Chapter 7
Liquid Biopsy in Hepatocellular Carcinoma

Irun Bhan, Daniel A. Haber, Raymond T. Chung, and David T. Ting

Introduction

Liquid biopsy, the sampling of cellular material from a solid tumor that has actively or passively entered the bloodstream, is an exciting area of research in cancer diagnostics. Tumor-derived components amenable to liquid biopsy include circulating tumor cells, exosomes, and circulating nucleic acids such as cell-free DNA and noncoding RNA. While the detection of secreted proteins might technically fit the definition of liquid biopsy, the term is usually used to refer to newer techniques focusing on other cellular products or cells themselves. The concept of sampling a tumor through phlebotomy is inherently attractive due to the risks of sampling the primary tumor which, in the case of hepatocellular carcinoma, include tumor seeding along the biopsy tract, hemoperitoneum, pneumothorax, bile peritonitis and sampling error leading to false negative results. The risk of tumor seeding with tissue biopsy of suspected HCC is reported to be 2.7% [1]. In the setting of cirrhosis, major complications of liver biopsy occur in 1.5–2.6% of cases [2, 3]. Liquid biopsy also offers the patient the convenience of a blood draw over procedures with conscious sedation which require hours from preparation to recovery.

I. Bhan (✉)
Division of Gastroenterology, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA

Massachusetts General Hospital Cancer Center, Harvard Medical School, Charlestown, MA, USA
e-mail: ibhan1@mgh.harvard.edu

D. A. Haber · D. T. Ting
Massachusetts General Hospital Cancer Center, Harvard Medical School, Charlestown, MA, USA

R. T. Chung
Division of Gastroenterology, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA

Y. Hoshida (ed.), *Hepatocellular Carcinoma*, Molecular and Translational Medicine, https://doi.org/10.1007/978-3-030-21540-8_7

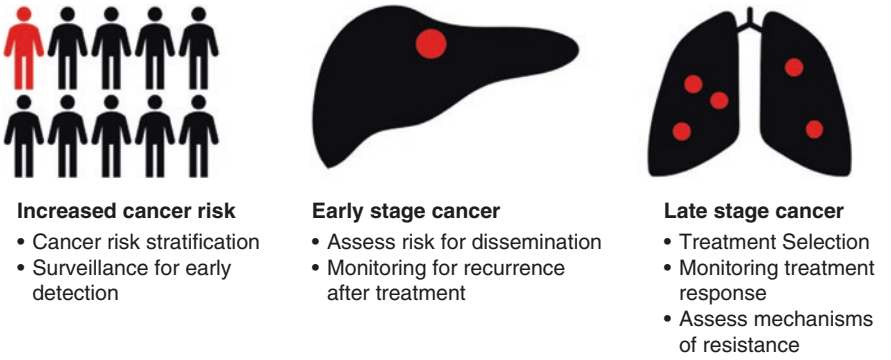

Fig. 7.1 The role of liquid biopsy according to disease stage in HCC. Blood-based biomarkers have the potential to improve each stage of cancer care

For these reasons, the application of liquid biopsy to biomarker development in hepatology and oncology has been an intense and rapidly expanding area of research in recent years.

Liquid biopsy development has focused on key areas of need in oncology that apply to each stage of cancer (Fig. 7.1). In individuals at high risk for developing HCC, such as those with cirrhosis or chronic hepatitis B infection, early detection of HCC through surveillance may facilitate curative treatment and improve long-term outcomes. However, commonly used surveillance tools including serum AFP and liver ultrasonography are suboptimal, suffering from low sensitivity for early lesions. Current areas of research include adapting liquid biopsy platforms to detect HCC lesions at an early, curable stage. For diagnosed early-stage cancer, liquid biopsy could play a role in risk stratification and detection of recurrence after treatment. In the setting of metastatic cancer, liquid biopsy could aid in treatment selection, monitoring of response, and understanding mechanisms of resistance. Each cellular component targeted by liquid biopsy has both strengths and weaknesses in addressing these clinical needs.

Circulating Tumor Cells

Background

Circulating tumor cells (CTCs) are cells shed from primary and metastatic sites of solid tumors into the bloodstream. CTCs were initially identified in 1869 during an autopsy of a woman with metastatic breast cancer [4]. Over 100 years later, the mechanism of entry of these cells into the vasculature is not well-understood but may involve both passive shedding of tumor cells facilitated by abnormal tissue vasculature and active migration of tumor cells as part of epithelial-to-mesenchymal transition. Upon entry into the circulation, many of these cells do not survive, but a

subset may carry additional functional gains required to persist including resistance to anoikis (apoptosis occurring when anchorage-dependent cells detach from the extracellular matrix) and evasion of the immune system [5]. The lifespan of these cells is likely several hours as most patients with localized cancer have no detectable CTCs at 24 hours after curative tumor resection [6]. Some CTCs gain the ability to intravasate into distant organs and coopt local tissues to create a supportive niche. Due to their varied mutational status, circulating tumor cells are heterogenous. The heterogeneity of CTCs is highlighted by comparisons of their morphology, proliferative index by Ki67 staining [6], and transcriptional profiling [7–9]. CTCs can circulate individually or in clusters. In the latter case, multicellular groupings are oligoclonal cells that may perform complementary metastatic functions [10].

Technology

The rarity of CTCs in the bloodstream creates challenges in the isolation of these cells. While a small number of patients may have high blood concentration of CTCs, even patients with metastatic cancer, who tend to have higher CTC concentrations, generally have fewer than 10 CTCs per mL of blood [11]. In a typical blood sample of a cancer patient, there may be one million-fold more white blood cells and one billion-fold more red blood cells than CTCs. CTC isolation technologies must balance achieving a high sensitivity for these rare and heterogenous cells while limiting contamination with white and red blood cells. CTC storage and processing must preserve the integrity of the cells and their informative cargo, including DNA, RNA, and protein. These technologies incorporate one or more of the following approaches to the isolation of CTCs: (1) size-based positive selection of CTCs, (2) positive selection of CTCs based on expected cell-surface marker expression, or (3) depletion of blood cells with collection of untagged CTCs (negative selection).

CTCs, consistent with their epithelial origin, tend to be larger than leukocytes (median diameter 15 μm vs. 10 μm) [12, 13]. Devices that filter CTCs based on size are attractive in their simplicity and ease of use. However, the hemodynamic forces required for filtration can cause cellular stress and damage, reducing cell viability and altering cell phenotype. Furthermore, due to heterogeneity in CTC size and the presence of large hematopoietic cells (such as bone marrow-derived megakaryocytes in patients undergoing chemotherapy), these devices could suffer from both reduced sensitivity and specificity [13–15]. Devices that include both size-based filtration and sorting based on additional physical properties such as deformability are under development and could provide superior results. Other physical properties that have been exploited for CTC isolation include photoacoustic resonance [16, 17], electrical charge [18], and differential density [19].

An alternative to a filtering-predominant approach is to detect the CTCs among the WBCs. One such platform involves plating CTCs and WBCs on an adherent surface, immunofluorescent staining of epithelial or tumor cell surface markers, and high-throughput microscopic scanning to identify CTCs [20]. Such an approach

facilitates enumeration of CTCs unbiased by cell size but may not allow for molecular analyses of the cells.

A popular approach involves the isolation and detection of CTCs using antibodies targeted against epithelial cell surface markers. This is the strategy employed by CellSearch (Menarini Silicon Biosystems), which, at the time of this writing, is the only platform for CTC assessment approved by the US Food and Drug Administration. In this assay, blood samples are fixed and CTCs are extracted in a magnetic field after being tagged with magnetic anti-EpCAM antibodies. Isolated cells are stained for DAPI to identify cell nuclei, additional epithelial markers (cytokeratins 8/18/19) to confirm identity of CTCs, and CD45 to highlight contaminating WBCs. Cells are imaged and candidate CTCs (DAPI-positive, cytokeratin-positive, CD45-negative) are displayed for final review by a human operator. The test is FDA-approved based on clinical studies demonstrating that CTCs identified by CellSearch are an independent predictor of overall and progression-free survival in metastatic breast [21], prostate [22], and colorectal cancer [23]. Limitations include an inability to interrogate cellular cargo (such as DNA or RNA) and reduced detection sensitivity due to cell loss through multiple processing steps and the requirement of EpCAM expression. Importantly, CTCs may lose expression of epithelial cell surface markers through epithelial-to-mesenchymal transition and may have particularly diminished or absent EpCAM expression in the setting of HCC, which is known to have low EpCAM expression [24, 25]. Other technologies use anti-EpCAM antibodies bound to various scaffolds to capture and eventually release CTCs but are liable to the same limitation of epithelial marker expression [26, 27].

Newer technologies rely on negative selection of CTCs through the depletion of white blood cells. The rationale is that WBCs have well-characterized cell surface markers that can be targeted for WBC removal, whereas targetable CTC antigens are incompletely understood. This strategy is employed by the CTC-iChip which is an integrated microfluidic device that removes red blood cells and platelets by size-based sorting and then deflects WBCs tagged with magnetic antibodies. CTCs, which remain untagged, are then collected for downstream bulk or single-cell analyses including enumeration, molecular characterization, and cell culture [15, 28]. Negative selection increases the sensitivity of CTC detection at the risk of contamination with WBCs that escape removal.

Applications in HCC

One of the first published works demonstrating the association of clinical outcomes and CTCs in HCC was by Matsumura et al. in 1999 who isolated peripheral blood nucleated cells by density centrifugation and identified CTCs among these cells by RT-PCR of alpha-fetoprotein mRNA as a marker of HCC origin [29]. The study followed 81 patients with biopsy-confirmed HCC confined to the liver who were undergoing locoregional therapy. The group found 64% of HCC patients tested positive for peripheral blood AFP mRNA which was associated with poorly

differentiated tumors and an increased incidence of extrahepatic metastasis. Patients with negative AFP mRNA after treatment demonstrated improved survival compared to patients with persistently positive levels. The utility of AFP mRNA as a prognostic marker was supported by subsequent studies [30, 31] but was also found to be positive in some patients with benign liver disease or cancers of non-hepatic origin [31, 32]. Other groups have studied various mRNA markers of HCC CTCs including MAGE 1, MAGE 3, GPC-3, CD44, and hTERT with variable success [33].

Studies employing the CellSearch system have generally demonstrated a sensitivity of 20–50% in identifying CTCs in HCC patients. Sun et al. enumerated CTCs using CellSearch in HCC patients undergoing potentially curative resection [34]. Using a threshold of 2 CTCs per 7.5 mL of peripheral blood, the group detected CTCs in 41% of patients preoperatively. CTC concentrations over this threshold were independently associated with postoperative tumor recurrence, even in subgroups thought to be otherwise at low risk for tumor recurrence such as those with Barcelona Clinic Liver Cancer stage 0 or A. The captured CTCs displayed stem cell-like phenotypes with expression of markers of cancer stem cell (CD133 and ABCG2), epithelial-mesenchymal transition, and Wnt pathway activation.

Emerging technologies have focused on depletion of white blood cells rather than positive selection of CTCs [15, 35, 36]. These technologies, which include the CTC-iChip, have the benefit of isolating unperturbed viable CTCs with high-quality RNA [7]. Our group combined the CTC-iChip with droplet digital PCR to create a CTC score based on the expression of liver-specific mRNA transcripts [28]. The CTC score had a sensitivity of 56% at a specificity of 95% for detecting HCC of any stage when using at-risk patients with chronic liver disease as controls. Notably, the CTC score decreased in HCC patients receiving therapy, suggesting a role for the platform in monitoring treatment response. Our follow-up study identified circulating cells of hepatic origin in patients with chronic liver disease (without hepatocellular carcinoma) using immunofluorescence and RNA sequencing after depletion of blood cells by the iChip [37]. These results suggest that liquid biopsy can detect preneoplastic "circulating epithelial cells." We created a machine learning algorithm based on RNA expression data to distinguish circulating epithelial cells of chronic liver disease and those of HCC (including early-stage disease), with an AUC of 0.927. The detection of circulating epithelial cells in preneoplastic disease has been noted in other solid organ disease including intraductal papillary mucinous neoplasm of pancreas [38–40]. The detection and analysis of these cells as they evolve from premalignant to malignant may facilitate using liquid biopsy for the early detection of cancer.

A large multicenter trial examined another negative enrichment strategy, RosetteSep Human CD45 Depletion Cocktail (STEMCELL Technologies), for CTC isolation in combination with RT-PCR of a panel of nine putative cancer stem cell mRNA transcripts for CTC detection in discovery, training, and validation cohorts (total of 1006 patients) [41]. In this study, Guo et al. narrowed the panel to four transcripts (EpCAM, CD90, CD133, CK19), which, when combined in a logistic regression model, demonstrated an AUC of 0.93 (sensitivity 82.1%, specificity 94.2%) in the validation cohort to differentiate HCC from a control

population of healthy blood donors and individuals with chronic hepatitis B, cirrhosis, or benign hepatic lesions. The platform's performance was similar in the detection of early-stage HCC and was superior to that of the standard serum biomarker AFP. Among patients undergoing resection for HCC, postoperative recurrence was associated with persistently positive CTC testing after surgery and higher preoperative CTC concentrations.

Studies to date of liquid biopsy in HCC have demonstrated the feasibility of detection and clinical relevance of CTCs. Research in HCC CTCs has been hampered by the use of multiple isolation and detection technologies, limiting the ability to compare results and draw definitive conclusions from the body of work. A meta-analysis of 20 studies with heterogenous methods calculated a pooled sensitivity of 67% at a specificity of 98% implying the included technologies would be inadequate to use alone for HCC diagnosis [42]. Whether CTCs can be useful as part of a multi-analyte surveillance regimen for the early diagnosis of HCC has not been adequately studied prospectively. On the other hand, enumeration of CTCs could play a role in monitoring response to therapy and evaluation of minimal residual disease given the demonstrated prognostic value of CTC testing. Studies successfully using molecular analysis of HCC CTCs raise hope that further research may lead to a better understanding of the mechanisms of metastasis and could allow for patient selection for emerging treatments such as immunotherapy. As new medical therapies for HCC develop, liquid biopsy could also facilitate personalized treatment through ex vivo culturing and treatment testing of CTCs.

Circulating Tumor DNA

Background

Cell-free DNA (cfDNA) are fragments of nucleic acids shed into the bloodstream from cells undergoing necrosis, apoptosis, or other forms of cell death [43]. ctDNA refers to the subset of cfDNA that is derived from tumors, whether from the primary site, metastatic deposits, or circulating tumor cells. cfDNA was first described in 1948 [44], but the identification of tumor-derived fragments was subsequently noted in the 1970s [45]. cfDNA typically circulates in fragments of approximately 180 bps which corresponds to the unit of chromatin protected by nucleosomes [46]. The plasma of healthy individuals typically carries less than 25 ng cfDNA per mL (the equivalent of several genomes) while in certain physiologic states, such as in the setting of inflammation or cancer, cfDNA concentrations are often several fold higher [45, 47]. Between cancer patients, the portion of cfDNA that is ctDNA is variable (ranging from <0.1% to >10%) which may reflect the underlying rate of cell turnover and cancer stage. However, within individuals, ctDNA fraction may track with tumor burden and response to treatment [48]. Tumor-specific genetic alterations, including point mutations, copy number changes, and gene rearrangements,

can be detected in ctDNA and reflect alterations found in the primary tumor in individual patients [49]. As ctDNA is shed from the entire tumor but tissue biopsies only sample a small portion, liquid biopsy of ctDNA may have an increased sensitivity for informative mutations which may only be present in a subpopulation of tumor cells. Liquid biopsy also allows for longitudinal sampling of an evolving cancer when repeat tissue biopsy carries prohibitive risk. These mutational analyses are helpful for cancers for which actionable mutations have been established. For example, specific ctDNA tests have been FDA-approved to detect BRAF V600E or V600K mutations (in melanoma to determine whether patients are candidates for targeted therapy), KRAS mutations (to identify colorectal cancer patients who may be ineligible for EGFR-targeted therapies), and EGFR mutations (to identify patients with non-small cell lung cancer who may benefit from targeted therapies). No specific tests have been approved to date for hepatocellular carcinoma, reflecting a lack of effective targeted therapies. Commercial tests for the detection of multiple tumor-associated mutations in ctDNA have also been approved. In such broad testing, a potential source of false positive results may be cfDNA derived from clonal expansions of nonmalignant cells that harbor typical tumor driver mutations. In blood cells, such nonmalignant proliferation has been termed "clonal hematopoiesis of indeterminate potential" as their mutations are associated with myeloid malignancy, but they generally do not progress to cancer [50]. A similar phenomenon has been described in solid organs [51, 52]. That cfDNA is a mixture of DNA derived throughout the body is both a strength and a weakness: it allows the sampling of all tumor sites (primary and metastatic as well as subpopulations therein) but makes identification of the tissue origin challenging. The analysis of DNA methylation patterns may allow for better tissue specificity [53].

Technologies

Compared to the isolation of circulating tumor cells, isolation of ctDNA is straightforward. Five to twenty milliliters of peripheral blood is drawn in a collection tube containing anticoagulant and preservatives. Cells are removed by centrifugation leaving plasma from which cfDNA can then be extracted using commercially available kits. cfDNA is generally procured from plasma rather than serum, due to the risk of increased non-tumor cfDNA from cell lysis when serum separator tubes are used. Distinguishing ctDNA from non-tumor cfDNA is a major challenge in liquid biopsy research, analogous to the "needle-in-a-haystack" problem of identifying CTCs among blood cells. Success depends on the sensitivity of the method employed for DNA analysis. While traditional RT-PCR has been used to identify point mutations, newer higher sensitivity methods with the potential for absolute quantification include digital PCR in which samples are diluted to one template per PCR reaction. Modifications of this approach include droplet digital PCR [54, 55] in which PCR reactions occur in separate droplets. Alternatively,

next-generation sequencing has become commonly used in the study of ctDNA for the detection of targeted mutations, whole exome sequencing or whole genome sequencing. Chromosomal rearrangements can also be detected by next-generation sequencing but requires the distinction of tumor-associated structural changes from germline copy number variants which can be facilitated by established bioinformatic filters [56]. While next-generation sequencing of ctDNA is performed clinically with FDA-approved tests, the concordance between assays has been called into question, a potential issue that will require further investigation [57]. An additional concern is that the amount of tumor-derived cfDNA varies as a function of total tumor burden. Most studies to date have focused on patients with advanced metastatic cancer, but in individuals with localized and potentially curable cancers, the ratio of signal to background in ctDNA-based mutation detection is less reliable.

Applications in HCC

Proof of principle for the detection of HCC point mutations in peripheral blood was demonstrated by Szymanska et al. who examined cfDNA for the presence of p53 R249S, a mutation described in patients with aflatoxin-associated HCC [58]. Plasma was collected from a longitudinal cohort monitored for the subsequent onset of HCC. Eight of fourteen patients carried the mutation in the tumor, and 9 carried R249S in cfDNA (as detected by short oligonucleotide mass analysis) with a concordance of 64%. Another study found a 22.2% concordance of HCC hotspot mutations in 27 matched resected tumor tissue specimens and plasma ctDNA samples [59]. The ctDNA samples were analyzed by digital droplet PCR which was noted to have a detection limit of 0.01%. Chan et al. demonstrated that copy number variations in four HCC patients could be detected in cfDNA prior to treatment but not after surgical tumor resection [60].

Cohen et al. developed a multi-analyte blood test for resectable cancer called CancerSEEK that incorporates ctDNA detection [61]. The platform determines a probability of cancer using plasma-based sequencing of 16 cancer-associated genes and the measurement of 8 cancer-associated serum proteins. A machine learning algorithm then combines these data with the measurement of a 31-protein panel to predict the tissue of origin of the cancer. The assay was developed to detect eight different solid cancers, including hepatocellular carcinoma. The initial portion of the test, which indicates the presence of any cancer, demonstrated a sensitivity of 100% for stage I hepatocellular carcinoma at a specificity of 99% when healthy individuals were used as the control population. In their cohort of eight cancer types, when cancer was detected in HCC patients, the machine learning algorithm was able to predict the tissue of origin with an accuracy of 44%. The assay is promising but will need to be evaluated in patients with chronic liver disease at high risk for HCC, the population who would most benefit from surveillance. It is possible the specificity may be reduced in this setting as the positive analytes could reflect the underlying liver disease rather than the HCC itself, a concern with any study that

relies on healthy control populations, rather than at-risk individuals who have cancer-predisposing conditions.

Several studies have examined the diagnostic potential of DNA methylation patterns which have been shown to be highly tissue-specific [62]. Kisiel et al. examined the accuracy of HCC detection using methylation patterns in cfDNA [53]. Candidate markers were identified by reduced representation bisulfite whole methylome sequencing on tissue DNA from HCC and control tissues. The candidate markers were evaluated in cfDNA from patients with HCC and cirrhotic controls. In the final assay, a six-marker panel (HOXA1, EMX1, AK055957, ECE1, PFKP, and CLEC11A) was scored using recursive partitioning decision analysis. In a cross-validated analysis of 95 HCC patients and 51 cirrhotic controls, the assay yielded a sensitivity of 95% at a specificity of 92%. The sensitivity for Barcelona Clinic Staging Criteria stage 0 was 75% and 93% for stage A. The selected genes were identified agnostic of biological significance but were subsequently noted to be involved in carcinogenesis.

Studies examining ctDNA in HCC have not yet explored the full technological power available in the field, which may partly be due to the lack of actionable mutations in HCC. There is, however, strong early data supporting the role of methylated ctDNA in the diagnosis of early HCC. Further validation is required.

Cell-Free Noncoding RNA

Background

Noncoding RNA (ncRNA) molecules are involved with various cellular processes including the regulation of gene expression. They are often categorized by their length as short and long species. The most studied variety of short noncoding is microRNAs (miRNAs) which are typically 21–25 nucleotides in length and regulate protein expression by binding mRNA at the 3'UTR, targeting the mRNA molecule for degradation or blocking translation. miRNAs have been shown to play roles in fundamental cell processes including differentiation, metabolism, and death. Long noncoding RNAs (lncRNAs) are >200 nucleotides in length and can modulate gene transcription through binding of regulatory proteins and complementary RNA or DNA. After cell lysis or from active secretion from cells, ncRNAs access the circulation as free nucleic acids or in membrane-bound extracellular vesicles [63].

Technology

The field of cell-free ncRNA is relatively new with the first description of circulating miRNA as a biomarker for solid tumors published in 2008 [64]. While extracellular RNA is susceptible to degradation by nucleases, miRNA is relatively resistant

to digestion compared with mRNA [65]. Studies examining circulating noncoding RNAs generally use commercially available kits for isolation of total RNA or miRNA from plasma followed by identification of RNA species by various methods including RT-PCR, digital PCR, microarray, NanoString (NanoString Technologies), or next-generation sequencing. While analysis of ctDNA focuses on the presence or absence of cancer-specific alterations, liquid biopsy of ncRNA relies on quantification of cancer-associated transcripts. Thus, ncRNA assessment requires either absolute transcript quantification or appropriate normalization to accurately compare levels between samples.

Applications in HCC

Numerous studies have examined the diagnostic capability of circulating miRNA in HCC. Okajima et al. identified candidate miRNAs with high expression in HCC tissue including miR-224 which was upregulated in HCC and HCC cell lines compared to normal tissues [66]. They found that miR-224 plasma levels detected by RT-PCR correlated with HCC tissue levels, decreased after resection of the primary tumor, correlated with tumor size and tumor recurrence, and was elevated in HCC patients compared to those with chronic liver disease without HCC. A meta-analysis suggested that panels of miRNAs may have better diagnostic characteristics than individual markers with miR-21, miR-199, and miR-122 providing improved specificity when using patients with liver disease as the control population [67]. Several studies have examined the utility of miRNA panels in HCC diagnosis, including a subsequently published work by Moshiri et al. [68]. Using RNA-seq, the group identified potential miRNA markers based on an initial analysis comparing plasma samples from patients with HCC, cirrhosis, and no known liver disease. They validated their findings in three small cohorts of HCC patients, cirrhotic patients, and healthy controls using droplet digital PCR to evaluate marker expression. A panel of three miRNA markers (miR-101-3p, miR-1246, and miR-106b-3p) scored by logistic regression performed with an AUC of 0.99 for distinguishing HCC and cirrhosis. The group demonstrated the feasibility of combining the discovery of miRNA markers of HCC by RNA-seq with subsequent validation using the higher throughput and more economical approach of droplet digital PCR.

The role of circulating lncRNA in liquid biopsy has only been studied in a limited fashion. Based on studies showing overexpression of the lncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) in solid cancer including HCC [69], Konishi et al. investigated its role as a plasma biomarker of HCC [70]. While some HCC patients had elevated plasma MALAT-1 levels, many had levels similar to healthy controls or controls with chronic liver disease, limiting the sensitivity of using MALAT-1 alone as a diagnostic tool. Yan et al. performed quantitative RT-PCR for circulating lncRNAs reported to be associated with HCC

and found that a panel of three lncRNAs (LINC00152, XLOC014172, and RP11-160H22.5) and serum AFP in a logistic regression model was able to distinguish HCC patients from HBV chronic hepatitis patients with an AUC of 0.986 [71]. The ability of the panel to distinguish HCC patients and cirrhotic patients was not examined.

The detection of ncRNA as a liquid biopsy is a nascent field with numerous heterogenous studies suggesting different biomarkers may be useful in HCC diagnosis and prognosis. The relative ease of miRNA isolation, storage, and detection will facilitate a rapid accumulation of new data that will identify the most promising candidates.

Extracellular Vesicles

Background

Extracellular vesicles (EVs) are lipid bilayer-enclosed particles that are released by both normal and diseased cells and carry various cellular molecules. They are categorized as apoptotic bodies, microvesicles (also called microparticles or ectosomes), and exosomes depending on their mechanism of generation. Apoptotic bodies are large cell fragments (usually with a diameter of over 500 nm) formed by blebbing during programmed cell death. Microvesicles are formed directly from blebbing of cell membranes and released into the extracellular space. Their diameter can range from 50 nm to 1000 nm, although in the setting of cancer, some larger microvesicles (termed "large oncosomes") have been observed. Exosomes are formed in a more complex manner from the endolysosomal pathway. First, the membrane of intracellular endosomes bulges inward and forms "intraluminal vesicles." The endosome subsequently fuses with the cell membrane, releasing the intraluminal vesicles as exosomes into the extracellular space. Exosomes are typically smaller than microvesicles, with a diameter of 30–100 nm. Biologically, EVs play a role in cell-to-cell communication, transferring various cellular molecules including proteins and nucleic acids to near or remote cells [72].

EVs have been reported to play an important role in carcinogenesis and metastasis. In a mouse model of pancreatic ductal adenocarcinoma, cancer-derived exosomes travel to the liver and induce changes in the hepatic microenvironment that facilitate liver metastases [73]. The role of EVs in hepatocarcinogenesis is less well studied. Wei et al. found that exosomes shuttle oncogenic miRNA between HCC cells, a process inhibited by the potential tumor suppressor Vps4a [74].

EVs have been detected in various body fluids, where they can be sampled for diagnostic means. The cargo can be interrogated to evaluate the cell of origin and query possible underlying disease states analogous to the analysis of circulating tumor cells.

Technology

As with circulating tumor cells, the technology for isolation of EVs is evolving. The associated techniques have not yet been standardized. Isolation techniques include differential ultracentrifugation, density gradient separation, immunoaffinity purification, and size-exclusion chromatography. EV research has largely used differential centrifugation with or without size-based exclusion by an additional filtration step, although the established protocols are lengthy and not conducive to high-throughput workflows for clinical care. Importantly, the particles isolated as well as the cargo detected can vary by technique [75]. While these factors have complicated the advancement and validation of research in EVs as biomarkers, several factors make them attractive candidates for ongoing study including the availability of newer commercial kits for EV isolation and the relative stability of these particles and their membrane-protected contents.

Applications in HCC

There has been hope that ncRNA isolated from EVs may show improved reproducibility as biomarkers over free circulating ncRNA due to the increased stability of nucleic acids in lipid-bound packaging where they are protected from endogenous nucleases [76]. Sohn et al. investigated the expression of several exosomal miRNAs in patients with HCC, cirrhosis, or chronic hepatitis B [77]. Exosomes were isolated from patient plasma using a kit-based system and the expression of ten miRNAs, selected based on published data on their expression in HCC tissue, was evaluated by RT-PCR. Levels of miR-18a, miR-221, and miR-222 were significantly elevated in HCC patients compared to patients with cirrhosis or chronic hepatitis but individually did not sufficiently discriminate between the patient populations for the purposes of diagnostic testing. Wang et al. examined miR-21 expression, which was previously shown to be elevated in solid tumors including HCC, using an exosome isolation kit and RT-PCR for quantification [78]. They found exosomal and free serum miR-21 expression were significantly elevated in HCC patients compared to those from chronic hepatitis B patients with overall higher expression and better discrimination using exosomal miRNA.

The possibility of using differentially expressed exosomal proteins as biomarkers was studied by Arbelaiz et al., who used mass spectrometry to examine the proteomes of exosomes derived from patients with primary sclerosing cholangitis, cholangiocarcinoma, or hepatocellular carcinoma [79]. Several potential exosomal protein biomarkers were identified including LG3BP which distinguished HCC patients and healthy controls with an AUC of 0.904 and FIBG which distinguished intrahepatic cholangiocarcinoma from HCC with an AUC of 0.894.

Given their mechanistic role in carcinogenesis, their multimolecular contents, and their stability, EVs have an exciting future in liquid biopsy; however, little is known about their role in HCC specifically at this time.

Conclusions

In recent years, there has been rapid development in blood-based analysis of solid tumors. The application of these technologies lags in HCC compared to other cancers, but as liver disease becomes a growing worldwide menace, we will see increasing applications to liver cancer. Advancement in the field will require standardization of isolation and analysis techniques and large-scale prospective studies with appropriate controls. The latter point regarding controls is especially important in the focus on early diagnosis: even screening tests with near-perfect sensitivity and specificity will suffer from poor positive predictive value if deployed in populations with low cancer incidence and prevalence. Thus, evaluating and employing diagnostic tests in high-risk populations (e.g., those with cirrhosis or chronic hepatitis B) is key. However, designing tests that distinguish HCC patients from chronic liver disease patients without HCC will likely be challenging. Many analytes that are elevated in HCC reflect an underlying proinflammatory or diseased state and may be elevated in nonmalignant liver disease as well. The role of liquid biopsy in HCC is currently strongest in cancer detection, monitoring, and prognostication but could evolve as novel therapies emerge that require identification of patient subgroups most likely to benefit. Technological issues that will continue to require attention include improvement in signal to noise due to the low concentration of tumor-derived components in the blood. As the sensitivity improves to detect subtle abnormalities, assessing cancer risk or detecting microscopic cancer and not just the presence of macroscopic cancer may be possible. The detection of invisible cancer will raise new questions in patient management for a disease that lacks effective chemoprevention or systemic therapy and for which curative treatment relies on the identification of radiographically detectable lesions.

Overall, each form of liquid biopsy has strengths and weaknesses in the noninvasive assessment of HCC. It is likely that not one but a combination of tests, perhaps both novel and conventional, will be required to improve detection and monitoring of HCC. The current body of work highlights exciting leads but is limited by heterogenous techniques, the need for further prospective validation, and the risk of false discovery associated with testing numerous markers simultaneously. Further work is still required before any of these technologies are ready for routine clinical application in HCC, but this is worthy of study: blood-based testing of a large and growing at-risk populations with liver disease could have major implications for the management of a deadly cancer.

References

1. Silva MA, Hegab B, Hyde C, Guo B, Buckels JAC, Mirza DF. Needle track seeding following biopsy of liver lesions in the diagnosis of hepatocellular cancer: a systematic review and meta-analysis. Gut. 2008;57(11):1592–6.
2. Maharaj B, Bhoora IG. Complications associated with percutaneous needle biopsy of the liver when one, two or three specimens are taken. Postgrad Med J. 1992;68(806):964–7.
3. Piccinino F, Sagnelli E, Pasquale G, Giusti G. Complications following percutaneous liver biopsy. A multicentre retrospective study on 68,276 biopsies. J Hepatol. 1986;2(2):165–73.
4. Ashworth TR. A case of cancer in which cells similar to those in the tumours were seen in the blood after death. Aust Med J. 1869;14:146.
5. Celià-Terrassa T, Kang Y. Distinctive properties of metastasis-initiating cells. Genes Dev. 2016;30(8):892–908.
6. Stott SL, Lee RJ, Nagrath S, Yu M, Miyamoto DT, Ulkus L, et al. Isolation and characterization of circulating tumor cells from patients with localized and metastatic prostate cancer. Sci Transl Med. 2010;2(25):25ra23–3.
7. Ting DT, Wittner BS, Ligorio M, Vincent Jordan N, Shah AM, Miyamoto DT, et al. Single-cell RNA sequencing identifies extracellular matrix gene expression by pancreatic circulating tumor cells. Cell Rep. 2014;8(6):1905–18.
8. Miyamoto DT, Lee RJ, Stott SL, Ting DT, Wittner BS, Ulman M, et al. Androgen receptor signaling in circulating tumor cells as a marker of hormonally responsive prostate cancer. Cancer Discov. 2012;2(11):995–1003.
9. Heitzer E, Auer M, Gasch C, Pichler M, Ulz P, Hoffmann EM, et al. Complex tumor genomes inferred from single circulating tumor cells by array-CGH and next-generation sequencing. Cancer Res. 2013;73(10):2965–75.
10. Aceto N, Bardia A, Miyamoto DT, Donaldson MC, Wittner BS, Spencer JA, et al. Circulating tumor cell clusters are oligoclonal precursors of breast cancer metastasis. Cell. 2014;158(5):1110–22.
11. Haber DA, Velculescu VE. Blood-based analyses of cancer: circulating tumor cells and circulating tumor DNA. Cancer Discov. 2014;4(6):650–61.
12. Lin HK, Zheng S, Williams AJ, Balic M, Groshen S, Scher HI, et al. Portable filter-based microdevice for detection and characterization of circulating tumor cells. Clin Cancer Res. 2010;16(20):5011–8.
13. Mohamed H, Murray M, Turner JN, Caggana M. Isolation of tumor cells using size and deformation. J Chromatogr A. 2009;1216(47):8289–95.
14. Coumans FAW, van Dalum G, Beck M, Terstappen LWMM. Filter characteristics influencing circulating tumor cell enrichment from whole blood. Secomb TW, editor. PLoS One. 2013;8(4):e61770.
15. Ozkumur E, Shah AM, Ciciliano JC, Emmink BL, Miyamoto DT, Brachtel E, et al. Inertial focusing for tumor antigen-dependent and -independent sorting of rare circulating tumor cells. Sci Transl Med. 2013;5(179):179ra47–7.
16. Weight RM, Viator JA. Detection of circulating tumor cells by photoacoustic flowmetry. Methods Mol Biol. 2014;1102(Chapter 35):655–63.
17. Galanzha EI, Shashkov EV, Kelly T, Kim J-W, Yang L, Zharov VP. In vivo magnetic enrichment and multiplex photoacoustic detection of circulating tumour cells. Nat Nanotechnol. 2009;4(12):855–60.
18. Gascoyne PRC, Noshari J, Anderson TJ, Becker FF. Isolation of rare cells from cell mixtures by dielectrophoresis. Electrophoresis. 2009;30(8):1388–98.
19. Gertler R, Rosenberg R, Fuehrer K, Dahm M, Nekarda H, Siewert JR. Detection of circulating tumor cells in blood using an optimized density gradient centrifugation. Recent Results Cancer Res. 2003;162:149–55.

20. Krivacic RT, Ladanyi A, Curry DN, Hsieh HB, Kuhn P, Bergsrud DE, et al. A rare-cell detector for cancer. Proc Natl Acad Sci U S A. 2004;101(29):10501–4.
21. Hayes DF, Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Miller MC, et al. Circulating tumor cells at each follow-up time point during therapy of metastatic breast cancer patients predict progression-free and overall survival. Clin Cancer Res. 2006;12(14 Pt 1):4218–24.
22. de Bono JS, Scher HI, Montgomery RB, Parker C, Miller MC, Tissing H, et al. Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. Clin Cancer Res. 2008;14(19):6302–9.
23. Cohen SJ, Punt CJA, Iannotti N, Saidman BH, Sabbath KD, Gabrail NY, et al. Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. J Clin Oncol. 2008;26(19):3213–21.
24. de Boer CJ, van Krieken JH, Janssen-van Rhijn CM, Litvinov SV. Expression of Ep-CAM in normal, regenerating, metaplastic, and neoplastic liver. J Pathol. 1999;188(2):201–6.
25. Went PT, Lugli A, Meier S, Bundi M, Mirlacher M, Sauter G, et al. Frequent EpCam protein expression in human carcinomas. Hum Pathol. 2004;35(1):122–8.
26. Talasaz AH, Powell AA, Huber DE, Berbee JG, Roh K-H, Yu W, et al. Isolating highly enriched populations of circulating epithelial cells and other rare cells from blood using a magnetic sweeper device. Proc Natl Acad Sci U S A. 2009;106(10):3970–5.
27. Nagrath S, Sequist LV, Maheswaran S, Bell DW, Irimia D, Ulkus L, et al. Isolation of rare circulating tumour cells in cancer patients by microchip technology. Nature. 2007;450(7173):1235–9.
28. Kalinich M, Bhan I, Kwan TT, Miyamoto DT, Javaid S, LiCausi JA, et al. An RNA-based signature enables high specificity detection of circulating tumor cells in hepatocellular carcinoma. Proc Natl Acad Sci U S A. 2017;114(5):1123–8.
29. Matsumura M, Shiratori Y, Niwa Y, Tanaka T, Ogura K, Okudaira T, et al. Presence of alpha-fetoprotein mRNA in blood correlates with outcome in patients with hepatocellular carcinoma. J Hepatol. 1999;31(2):332–9.
30. Cillo U, Navaglia F, Vitale A, Molari A, Basso D, Bassanello M, et al. Clinical significance of alpha-fetoprotein mRNA in blood of patients with hepatocellular carcinoma. Clin Chim Acta. 2004;347(1–2):129–38.
31. Jeng K-S, Sheen I-S, Tsai Y-C. Does the presence of circulating hepatocellular carcinoma cells indicate a risk of recurrence after resection? Am J Gastroenterol. 2004;99(8):1503–9.
32. Lemoine A, Le Bricon T, Salvucci M, Azoulay D, Pham P, Raccuia J, et al. Prospective evaluation of circulating hepatocytes by alpha-fetoprotein mRNA in humans during liver surgery. Ann Surg. 1997;226(1):43–50.
33. Okajima W, Komatsu S, Ichikawa D, Miyamae M, Ohashi T, Imamura T, et al. Liquid biopsy in patients with hepatocellular carcinoma: circulating tumor cells and cell-free nucleic acids. World J Gastroenterol. 2017;23(31):5650–68.
34. Sun YF, Xu Y, Yang XR, Guo W, Zhang X, Qiu SJ, et al. Circulating stem cell–like epithelial cell adhesion molecule–positive tumor cells indicate poor prognosis of hepatocellular carcinoma after curative resection. Hepatology. 2013;57(4):1458–68.
35. Guo W, Yang XR, Sun YF, Shen M-N, Ma X-L, Wu J, et al. Clinical significance of EpCAM mRNA-positive circulating tumor cells in hepatocellular carcinoma by an optimized negative enrichment and qRT-PCR-based platform. Clin Cancer Res. 2014;20(18):4794–805.
36. Sollier E, Go DE, Che J, Gossett DR, O'Byrne S, Weaver WM, et al. Size-selective collection of circulating tumor cells using Vortex technology. Lab Chip. 2014;14(1):63–77.
37. Bhan I, Mosesso K, Goyal L, Philipp J, Kalinich M, Franses JW, et al. Detection and analysis of circulating epithelial cells in liquid biopsies from patients with liver disease. Gastroenterology. 2018;155:2016–8.
38. Rhim AD, Thege FI, Santana SM, Lannin TB, Saha TN, Tsai S, et al. Detection of circulating pancreas epithelial cells in patients with pancreatic cystic lesions. Gastroenterology. 2014;146(3):647–51.

39. Rhim AD, Mirek ET, Aiello NM, Maitra A, Bailey JM, McAllister F, et al. EMT and dissemination precede pancreatic tumor formation. Cell. 2012;148(1–2):349–61.
40. Franses JW, Basar O, Kadayifci A, Yuksel O, Choz M, Kulkarni AS, et al. Improved detection of circulating epithelial cells in patients with intraductal papillary mucinous neoplasms. Oncologist. 2018;23:121–7.
41. Guo W, Sun YF, Shen M-N, Ma X-L, Wu J, Zhang C-Y, et al. Circulating tumor cells with stem-like phenotypes for diagnosis, prognosis, and therapeutic response evaluation in hepatocellular carcinoma. Clin Cancer Res. 2018;24(9):2203–13.
42. Sun C, Liao W, Deng Z, Li E, Feng Q, Lei J, et al. The diagnostic value of assays for circulating tumor cells in hepatocellular carcinoma: a meta-analysis. Medicine (Baltimore). 2017;96(29):e7513.
43. Stroun M, Lyautey J, Lederrey C, Olson-Sand A, Anker P. About the possible origin and mechanism of circulating DNA apoptosis and active DNA release. Clin Chim Acta. 2001;313(1–2):139–42.
44. Mandel P, Seances CR. Les acides nucleiques du plasma sanguin chez l'homme. Soc Biol Fil. 1948;142:241–3.
45. Leon SA, Shapiro B, Sklaroff DM, Yaros MJ. Free DNA in the serum of cancer patients and the effect of therapy. Cancer Res. 1977;37(3):646–50.
46. Fan HC, Blumenfeld YJ, Chitkara U, Hudgins L, Quake SR. Analysis of the size distributions of fetal and maternal cell-free DNA by paired-end sequencing. Clin Chem. 2010;56(8):1279–86.
47. Perkins G, Yap TA, Pope L, Cassidy AM, Dukes JP, Riisnaes R, et al. Multi-purpose utility of circulating plasma DNA testing in patients with advanced cancers. Perez-Gracia JL, editor. PLoS One. 2012;7(11):e47020.
48. Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, et al. Circulating mutant DNA to assess tumor dynamics. Nat Med. 2008;14(9):985–90.
49. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA, Kinzler KW. Cancer genome landscapes. Science. 2013;339(6127):1546–58.
50. Steensma DP, Bejar R, Jaiswal S, Lindsley RC, Sekeres MA, Hasserjian RP, et al. Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes. Blood. 2015;126(1):9–16.
51. Martincorena I, Roshan A, Gerstung M, Ellis P, Van Loo P, McLaren S, et al. Tumor evolution. High burden and pervasive positive selection of somatic mutations in normal human skin. Science. 2015;348(6237):880–6.
52. Aghili L, Foo J, DeGregori J, De S. Patterns of somatically acquired amplifications and deletions in apparently normal tissues of ovarian cancer patients. Cell Rep. 2014;7(4):1310–9.
53. Kisiel JB, Dukek BA, Kanipakam RVSR, Ghoz HM, Yab TC, Berger CK, et al. Hepatocellular carcinoma detection by plasma methylated DNA: discovery, phase I pilot, and phase II clinical validation. Hepatology. 2019;69:1180–92.
54. Hindson BJ, Ness KD, Masquelier DA, Belgrader P, Heredia NJ, Makarewicz AJ, et al. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. Anal Chem. 2011;83(22):8604–10.
55. Pekin D, Skhiri Y, Baret J-C, Le Corre D, Mazutis L, Salem CB, et al. Quantitative and sensitive detection of rare mutations using droplet-based microfluidics. Lab Chip. 2011;11(13):2156–66.
56. Leary RJ, Sausen M, Kinde I, Papadopoulos N, Carpten JD, Craig D, et al. Detection of chromosomal alterations in the circulation of cancer patients with whole-genome sequencing. Sci Transl Med. 2012;4(162):162ra154–4.
57. Torga G, Pienta KJ. Patient-paired sample congruence between 2 commercial liquid biopsy tests. JAMA Oncol. 2018;4(6):868–70.
58. Szymańska K, Chen J-G, Cui Y, Gong YY, Turner PC, Villar S, et al. TP53 R249S mutations, exposure to aflatoxin, and occurrence of hepatocellular carcinoma in a cohort of chronic hepatitis B virus carriers from Qidong, China. Cancer Epidemiol Biomark Prev. 2009;18(5):1638–43.
59. Huang A, Zhang X, Zhou S-L, Cao Y, Huang X-W, Fan J, et al. Detecting circulating tumor DNA in hepatocellular carcinoma patients using droplet digital PCR is feasible and reflects intratumoral heterogeneity. J Cancer. 2016;7(13):1907–14.

60. Chan KCA, Jiang P, Zheng YWL, Liao GJW, Sun H, Wong J, et al. Cancer genome scanning in plasma: detection of tumor-associated copy number aberrations, single-nucleotide variants, and tumoral heterogeneity by massively parallel sequencing. Clin Chem. 2013;59(1):211–24.
61. Cohen JD, Li L, Wang Y, Thoburn C, Afsari B, Danilova L, et al. Detection and localization of surgically resectable cancers with a multi-analyte blood test. Science. 2018;359(6378):926–30.
62. Aryee MJ, Wu Z, Ladd-Acosta C, Herb B, Feinberg AP, Yegnasubramanian S, et al. Accurate genome-scale percentage DNA methylation estimates from microarray data. Biostatistics. 2011;12(2):197–210.
63. Schwarzenbach H, Hoon DSB, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. Nat Rev Cancer. 2011;11(6):426–37.
64. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A. 2008;105(30):10513–8.
65. Chim SSC, Shing TKF, Hung ECW, Leung T-Y, Lau T-K, Chiu RWK, et al. Detection and characterization of placental microRNAs in maternal plasma. Clin Chem. 2008;54(3):482–90.
66. Okajima W, Komatsu S, Ichikawa D, Miyamae M, Kawaguchi T, Hirajima S, et al. Circulating microRNA profiles in plasma: identification of miR-224 as a novel diagnostic biomarker in hepatocellular carcinoma independent of hepatic function. Oncotarget. 2016;7(33):53820–36.
67. Ding Y, Yan J-L, Fang A-N, Zhou W-F, Huang L. Circulating miRNAs as novel diagnostic biomarkers in hepatocellular carcinoma detection: a meta-analysis based on 24 articles. Oncotarget. 2017;8(39):66402–13.
68. Moshiri F, Salvi A, Gramantieri L, Sangiovanni A, Guerriero P, De Petro G, et al. Circulating miR-106b-3p, miR-101-3p and miR-1246 as diagnostic biomarkers of hepatocellular carcinoma. Oncotarget. 2018;9(20):15350–64.
69. Lin R, Maeda S, Liu C, Karin M, Edgington TS. A large noncoding RNA is a marker for murine hepatocellular carcinomas and a spectrum of human carcinomas. Oncogene. 2007;26(6):851–8.
70. Konishi H, Ichikawa D, Yamamoto Y, Arita T, Shoda K, Hiramoto H, et al. Plasma level of metastasis-associated lung adenocarcinoma transcript 1 is associated with liver damage and predicts development of hepatocellular carcinoma. Cancer Sci. 2016;107(2):149–54.
71. Yuan W, Sun Y, Liu L, Zhou B, Wang S, Gu D. Circulating LncRNAs serve as diagnostic markers for hepatocellular carcinoma. Cell Physiol Biochem. 2017;44(1):125–32.
72. Hirsova P, Ibrahim SH, Verma VK, Morton LA, Shah VH, LaRusso NF, et al. Extracellular vesicles in liver pathobiology: small particles with big impact. Hepatology. 2016;64(6):2219–33.
73. Costa-Silva B, Aiello NM, Ocean AJ, Singh S, Zhang H, Thakur BK, et al. Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. Nat Cell Biol. 2015;17(6):816–26.
74. Wei JX, Lv LH, le Wan Y, Cao Y, Li GL, Lin HM, et al. Vps4A functions as a tumor suppressor by regulating the secretion and uptake of exosomal microRNAs in human hepatoma cells. Hepatology. 2015;61(4):1284–94.
75. Witwer KW, Buzás EI, Bemis LT, Bora A, Lässer C, Lötvall J, et al. Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. J Extracell Vesicles. 2013;2(1):20360.
76. Cheng L, Sharples RA, Scicluna BJ, Hill AF. Exosomes provide a protective and enriched source of miRNA for biomarker profiling compared to intracellular and cell-free blood. J Extracell Vesicles. 2014;3:23743.
77. Sohn W, Kim J, Kang SH, Yang SR, Cho J-Y, Cho HC, et al. Serum exosomal microRNAs as novel biomarkers for hepatocellular carcinoma. Exp Mol Med. Nature Publishing Group. 2015;47(9):e184.
78. Wang H, Hou L, Li A, Duan Y, Gao H, Song X. Expression of serum exosomal microRNA-21 in human hepatocellular carcinoma. Biomed Res Int. 2014;2014:864894.
79. Arbelaiz A, Azkargorta M, Krawczyk M, Santos-Laso A, Lapitz A, Perugorria MJ, et al. Serum extracellular vesicles contain protein biomarkers for primary sclerosing cholangitis and cholangiocarcinoma. Hepatology. 2017;66(4):1125–43.