modify gene expression, genomic imprinting and chromosome structure and stability [26]. Altered gene methylation at cytosine residues in dinucleotide CpG sites in cancer may lead to chromosomal instability, inactivation of tumor suppressor genes and activation of oncogenes [12]. Importantly, cfDNA methylation has significant prognostic and diagnostic utility in gastrointestinal cancers, including HCC [27–29].

Apart from its extraction from different body fluids (such as plasma, serum and urine), DNA can also be isolated from free circulating tumor cells, or CTCs, in plasma to allow more specific clonal information about tumor cells independent of normal host cells [6,30]. CTCs provide a unique method for studying the effects of genetic and epigenetic changes on individual tumor cell phenotypes, which cannot be explored using cfDNA. While this technique has exciting potential for highly detailed information on tumor cell genotype and behavior, to date large volumes of blood (often in the order of 7.5–10 ml) are required from patients to isolate CTCs in sufficient numbers for quantification and DNA extraction compared with cfDNA, limiting its clinical practicality at the current time.

Diagnosis & screening in HCC

Currently, diagnosis of HCC is based on radiological criteria for lesions that are greater than 1–2 cm in size in patients with liver cirrhosis [3]. This is supplemented by HCC biopsy for lesions smaller than 1–2 cm and lesions occurring in noncirrhotic livers [3,31,32]. Diagnosis of lesions smaller than 2 cm is often difficult, requiring repeat imaging with multiple imaging modalities and invasive biopsy [3]. Furthermore, the strength of recommendations in international guidelines for management of lesions less than 1 cm in size is weaker and based on less conclusive evidence than for larger lesions (3D level evidence, 2B level recommendation) [3].

An additional concern is while 70–90% of HCC cases occur in patients with liver cirrhosis, 10–30% develop in patients with hepatitis B, nonalcoholic fatty liver disease and hepatitis C with advanced fibrosis in the absence of cirrhosis, and this group represents a greater diagnostic challenge [33,34]. There is a real clinical need for improved specificity of radiological diagnosis by the addition of biomarkers to diagnostic algorithms for small tumors less than 2 cm in size and lesions in noncirrhotic livers [35–38]. Improved sensitivity of HCC detection would also facilitate early diagnosis of HCC recurrence postresection and liver transplant.

Currently available blood-based tumor markers, such as α-fetoprotein [39], des-gamma-carboxy prothrombin [40], glypican 3 and α-frucosidase [41,42] show only moderate sensitivity and specificity for HCC diagnosis, particularly for early stage tumors. Alpha-fetoprotein (AFP) particularly performs poorly as a screening marker and has been dropped from international screening guidelines [3]. Therefore, there has been considerable interest in detecting genetic mutations and epigenetic changes in plasma or serum cfDNA as a potential biomarker for HCC diagnosis.

Prognosis in HCC

There is also a significant unmet need for prognostic biomarkers to predict outcomes in HCC [3]. Currently, treatment algorithms are defined by radiological tumor staging. However, there are significant limitations to the sensitivity of radiology for predicting outcomes in HCC, particularly post-transplant and resection [43]. Furthermore, the majority of HCC are not biopsied or resected as it is not required for diagnosis, limiting the utility of existing tissue-based prognostic factors in HCC [3]. The ability to predict tumor behavior at diagnosis, as well as the probability of responsiveness to treatment is an important yet currently unavailable tool for HCC management.

Cell-free DNA levels & integrity as diagnostic & prognostic markers in HCC

There have been several studies evaluating the diagnostic and prognostic utility of cfDNA and ctDNA in HCC management. A summary of available circulating DNA data in HCC is found in Table 1.

Several studies have shown that cfDNA levels are greater in patients with HCC compared with chronic liver disease and healthy controls. Moreover, cfDNA appears to have reasonable accuracy for distinguishing between HCC and chronic liver disease [42,44–48]. cfDNA and more specifically ctDNA quantification have also been evaluated as potential prognostic biomarkers in HCC, including postsurgical resection.

Table 1. Utility of cfDNA and ctDNA for diagnosis and prognosis in hepatocellular carcinoma.

Technique	Diagnosis	Prognosis	Clinical association
cfDNA levels	✓	✓	Higher in HCC compared with chronic liver disease and healthy controls [40–44]; shorter overall survival; distant metastases postresection [42]; tumor differentiation; tumor size [43,61]; 3-year DFS [61]
DNA integrity index	✓	✓	Long fragments more common HCC compared with healthy controls; shorter overall survival; tumor size; TMN stage; vascular and lymphatic invasion; metastases [46]
Relative telomere length	✓		Longer in HCC compared with chronic liver disease and healthy controls [47]
Copy number variations	✓		More common in HCC compared with healthy controls [28]
TP53 249 Ser mutation	✓		More common in HCC compared with chronic liver disease and healthy controls [44,50–52]
Deletion chromosome 8	✓		More common in HCC compared with healthy controls [62]
RASSf1A methylation	✓		More common in HCC compared with chronic liver disease and healthy controls [25,26,54–56]
p15 methylation	✓		More common in HCC compared with chronic liver disease and healthy controls [25,27]
p16 methylation	✓		More common in HCC compared with chronic liver disease and healthy controls [25,27]
APC methylation	✓		More common in HCC compared with healthy controls [27]
FHIT methylation	✓		More common in HCC compared with healthy controls [27]
E cadherin methylation	✓		More common in HCC compared with healthy controls [27]
TGR5 methylation	✓		More common in HCC compared with chronic liver disease and healthy controls [58]
LINE1 methylation	✓	✓	Hypomethylation in HCC compared with liver cirrhosis and healthy controls [59]' shorter overall survival; HBV infection; tumor size; high CLIP score [59]
Microsatellite instability marker D8S258		✓	Shorter overall survival and 3-year DFS; tumor stage; tumor differentiation; vascular invasion [62]
Microsatellite instability marker D8S264		✓	Shorter overall survival and 3-year DFS [62]

cfDNA: Circulating free DNA; ctDNA: Circulating free tumor DNA; DFS: Disease-free survival; HCC: Hepatocellular carcinoma.

Chen *et al.* [42] found significantly elevated levels of serum cfDNA in 22 of 39 HCC patients compared with 2 of 45 healthy controls ($p < 0.05$), however the sensitivity of cfDNA alone for HCC diagnosis was only 56.4%. In a further study of 96 HCV patients with HCC and 100 HCV patients without HCC, Tokuhisa *et al.* [45] reported significantly higher levels of cfDNA in patients with HCC compared with controls ($p < 0.0001$). They also found high serum cfDNA levels post-HCC resection were an independent predictor of shorter overall survival and distant metastases after hepatectomy on multivariate analysis. However, in another cohort of 96 HCV-related HCC and 99 chronic HCV controls not undergoing surgery, the same group found no association between cfDNA levels and tumor size, tumor stage or overall prognosis [48]. In this study, cfDNA levels correlated with inflammatory cytokine gene expression, aspartate aminotransferase and neutrophil levels [48], suggesting cfDNA levels may correlate with inflammation in the primary tumor. The contradictory nature of the reported findings means these data need validation in further studies.

In a study including 52 HCV-related HCC cases, 30 HCV without HCC patients and 16 healthy controls, Iizuka *et al.* [46] reported significantly higher levels of serum cfDNA in HCC patients compared with patients with HCV but without HCC, and healthy controls. They found a cut-off value of 73 ng/ml in serum cfDNA had a sensitivity of 69.2% and specificity of 93.3% for distinguishing HCV-related HCC from HCV liver disease (AUC 0.90, 95% CI 0.83–0.96) [46]. However, in this study and in the Tokuhisa study, no attempt to distinguish ctDNA from cfDNA was made. The same group also reported a significant association between serum ctDNA levels and degree of tumor differentiation and tumor size, but not TMN stage, age, gender or AFP level.

Studies have also reported significant associations between overall cfDNA and/ or ctDNA levels and tumor differentiation and size [46,49]. A further study of 79 HCC patients, 20 patients with liver cirrhosis but no HCC and 20 healthy controls also found ctDNA levels correlated with tumor size and TMN stage [49]. Additionally, in this study there was a negative correlation between ctDNA levels and 3-year disease-free survival and overall survival in HCC [49].

cfDNA levels have also been shown to discriminate HCC in patients with noncirrhotic liver disease. In a large, well-designed study, Fu *et al.* [50] reported relative telomere length in serum cfDNA was significantly higher in 140 Hepatitis B (HBV) related HCC cases without cirrhosis compared with 280 HBV-infected noncirrhotic controls [50], even after adjustment for potential clinical confounders. Additionally, aberrantly sized ctDNA fragments (both abnormally long and short segments) are more common in HCC compared with healthy controls and patients with liver disease [51].

Hypomethylation of noncoding regions and DNA integrity have also been evaluated as diagnostic markers in HCC [50,54]. Hypomethylation of LINE1 repeats in ctDNA was also shown to be an independent predictor of reduced overall survival in HCC in one study [52]. Moreover, LINE1 hypomethylation was also associated with HBV etiology of liver disease, large tumor size and advanced CLIP score [52]. High DNA integrity has also been shown to be an independent marker of shorter overall survival, tumor size, TMN stage, vascular and lymphatic invasion and distant metastases [53].

El-Shazly *et al.* [53] reported that longer cfDNA fragments were more common in HCC cases compared with healthy controls. However, DNA integrity index was more strongly associated with HCC diagnosis than cfDNA concentration in this study [53].

Two markers of microsatellite instability *D8S258* and *D8S264*, in combination with ctDNA concentration, were independent predictors of overall and 3-year disease-free survival in HCC in a study by Ren *et al.* [54]. D8S258 was independently associated with tumor stage, tumor differentiation and vascular invasion [54]. However, this study used allelic imbalance to determine the presence of microsatellite instability, which is not the most accurate method.

Cell-free DNA gene mutations as diagnostic & prognostic markers in HCC

Distinguishing tumor-derived cfDNA (ctDNA) from host-derived cfDNA using genetic mutations and epigenetic alterations provides a more specific biomarker for HCC diagnosis. However, a fundamental problem with gene mutation detection in ctDNA from HCC is that gene mutations are highly varied, with few 'hot spots' of frequent mutation. Tumor suppressor *TP53* 249Ser is one exception, a mutation frequently associated with aflatoxin exposure and HBV infection in HCC. It occurs predominantly in HCC patients from south Asia and sub-saharan Africa, where exposure to aflatoxin through groundnut consumption is common [47], however is very uncommon in Caucasian HCC populations [47,55]. Several high-quality genetic epidemiology studies published by Kirk *et al.* [47] have shown a strong association between detection of *TP53* mutation Ser249 in ctDNA and hepatitis B (HBV)-related HCC in Gambian patients. Furthermore, TP53 Ser249 mutations in ctDNA correlate closely with serum aflatoxin adduct levels [47,56–58]. The largest of these studies detected the mutation in 74 of 186 HCC cases; 15 of 98 patients with HBV-related liver cirrhosis and 12 of 348 HBV infected controls. The odds ratio for HCC diagnosis was 20.3 [47]. Collectively, these studies highlighted the potential utility of ctDNA for diagnosis of HCC in resource-poor settings. However, it must be borne in mind that this *TP53* mutation represents a marker of predisposition to HCC and predates HCC development, therefore it is unlikely to be a highly specific marker for HCC diagnosis and this has been recently shown [49]. Importantly, the authors also described confounding adjacent gene mutations that may affect the accuracy of *TP53* 249Ser mutation assays [47], highlighting an important potential cause of reduced diagnostic sensitivity of ctDNA point mutation analysis for HCC diagnosis.

There have been few studies of ctDNA gene mutations in HCC. One case-control study of Egyptian patients with and without HCC reported low levels of *TP53* detection (4.8%) and an absence of *CTNNB1* mutations in ctDNA of HCC patients of mixed etiology, and these findings were confirmed in primary tumor tissue specimens in a smaller subset of ten HCC patients [44]. Another group evaluated *hTERT* mutations in 66 HCC patients with HCV, 35 with HCV cirrhosis and 42 with HCV chronic

hepatitis [59]. They found though levels of ctDNA were higher in patients with cirrhosis and HCC compared with chronic hepatitis without cirrhosis, discriminative ability of ctDNA between cirrhosis and HCC was poor, with an AUC for HCC diagnosis of only 0.690 [59]. The same group found prognostic utility for *hTERT* mutations in ctDNA, with ctDNA levels noted to be higher in patients with multinodular HCC compared with early stage disease ($p = 0.05$) and higher ctDNA levels were associated with shorter overall survival ($p = 0.03$) [59].

With respect to prognostic utility of gene mutations in ctDNA, one study of 66 HCV-infected HCC patients used *hTERT* mutations to quantify ctDNA and found ctDNA levels were higher in patients with multinodular HCC compared with early stage disease ($p = 0.05$) and higher ctDNA levels were associated with shorter overall survival ($p = 0.03$) [59].

Cell-free DNA methylation as diagnostic markers in HCC

An alternative diagnostic marker in HCC is detection of epigenetic changes in ctDNA, and even genome-wide methylation profiling can be performed on ctDNA [60]. Methylation of the promotor region in *RASSF1A* occurs in up to 70% of HCC patients compared with patients with chronic liver disease and healthy controls [27,28,61–63]. Methylated *RASSF1A,* as well as *p15* and *p16*, *APC*, *FHIT* and *E cadherin* [27,29] are frequently elevated in ctDNA, and importantly these changes may predate HCC diagnosis by up to 9 years [27,28]. In one study by Zhang *et al.* [27] where serum ctDNA was quantified in 50 patients, overall accuracy of *RASSF1A, p15* and *p16* methylation detection in ctDNA for HCC diagnosis was 89% (sensitivity of 84% and specificity of 94%), after adjusting for confounding variables. Others have reported higher levels of Inhibitor of *INK4A* promotor methylation in cfDNA from HCC compared with non-HCC subjects [64].

In a reasonably large study by Han *et al.* [65], hypermethylation of *TGR5* in ctDNA was significantly more common in HCC cases (77/160) compared with chronic hepatitis B infection (12/88) and healthy controls (2/45). When combined with α-fetoprotein, *TGFR5* significantly improved sensitivity for diagnosis of HCC (81.25% for AFP cut-off of 20 ng/ml), however, this was at the expense of reduced specificity (38.64%). Interestingly, there was significantly greater methylation in those over 60 years of age, confirming the importance of adjusting for confounding factors such as age in gene methylation studies [65]. By contrast, hypomethylation of *LINE1* repeats in ctDNA are more common in HCC cases compared with cirrhosis and healthy controls, though diagnostic accuracy has not been assessed [52].

A recent paper by Vaca-Paniagua and colleagues [66] evaluated methylation of *VIM*, *FBLN1*, *LTBP2*, *HINT-2*, *h19* and *IGF-2* in plasma-derived cfDNA and peripheral blood mononuclear cells from eight patients with HCC. They found evidence for *h19*, *IGF2*, *VIM* and *FBLN1* methylation, but not *LTBP2* or *HINT2* methylation in HCC ctDNA [66]. Importantly, *VIM* methylation was higher in cfDNA from HCC patients compared with controls, and was also higher in ctDNA compared with PBMC-derived DNA from HCC patients, suggesting it might be a tumor-specific, potentially useful biomarker for HCC diagnosis. However, the primary aim of this very small study was to evaluate ION TORRENT semi-conductor sequencing for detecting methylation changes in cfDNA, rather than a clinical analysis of gene methylation in HCC, therefore utility for diagnosis, clinical disease stage and prognosis were not evaluated.

Meta-analysis of the diagnostic utility of cfDNA in HCC

Liao *et al.* [67] recently published a systematic review and meta-analysis to evaluate the diagnostic utility of cfDNA quantification and single gene methylation qualitative measurement in HCC patients, also in combination with AFP. They included 22 studies with a total of 1280 patients with HCC which met QUADRAS criteria for the study: seven quantification of cfDNA in HCC, 15 measuring cfDNA single gene methylation in HCC and six measuring cfDNA in combination with AFP levels [67]. Overall, they found reasonable pooled sensitivity and specificity for cfDNA quantification for HCC diagnosis, which was increased when combined with AFP levels (sensitivity 81.8%, specificity 96.0%) [67]. However, considerable heterogeneity among the studies and small study numbers for inclusion mean robustness of these findings is significantly limited. Despite this, these data are supportive of the potential utility of cfDNA as a diagnostic marker for HCC.

Utility of CTCs in HCC

Evaluation of the utility of HCC-related CTCs has only been recently described in the published literature. A summary of the main papers describing the role of CTCs in HCC is outlined in Table 2. EpCAM [68–70], asialoglycoprotein receptor, carbamoyl phosphate synthetase 1 and pan-cytokeratin [71] have all been used as CTC enrichment markers. A preliminary study by Schulze *et al.* [68] including 59 patients with intermediate or advanced stage HCC and 19 controls with cirrhosis or benign liver tumors found EpCAM-positive CTCs were present in 18/59 HCC patients and 1/19 controls. Furthermore, EpCAM-positive CTC levels correlated with overall survival, AFP levels and vascular invasion [68]. However, the number of patients in these subgroups was small. A large, well-designed prospective study by Guo *et al.* [69] evaluated different detection methods for CTCs in HCC. In a cohort of 299 HCC patients, of whom 157 underwent resection, 76 underwent TACE and 66 underwent radiotherapy, they optimized a novel CTC-negative enrichment and PCR-based detection platform against the current commercial CTC isolation platforms, including the CellSearch system [69]. Using their system, they reported good sensitivity, specificity and most importantly a reduced blood volume requirement (5 ml) in comparison to current CTC isolation methods such as CellSearch (7.5–10 ml), with minimal loss in sensitivity [69]. However, the correlation between their platform and CellSearch, though positive, was only modest (9/17 HCC patients with CellSearch compared with 7/17 patients with novel the platform, 76.7% consistency, r = 0.54) and diagnostic equivalence was not statistically assessed [69].

EpCAM expression measurement in CTCs using mRNA isolation showed high specificity for HCC CTCs compared with normal cells [69]. Importantly, in several nested smaller substudies, CTC levels were associated with response to treatment, disease recurrence and time to recurrence [69]. Expression of EpCAM-positive CTCs before surgical resection was associated with higher recurrence rates and shorter time to recurrence, occurring in 49 of 122 patients with HCC. Indeed, EpCAM-positive CTCs were the only independent prognostic factor for disease recurrence [69]. In a smaller nested substudy of 35 patients with HCC, it was shown that recurrence rates postsurgery were higher in those who had an increase in CTCs compared with baseline postsurgery [69]. Recurrence was 50% if patients had a negative CTC baseline and then positive CTC detection postsurgery, or 75% if patients had positive CTCs both at baseline and after surgery [69].

In a further prospective substudy of 100 HCC patients who underwent TACE or radiotherapy, EpCAM-positive CTCs were shown to be more plentiful in patients with worse prognosis post-treatment, independent of BCLC stage [69]. This paper represents a very important and positive preliminary foray into the field of CTC detection in HCC clinical management. However, some of the substudies involved only small numbers of patients and the findings require validation.

Building on this work, a recent paper by Kelley *et al.* [70] described detection of HCC-related CTCs and next-generation sequencing of CTC DNA in 20 HCC patients and 10 chronic liver disease controls. DNA isolated from CTCs was amplified and sequenced using targeted ion semiconductor sequencing and compared with DNA sequenced from primary tumor specimens and peripheral blood mononuclear cells. Using next-generation sequencing, they found that CTCs were detected in 7 of 20 HCC patients, to a level of at least two cells per 7.5 ml, but not in patients with chronic liver disease without HCC [70]. They found detectable CTCs of at least one cell per 7.5 ml were associated with elevated AFP levels and vascular microinvasion [70]. However, a technical

Table 2. Utility of circulating-free tumor cells for diagnosis and prognosis in hepatocellular carcinoma.

Study authors	Study numbers	Clinical association	Ref.
Schulze *et al.*	59 intermediate/advanced HCC 19 cirrhosis or benign liver tumor controls	Diagnosis, overall survival, AFP levels, vascular invasion	[68]
Guo *et al.*	299 HCC	Diagnosis, response to treatment, disease recurrence, time to recurrence including postsurgery, BCLC stage	[69]
Kelley *et al.*	20 HCC 10 chronic liver disease controls	Diagnosis, elevated AFP, vascular microinvasion	[70]
Fang *et al.*	42 HCC	No association with response to TACE	[72]

AFP: Alpha-fetoprotein; HCC: Hepatocellular carcinoma.

issue with this technique was that read depth was limited in CTCs compared with tumor and PBMC origin DNA [70], which may impede the ability to detect significant mutations using CTCs as a source of malignant DNA. In contrast, a negative study of 42 HCC patients was published, where it was found that there was no significant difference in CTC levels in patients who underwent TACE therapy [72].

Collectively, these preliminary studies evaluating the role of CTCs in HCC are promising and further larger studies are needed. However, the large volume of blood currently required by standard technologies is a significant impediment to their clinical use in patients with HCC.

Limitations in the current literature & considerations for future studies

While this review highlights exciting developments in our understanding of the clinical utility of ctDNA in HCC, large well-designed prospective studies including patients with mixed disease etiologies and different tumor stages, with statistical adjustment for important clinical factors related to prognosis in HCC, are urgently required before the place of ctDNA in current clinical algorithms can be determined. Similarly, the utility of CTCs in HCC has also not been adequately established, either for diagnosis or prognosis. Diverse methodologies, difficulty isolating CTCs in early stage malignancy and the large volumes of blood required for sufficient cell isolation using current technologies limit the practicality of CTC use in clinical practice at this stage. Validation studies for these pilot data are imperative before ctDNA and CTCs can be translated to the bedside.

Despite preliminary evidence for the utility of ctDNA for diagnosis of HCC recurrence postsurgery, no studies to date have evaluated the utility of ctDNA quantification in liver transplantation for HCC. Similarly, the relationship between ctDNA levels and response to other treatment modalities, such as transarterial chemoembolization, radiofrequency ablation and systemic chemotherapy such as sorafenib also requires further investigation in larger studies.

There are also several methodological limitations common to many studies in this field. The lack of a standardized approach to isolation, detection and quantification of cfDNA levels or gene mutations and epigenetic changes in ctDNA is of ongoing concern, however increasingly methodological comparative papers are being published and a uniform approach is slowly being adopted. Another methodological issue is that most genetic mutation studies have employed candidate genes of interest based on *a priori* hypotheses, in part due to the cost of genetic sequencing and reduced sample quality. The potential for exploratory genome sequencing studies using next-generation sequencing will hopefully be further evaluated in the future. Not all studies have determined gene mutations and gene methylation patterns in both ctDNA and matched primary tumor specimens, and few have compared ctDNA sequence to germline sequence in nontumor cells within the same subject, which can be useful for identifying novel gene alterations of interest. For diagnosis, genetic variants ideally must only be present in ctDNA, not cfDNA from healthy cells. For prognosis, ctDNA must accurately reflect mutations currently present in the primary tumor.

Another limitation of many published studies in this field is their small sample size, limiting power to determine the effects of clinical confounding variables. For example, studies have shown DNA methylation is independently influenced by age, smoking, alcohol consumption and gender to name a few [73]. cfDNA levels are influenced by levels of inflammation and this needs to be controlled for in studies assessing cfDNA quantification as a marker in malignancy. Germline polymorphisms can also affect epigenetic methylation and tumor phenotype [74]. It is hoped that in the future, statistical analysis guidelines will be developed for studies in ctDNA akin to bioinformatical analysis standards developed for GWAS studies, in order to improve the quality, repeatability and accuracy of reported findings [75].

Conclusion & future perspective

Current evidence demonstrates that ctDNA levels are a promising diagnostic and prognostic biomarker in HCC patients and future large-scale validation studies assessing ctDNA biomarkers in combination with current management strategies are required. ctDNA provides a minimally invasive technique for providing detailed, dynamic information about tumor biology and behavior as well as clinical outcomes. Additionally, ctDNA and CTC detection have the potential to address several

important unmet clinical needs in HCC management, such as improved diagnosis of HCC lesions less than 1–2 cm in size, and predicting likely response to treatment. As costs of these techniques reduce over time, the 'liquid HCC biopsy' has the potential to revolutionize clinical management in HCC.

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