Review

JOURNAL OF HEPATOLOGY

Liver-derived cell-free nucleic acids in plasma: Biology and applications in liquid biopsies

Peiyong Jiang[1,2,3], K.C. Allen Chan[1,2,3], Y.M. Dennis Lo[1,2,3,*]

Summary

There is much global research interest surrounding the use of cell-free DNA (cfDNA) for liquid biopsies. cfDNA-based non-invasive prenatal testing for foetal chromosomal aneuploidies was the first successful application of cfDNA technology that transformed clinical practice – it has since been rapidly adopted in dozens of countries and is used by millions of pregnant women every year. Prompted by such developments, efforts to use cfDNA in other fields, especially for cancer detection and monitoring have been actively pursued in recent years. Cancer-associated aberrations including single nucleotide mutations, copy number aberrations, aberrations in methylation and alterations in DNA fragmentation patterns have been detected in the cfDNA of patients suffering from a wide variety of cancers. In addition, the analysis of methylation and fragmentomic patterns has enabled the tissue origin of cfDNA to be determined. In this review, different approaches for detecting circulating liver-derived nucleic acids and cancer-associated aberrations, as well as their potential clinical applications for the detection, monitoring and management of hepatocellular carcinoma, will be discussed.

Keywords: Liquid biopsy; Liver diseases; Hepatocellular carcinoma; fragmentomics.

Received 12 November 2018; received in revised form 1 April 2019; accepted 5 April 2019

Introduction

Liquid biopsy is increasingly used for the management of patients with cancer, ranging from the screening of early cancers to guiding treatment decisions in patients with advanced cancers. The potential application of liquid biopsy for the management of liver cancers has been actively explored recently, with the hope of overcoming the limitations of conventional tumour markers which are mainly proteins or glycoproteins. Although the term 'liquid biopsy' is frequently used to refer to the analysis of circulating cell-free DNA (cfDNA) in plasma and other bodily fluids, such as pleural fluid, ascitic fluid and cerebrospinal fluid, it also encompasses the analysis of circulating tumour cells (CTCs) and other nucleic acids (*e.g.* RNA and microRNA) in blood. However, CTCs are present at very low concentrations, typically less than 10 CTCs per milliliter of blood, even in patients with metastatic disease.[1] Given that there are some 10 million nucleated cells per ml of blood, there would be less than 1 CTC in a million nucleated blood cells. This low concentration greatly limits the diagnostic potential of CTC analysis. For example, Yu *et al.* showed that there were a mean of only 1.15 CTCs per 7.5 ml of blood in patients with operable hepatocellular carcinoma (HCC).[2] Patients with 2 or more CTCs per 7.5 ml of blood had inferior disease-free survival and overall survival than those with less than 2 CTCs per 7.5 ml blood.[2] Precise measurement of the number of CTCs at this level is impossible, even when only considering sampling errors. Compared with CTC, a substantially higher fraction of cfDNA is contributed by cancer cells. Using digital PCR analysis, Yung *et al.* showed that the absolute concentration of tumour-derived DNA fragments carrying *EGFR* mutations can be up to 34,000 copies per ml of plasma in a patient with metastatic non-small-cell lung cancer[3]. Cai *et al.* showed that in patients with advanced HCC, DNA fragments carrying cancer-specific mutations can constitute over 50% of the pool of circulating cfDNA.[4] Because of the higher fraction of tumoral constituents present when analysing cfDNA instead of cells in the blood, the analysis of cfDNA is more widely applied for the management of cancer than CTCs.

Recently, a number of approaches have been developed to analyse the tissue origin of cfDNA. In this review, we will focus on the strategies for detecting liver-derived nucleic acids and the different cancer-associated alterations in circulating cell-free nucleic acids, as well as their potential clinical applications in the management of patients with HCC (Fig. 1).

History of cfDNA analysis

The first discovery of the presence of extracellular nucleic acids in human blood can be dated back to 1948.[5] The biological properties and diagnostic applications of cfDNA molecules have been actively pursued in subsequent decades.[6] For example, Leon *et al.* demonstrated that the concentrations of cfDNA from patients with cancer were higher than those from healthy individuals,[7] motivating investigators to study potential diagnostic applications in oncology. However, a convincing demonstration that some of the cfDNA molecules in cancer patients were actually tumour-derived had to await the

[1]*Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong Special Administrative Region*;
[2]*Department of Chemical Pathology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong Special Administrative Region*;
[3]*State Key Laboratory of Translational Oncology, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong Special Administrative Region*

Key point

Human plasma contains a mixture of DNA and RNA molecules released from different tissues, including the placenta of a foetus, the liver and tumorous tissues (such as a hepatocellular carcinoma).

* Corresponding author.
Address: Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong, Shatin, New Territories, Hong Kong Special Administrative Region.
E-mail address: loym@cuhk.edu.hk (Y.M.D. Lo).

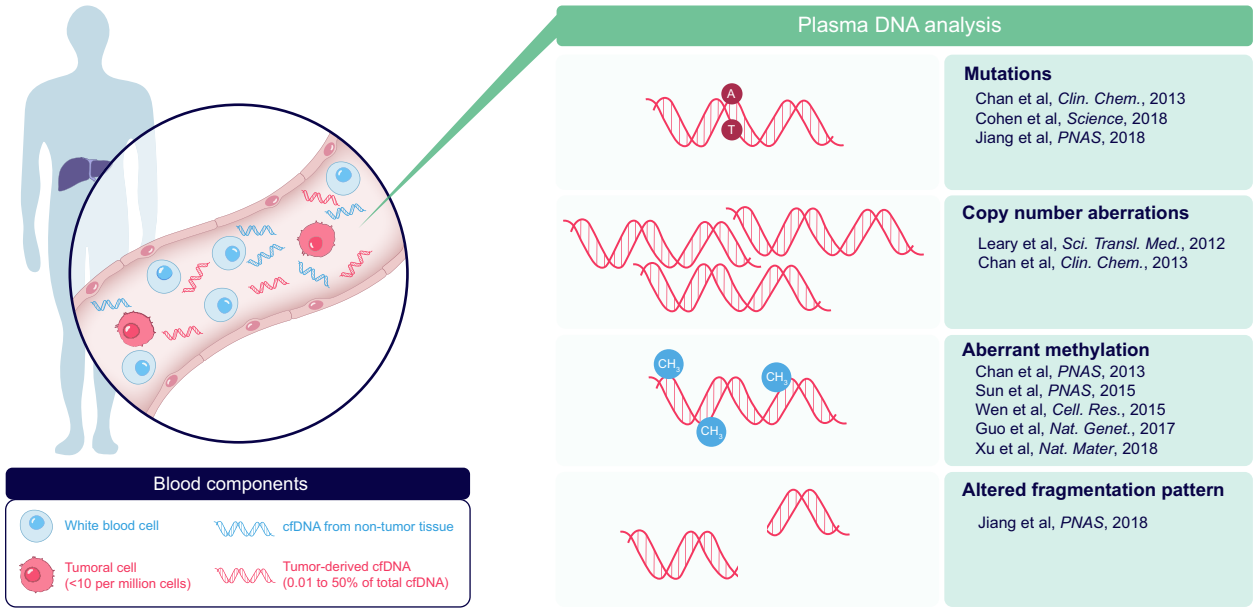

Fig. 1. Overview of liquid biopsy in the management of HCC. Liquid biopsy can refer to the analysis of circulating tumour cells or circulating cfDNA derived from cancer cells. Different cancer-associated changes can be detected through the analysis of circulating DNA including i) mutations, ii) chromosome copy number aberrations, iii) aberrations in DNA methylation and iv) altered DNA fragmentation patterns. cfDNA, cell-free DNA; HCC, hepatocellular carcinoma.

development of technologies that allowed the detection of tumour-associated molecular characteristics. With the development of PCR-based technologies, tumour-associated mutations and microsatellite alterations were detected in cfDNA in patients with cancer.[8–11] In 1997, inspired by the presence of tumour-derived cfDNA in the blood of patients with cancer,[10,11] Lo *et al.* made a parallel discovery that cell-free foetal DNA was present in the plasma and serum of pregnant women.[12] This development has paved the way for the development of cfDNA-based non-invasive prenatal testing (NIPT). Indeed, the multiple similarities between circulating tumour DNA and circulating foetal DNA have inspired many analogous developments in the field. The conceptual understanding of these 2 types of cfDNA molecules and the related analytical strategies could, in general, be used by researchers in both fields and serve as catalysts for advances in both areas. Notably, cfDNA-based NIPT for foetal chromosomal aneuploidies[13,14] was the first major success in using cfDNA technology to transform clinical practice. NIPT is now used by millions of pregnant women in dozens of countries every year.[15,16] The success of NIPT has, in turn, aroused global research interest, with researchers trying to recreate this success using cfDNA in liquid biopsies for cancer. With such efforts, multiple types of cancer-related molecular entities, including single nucleotide variations,[17–20] copy number aberrations[17,21,22], translocations,[23] methylomic changes,[22,24] gene expression alterations,[25,26] as well as viral DNA[27,28] have been detected in cell-free nucleic acids.

Key point

The global success of detection of foetal DNA in the plasma of pregnant women, enabling non-invasive prenatal testing, has inspired researchers worldwide to look for similar success in other fields, especially the liquid biopsies of cancer.

Sample preparation for cfDNA analysis

cfDNA molecules consist of a mixture of DNA shed from different tissues within the body, some of which will be from tumour cells in patients with cancer. One key factor affecting the accuracy of liquid biopsy is the fraction of cfDNA contributed by cancer cells. As the concentration of cfDNA is much lower than the number of blood cells, contamination with DNA from blood cells would significantly dilute the tumour-derived DNA in any plasma sample, affecting the downstream analysis. In this regard, two key strategies have been introduced to minimise the contamination of cfDNA with DNA from blood cells. First, the lysis of blood cells during blood collection and storage should be minimised. Haemolysis during blood collection needs to be avoided and blood samples with significant haemolysis are not suitable for cfDNA analysis. Blood samples should be processed within 6 h of collection to separate plasma from blood cells, as previous studies have shown that delaying processing beyond 6 h leads to the release of a significant amount of DNA from blood cells. In the last few years, blood collection tubes designed for cfDNA analysis have been introduced. These cfDNA blood collection tubes contain additives to stabilise blood cells so that the release of DNA from blood cells is minimal for up to 6 days. Another strategy for minimising blood cell DNA contamination is to ensure thorough removal of blood cells from plasma. To achieve this, a double centrifugation protocol is commonly adopted. A blood sample would be centrifuged at low speed, for example 3,000 g, to allow separation of plasma from blood cells. The harvested plasma would then be centrifuged at high speed, for example 30,000 g, to remove residual blood cells. There are a number of commercially available DNA extraction kits that are specifically designed for the extraction of cfDNA. Compared with kits for extraction of genomic DNA, these kits are capable of retaining short

DNA fragments. Previous studies have shown that most cfDNA molecules are short DNA fragments of below 200 base pairs. Moreover, it was shown that the size distribution for DNA fragments derived from cancer cells was shorter than those from non-malignant tissues. Therefore, retaining short DNA fragments is essential for the detection of signals from cancer cells. The significance of the size distribution of plasma DNA will be discussed in more detail in a subsequent section.

Detection of cancer-associated changes in cfDNA

In the following section, the methods for the detection of different cancer-associated alterations in plasma will be discussed.

Single nucleotide mutations

Significant efforts have been devoted to identifying and characterising somatic genetic mutations in HCC.[29–34] The prevalence of a mutated gene (*i.e.* the percentage of HCC samples carrying such a mutation) involving major signalling pathways associated with HCC was shown to vary greatly, from less than 1%, to over 30%.[35] The majority of those mutations are present in less than 10% of HCC samples.[35] Therefore, the simultaneous analysis of multiple genes or a survey of a relatively large genomic region is required to achieve the desired coverage of mutations present in different patients with HCC. In practice, these goals can be achieved using massively parallel sequencing (also called next-generation sequencing). The genes or regions of interest can be enriched for sequencing through PCR amplification or hybridisation with capture probes and these 2 approaches are termed amplicon-sequencing and capture sequencing, respectively.

As tumour-derived DNA only constitute a fraction, usually a small fraction, of cfDNA, the efficacy of detecting cancer-associated mutations is limited by errors generated during PCR amplification or sequencing when a large number of nucleotides have to be sequenced to look for a small number of DNA fragments carrying the mutations. To overcome this, the technique of unique molecule identifiers (UMI) was introduced.[36] The principle of UMI is illustrated in Fig. 2. In this method, each plasma DNA is tagged with a randomly generated sequence, usually of 6–12 nucleotides, *i.e.* the UMI code for the particular molecule.[36–38] As the sequences of the UMI are randomly generated, the UMI codes for different plasma DNA molecules are different. After PCR amplification, all copied PCR products originating from the same plasma DNA molecule share the same UMI code. After sequencing, all sequenced reads with the same UMI code are analysed together so that a consensus sequence can be generated based on the majority of the sequenced reads.[36] With this arrangement, errors generated at the later cycles of PCR or during sequencing can be eliminated. Using UMI, single nucleotide mutations present at a fraction of as low as 1 in 1 million molecules can be detected.[36–38]

Chromosomal copy number aberrations

Chromosomal amplification and deletions are commonly observed in HCC and other types of cancers. For example, chromosomes 1q and 8q amplification and 1p and 8p deletions are frequently observed in HCC. Cancer-associated copy number aberrations (CNAs) can be accurately detected through cfDNA analysis using a technique modified from the approach widely used for the NIPT for foetal chromosomal aneuploidies.[13,14,17,23] For the detection of foetal chromosomal aneuploidies, for example Down syndrome, plasma DNA from a pregnant woman is sequenced using massively parallel sequencing.[13,14] Then, the sequenced reads are aligned to the reference human genome to determine the chromosomal origin of each DNA fragment. The number of reads mapping to different chromosomes is counted and compared to a reference group of pregnant women known to be carrying normal foetuses. In a pregnant mother carrying a trisomic foetus, the extra copy of the chromosome in the foetus releases an additional dosage of DNA into the maternal plasma so that such a chromosome is over-represented in the plasma DNA. This sequencing-based method has proven to be very accurate for the detection of foetal chromosomal aneuploidies, with both sensitivity and specificity of above 99%.[39–41]

The detection of cancer-associated CNAs is more challenging than the detection of foetal chromosomal aneuploidies. First, chromosomal amplification and deletion in cancers typically only involve part of a chromosome instead of a whole chromosome. Moreover, in contrast to foetal chromosomal aneuploidies which frequently involve chromosomes 13, 18 and 21, cancer-associated CNAs are more variable. In addition to the commonly affected chromosomal arms in HCC, for example, chromosomes 1p, 1q, 8p and 8q, CNAs can occur across the whole genome. Therefore, several important modifications to the method have been made for cancer detection. To detect sub-chromosomal alterations, the genome is divided into smaller regions instead of chromosomes. For example, the genome can be divided into approximately 3,000 regions of 1 Mb in size and the representation of each 1 Mb region in cfDNA is compared with a reference group of healthy individuals. Bioinformatic algorithms are then employed to identify contiguous regions that share the same type of aberration, either over- or under-represented. These modified methods can more efficiently pick up sub-chromosomal CNAs.[42,43]

Aberrations in DNA methylation

In addition to genetic alterations, epigenetic changes also contribute significantly to carcino-

Key point

Circulating nucleic acids molecules can be classified by their origin based on their sequences (*e.g.* containing tumour-associated mutations, alleles differentiating donor and recipient in a liver transplantation context), fragmentation patterns, methylation status and correlation with gene expression status (*e.g.* mRNA and miRNA).

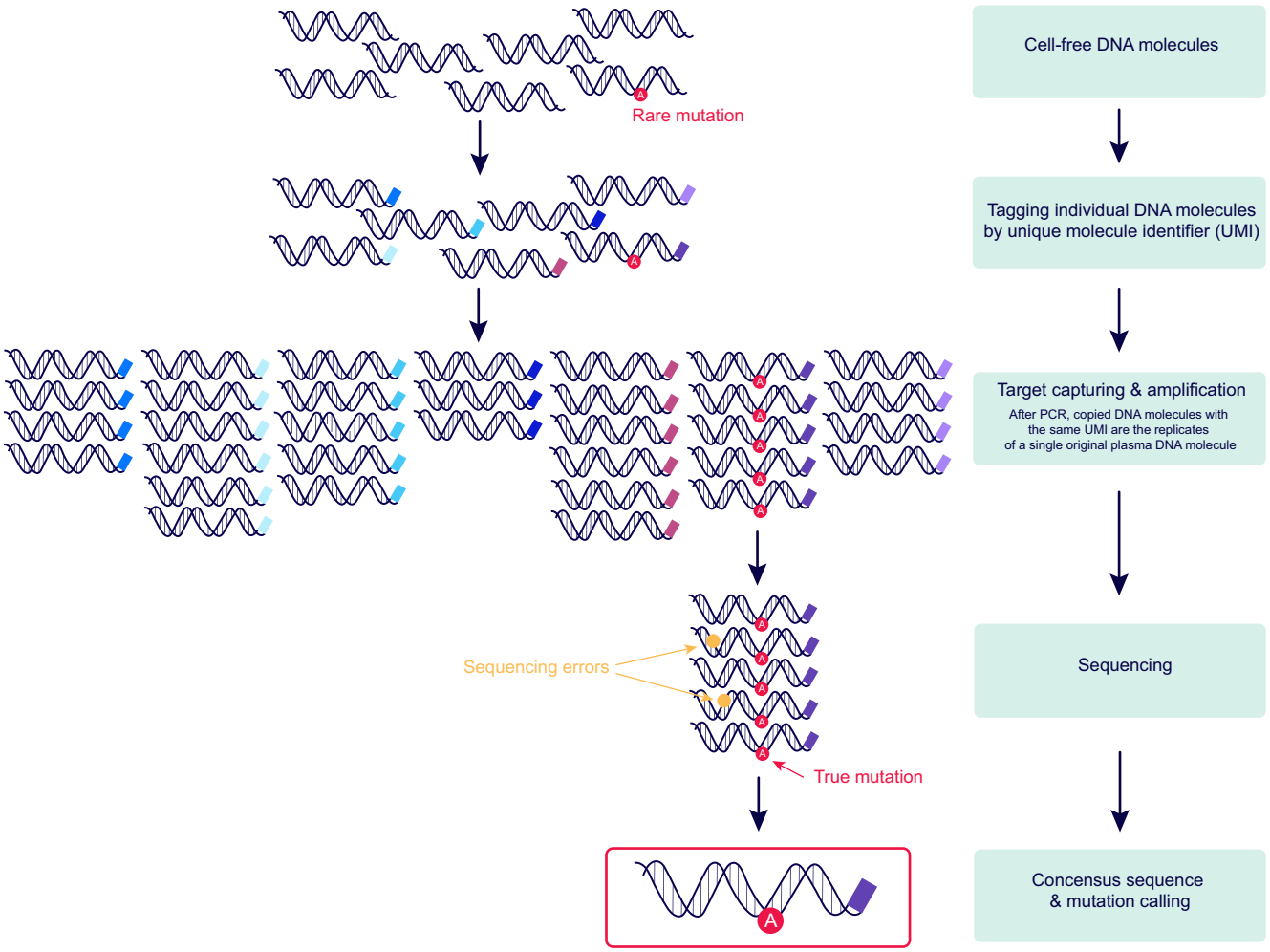

Fig. 2. UMI coding for individual plasma DNA molecules to enhance the accuracy of detecting tumour-associated mutations in cfDNA. The sequencing errors inevitably occurring in different sequencing instruments prevent the mutations with an extremely rare frequency which commonly exist in the blood of a patient with early-stage cancer. In an attempt to revert those sequenced reads carrying sequencing errors occurring in sequencing step back to the original molecules, each original molecule will be tagged with a unique molecular sequence (also referred to as unique identifier, unique molecular identifier, unique molecular barcode) before amplification and sequencing. Then, after amplification and sequencing, the molecules sharing the same molecular barcode will be considered as derived from 1 original DNA molecule, termed a "one molecule family". The true mutation in this family is expected to be shown in the majority of molecules (*e.g.* >95%) belonging to this family while sequencing errors would scatter in a small subset of molecules (*e.g.* <5%).[36] Therefore, the mutation might be identified by constructing the consensus sequence from the molecule family. A consensus sequence refers to a nucleotide sequence shared by the majority of the sequenced reads (*e.g.* >95%). The molecular barcode-based analysis is widely explored for its diagnostic value nowadays. It is worth noting that this method usually needs to guarantee the high-depth coverage to form the molecule family, likely being restricted to target sequencing because the current cost of sequencing is still prohibitive for each identical molecule in the entire genome being sequenced up to, for example 30×. cfDNA, cell-free DNA; UMI, unique molecular identifier.

genesis.[44–46] Aberrant methylation of the promoters of tumour suppressor genes plays a pivotal role in the inactivation of such genes involved in hepatocarcinogenesis.[47,48] Targeted analysis of tumour suppressor gene hypermethylation in cfDNA was first explored using methylation-specific PCR.[49–51] In this method, DNA samples were first treated with bisulfite which converts unmethylated cytosine to uracil while leaving methylated cytosine unchanged. After bisulfite conversion, methylation-specific primers can be used to amplify sequences that have altered and unaltered cytosines to detect unmethylated and methylated DNA, respectively. In addition to the hypermethylation of the promoters of tumour suppressor genes, a cancer genome is usually globally hypomethylated. To detect this methylation alteration across the genome, massively parallel sequencing can be performed on bisulfite converted DNA and the methylation status of the DNA fragments can be deduced based on whether cytosine is converted to uracil. This method is called bisulfite sequencing.[22] Recently, it was shown that the profiling of the methylation status of cfDNA in a genome-wide manner, *i.e.* the methylomic approach, enables the relative contributions of different tissues and organs to the cfDNA pool to be deduced.[52] In this method, the cfDNA methylome is compared to the methylation profiles of different tissues and the contributions of individual tissues to cfDNA can be deduced mathematically (Fig. 3).

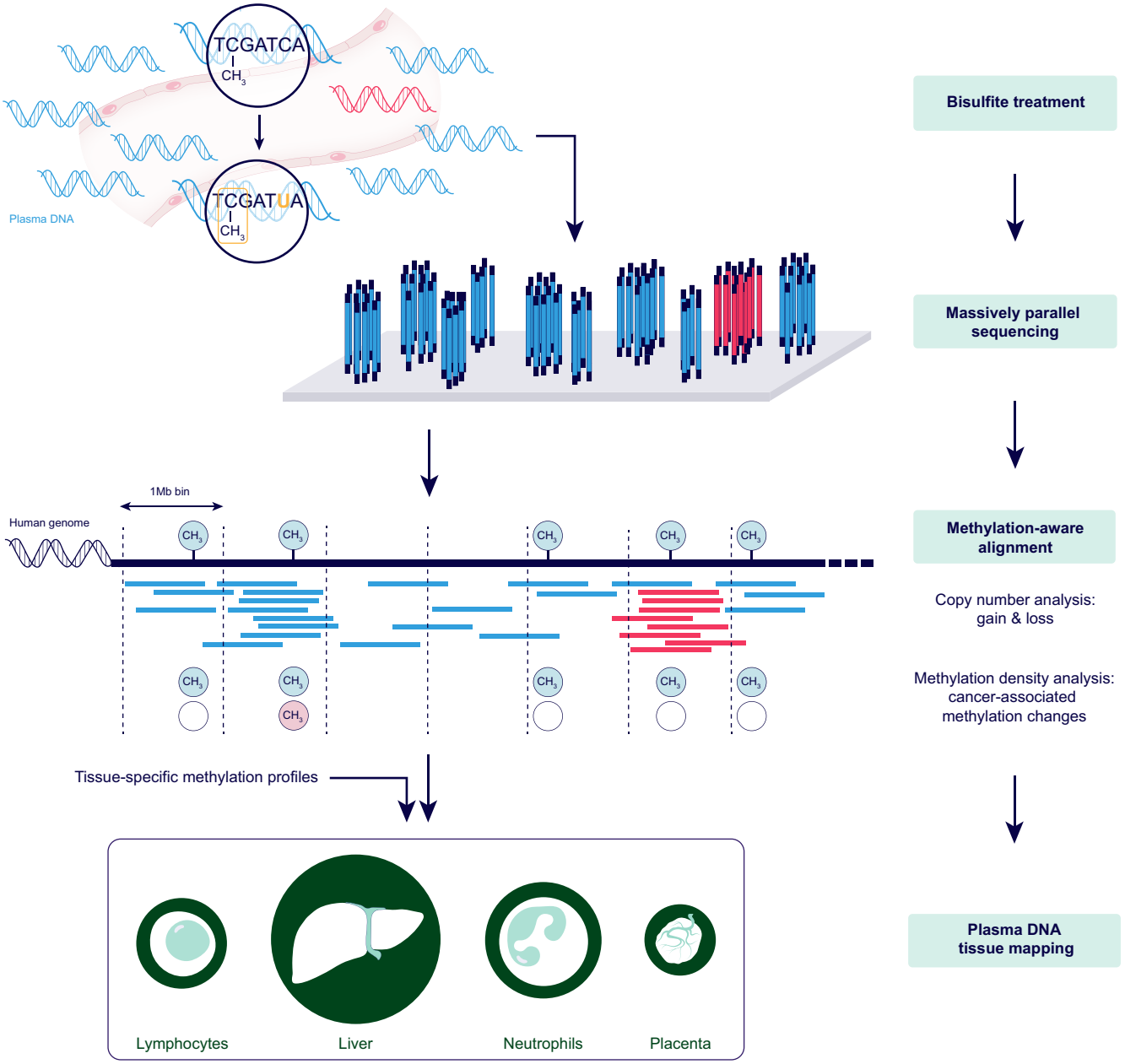

Fig. 3. Methylomic analysis of plasma DNA. Plasma DNA consists of DNA molecules originating from different tissues/organs during cell death and apoptosis. The tissue-specific methylation signatures or cancer-associated methylation changes can be reflected in the plasma DNA of patients with cancer. Massively parallel bisulfite sequencing allows us to differentiate the methylated and unmethylated DNA molecules because the bisulfite treatment would convert unmethylated CG dinucleotides to TG but leave methylated CG unchanged. Therefore, the methylation density (*i.e.* methylation level) of a particular genomic region (*e.g.* a 1 Mb bin) could be expressed as the percentage of methylated fragments (or CG sites) being sequenced. The degree of hypomethylation for a 1 Mb bin could be quantified by a negative z-score metric indicating the number of standard deviations below the mean of methylation levels in a healthy control group. A z-score that is below −3 suggests a genomic window showing hypomethylation. On the other hand, the number of fragments locating in each 1 Mb window could further be used to determine copy number aberrations. By comparing the plasma DNA methylation profile against a series of tissue methylation profiles, it is feasible to deduce the proportional contributions of different tissues to the plasma DNA pool,[52] opening a new avenue for cancer detection, determination of tissue of origin for aberrations seen in plasma DNA and monitoring of organ DNA damage *etc.*

mRNA and non-coding RNA

Parallel to the exploration of cfDNA, much effort has been devoted to interrogating messenger RNA (mRNA) and non-coding RNA species in cell-free nucleic acids.[53,54] About 2 decades ago, researchers used reverse transcription-PCR (RT-PCR) and oligonucleotide hybridisation to reveal the specific presence of Epstein-Barr virus-specific EBER-1 RNA species in the plasma of patients with nasopharyngeal carcinoma, and their absence in healthy controls.[54] These 2 papers reported for the first time that cell-free tumour-derived RNA could be detected in the plasma of patients with cancer and might hold promise for the non-invasive detection of cancer. In this regard, it has been reported that elevated levels

of the mRNAs of alpha-fetoprotein and glypican-3 were observed in the extracellular vesicles of patients with HCC compared to healthy controls.[55] The analysis of circulating mRNA has also been shown to be useful for identifying non-malignant liver conditions. Chan *et al.* observed significant increases in the concentration of liver-derived albumin mRNA in the plasma of individuals with various liver pathologies including chronic hepatitis, cirrhosis and non-alcoholic fatty liver disease.[56] The analysis of plasma albumin RNA is more sensitive than alanine aminotransferase in detecting these liver conditions. Recently, profiling of the plasma RNA transcriptome using massively parallel sequencing was shown to be useful for determining the relative contributions of placental and hematopoietic cells,[25] and even the contribution of different tissues to plasma nucleic acids.[26]

In addition to circulating mRNA species, other forms of tissue-specific RNA species such as plasma microRNA (miRNA) and long non-coding RNA (lncRNA) have attracted much research interest. Chim *et al.* studied the use of a panel of real-time PCR-based miRNA assays in the plasma of pregnant women. They demonstrated the existence of placenta-specific miRNA species in maternal plasma which appeared to have a higher stability than plasma mRNA.[57] Later, Williams *et al.*[58] confirmed these findings using deep sequencing of circulating miRNA in plasma and serum. Pregnant and non-pregnant individuals can be differentiated based on the profile of circulating miRNA in plasma.[58] It is believed that the placenta could be viewed as a model for a solid tumour, inspiring many subsequent publications aimed at detecting various miRNA species in the circulation of patients with cancer.[59,60] Alterations in the levels of miRNAs have also been reported in non-malignant conditions. For example, the elevation of miR-122 has been associated with non-alcoholic fatty liver disease and liver fibrosis.[61–63]

Clinical applications of liquid biopsy in the management of HCC

In the following sections, we discuss the clinical applications of detecting cancer-associated changes in cfDNA in the management of HCC. Table 1 summarises the results of the key studies.

Diagnosis and screening

Key point

The detection of cancer-associated aberrations is useful for the diagnosis, screening, prognostication and monitoring of patients with hepatocellular carcinoma, as well as for guiding treatment decisions.

HCC is the sixth most common cancer worldwide but the second leading cause of cancer-related deaths. One key reason for the poor prognosis of HCC is that most of the patients present with advanced cancers because early HCC is relatively asymptomatic. In this regard, liquid biopsy may be useful for the surveillance and diagnosis of HCC. Liao *et al.* used amplicon-based sequencing to study 22 hotspot mutations spanning 3 genes including *TERT*, *CTNNB1*, and *TP53* in patients with HCC. They showed that 19.5% (8 out of 41 patients with HCC, among them 38 were HBeAg positive) plasma samples from patients with HCC had at least 1 tumour-associated mutation.[64] The hotspots in the *TERT*, *CTNNB1*, and *TP53* genes were found to be present in only 3.9%, 7.8% and 3.9% of patients with HCC,[64] suggesting that expanding the targeted regions to detect mutations could improve the sensitivity. However, it seemed that a total of 6 controls carried at least 1 mutation among these 3 genes when scrutinising all nucleotides covering the amplicons instead of restricting analyses to an *a priori* list of mutations.[64] These results suggest that one would face significant challenges regarding both the sensitivity and specificity of this approach if one tried to determine whether a patient has cancer based solely on it. This is worth noting as most individuals with mutations occurring during clonal haematopoiesis never develop cancers during their lifetime,[65] age-associated clonal haematopoiesis with somatic mutations is an important confounding factor that might adversely affect the specificity of cfDNA mutation analysis.

To improve the overall sensitivity and specificity for early cancer detection, Cohen *et al.* integrated a UMI-based mutation detection system and 8 conventional protein biomarkers such as cancer antigen 125, carcinoembryonic antigen, cancer antigen 19-9, prolactin, hepatocyte growth factor *etc.* into a risk assessment test, called CancerSEEK.[19] They analysed a total of 1,005 patients with 8 common cancer types and 812 healthy controls. A median of 70% of patients with cancer tested positive, with a specificity of 99%. Among these patients, 98% of patients with liver cancer (43 out of 44) were detected. Compared with the other cancer types in this study, including stomach, pancreas, oesophagus, colorectum, lung, and breast, these initial data seem to suggest a better performance of CancerSEEK in detecting liver cancer. It is worth noting that only 5 patients had stage I cancer (11%).[19] It would be important to validate the specificity of this test in chronic hepatitis B carriers and cirrhotic patients because HCC in the majority (70 to 80%) of patients in endemic areas is causally related to hepatitis B infection.

Using genome-wide sequencing, Chan *et al.* showed that concordant copy number variants and mutations could be detected in cfDNA and the corresponding resected tumour tissues.[17] Furthermore, these changes disappear after the surgical resection of the tumour. This observation suggests that these cancer-derived aberrations could be used for the monitoring of the clinical progress of the patient and the detection of residual tumour cells after treatment. In a follow-up study, they showed that 76 (84.4%) of the 90 patients with HCC had at least 1 chromosomal arm-level CNA on chromosomes 1 and 8 in plasma.[43] For hepatitis B carriers with and without liver cirrhosis, the detection rates of these CNAs were 22.2% and 4.5%, respectively. Interestingly,

Table 1. Clinical applications of cancer-associated aberrations in the cfDNA of HCC patients.

Type of aberrations	Methodology	Advantage/limitation	Example
Detection or screening			
Single nucleotide mutations	Amplicon sequencing or capture sequencing of hotspot mutations/ whole exome	Advantage: Mutational profile may be useful for choosing treatment Limitation: A lack of hotspot mutations in HCC; the sensitivity would be largely affected by tumour heterogeneity; the specificity would be adversely affected by clonal haematopoiesis.	19.5% of patients with HCC had at least 1 tumour-associated mutation[64] Using a combination of mutational analysis and conventional protein markers, 43 out of 44 HCC patients were tested positive[19]
CNA	Random sequencing to detect over-/under-representation of chromosomal regions	Advantage: CNA is frequently observed in HCC Limitation: As CNA is also observed in other types of cancers, there is a lack of cancer-type specificity	Of 90 patients with HCC, 76 (84%) had at least 1 chromosomal arm-level CNA on chromosomes 1 or 8[43] Two hepatitis carriers who had CNA detected, but were not known to have HCC at the time of blood collection, were diagnosed with HCC 3 and 4 months later[43]
Aberrant methylation	MSP for detecting tumour suppressor gene hypermethylation	Advantage: Tumour suppressor gene methylation is frequently observed in HCC Limitation: These methylation changes may occur in pre-malignant liver tissues in chronic hepatitis carriers	By targeting 1,000 markers, Xu *et al.* achieved a sensitivity of 83.3% and specificity of 90.5% in a cohort of 383 HCC and 275 normal samples[68]
	Genome-wide bisulfite sequencing to detect global methylation alteration, *e.g.* hypomethylation	Advantage: Global hypomethylation is frequently observed in HCC Limitation: A lack of cancer-type specificity	Combining genome-wide hypomethylation and CNA, led to a sensitivity of 92% and specificity of 88% for differentiating patients with operable HCC from chronic hepatitis B carriers[22]
Plasma DNA tissue mapping	Genome-wide bisulfite sequencing and comparison with tissue-specific methylation profile	Advantage: Can provide tissue specificity Limitation: A lack of large-scale validation study	The contribution of liver and other tissues can be determined using plasma DNA tissue mapping[52,70]
Risk prediction			
Aberrant methylation	MSP for detecting tumour suppressor gene hypermethylation	Advantage: Might be useful for modelling risk prediction Limitation: The modelling power with a small set of tumour suppressor genes might also be affected by tumour heterogeneity.	The presence of TBX methylation in cfDNA was associated with an increased risk for future development of HCC with an odds ratio of 5.35[72]
Guiding treatment			
Driver mutations	Amplicon sequencing or Capture sequencing of hotspot mutations	Advantage: Reflect tissue mutational profile Limitation: A lack of predictive marker for the targeted therapy for HCC	The mutational profile of cfDNA was further shown to reflect the status in the corresponding tumour tissues[77]
Tumour mutational burden	Amplicon sequencing of cancer-related genes	Advantage: May predict response to immunotherapy Limitation: A lack of data on HCC patients	Tumour mutational burden in cfDNA predicts anti-PD-1 immunotherapy in lung cancer patients[79]
Prognostication and monitoring			
Single nucleotide mutations	Amplicon sequencing for hotspot mutations	Limitation: A lack of hotspot mutations in HCC	The detection of p53 mutations in the cfDNA of patients with HCC treated with liver transplantation was associated with tumour recurrence[81]
	Targeted analysis of patient-specific mutation	Advantage: Applicable to all patients Limitation: Costly and time-consuming to individualised mutation detection assays	The level of mutations detected in cfDNA correlated positively with clinical status of patients with HCC[4]
CNA	Random sequencing	Advantage: Applicable for almost all patients Limitation: A lack of large-scale study on the sensitivity for detection low-level residual disease	Disappearance of all CNA after surgical resection of HCC[17]

cfDNA, cell-free DNA; CNAs, copy number aberrations; HCC, hepatocellular carcinoma; MSP, methylation specific PCR.

2 hepatitis B carriers who exhibit CNAs in plasma, but were not known to have HCC at the time of blood collection, were diagnosed with HCC 3 and 4 months afterward. This suggests that CNA analysis may be useful for the surveillance or screening of HCC.

Using methylation-specific PCR, Wong *et al.* demonstrated that aberrantly methylated *CDKN2A* (coding for p16) and *CDKN2B* (coding for p15) were readily detected in the blood of patients with HCC.[48,51] Tumour-associated *RASSF1A* hypermethylation analysis was shown to have a 70–93% sensitivity for detecting patients with HCC.[66,67] The aberrant methylation signal in cfDNA molecules enabled the detection of alpha-fetoprotein-negative HCC.[67] Recently, it was shown that the diagnostic accuracy could be improved by including a larger number of methylation markers. By targeting 1,000 markers, Xu *et al.* achieved a sensitivity of 83.3% and specificity of 90.5% in a cohort of 383 HCC and 275 normal samples.[68] By combining the detection of genome-wide hypomethylation and CNA, Chan *et al.* were able to achieve a sensitivity of 92% and specificity of 88% in differentiating patients with operable HCC from chronic hepatitis carriers.[22]

While most of the above methods can be used to detect various types of cancers instead of HCC alone, it is useful for future investigations to know the probable anatomical location of the tumour when an aberration is detected in the cfDNA. Such anatomical localisation could be performed, for example, using various non-nucleic acid-based biomarkers (*e.g.* protein markers) and various imaging modalities. There is also much recent interest in the exploration of the use of cfDNA for such anatomical localisation, referred to as "plasma DNA tissue mapping". Plasma DNA tissue mapping through genome-wide methylomic analysis can serve as a "whole body molecular imaging" approach for identification of the potential tissue origin of the aberrations detected in cfDNA (Fig. 3).[52,69–71]

Risk prediction for HCC

Genetic and epigenetic aberrations occurring in the early stages of HCC carcinogenesis may be detectable in cfDNA before the actual formation of a cancer. In this regard, Wu *et al.* analysed the methylation levels of 6 genes (*CDKN2A, RASSF1A, STEAP4, TBX2, VIM* and *ZNF154*) in the pre-diagnostic cfDNA of 237 patients with HCC and 257 matched controls.[72] Those studied were part of a community-based cancer screening program involving over 23,000 individuals. They demonstrated that the presence of TBX methylation in cfDNA was associated with an increased risk of developing HCC in the future, with an odds ratio of 5.35. In addition to cfDNA, Huang *et al.* identified a set of miRNA signatures consisting of 16 miRNAs which could predict the risk of HCC development in chronic hepatitis C carriers.[73]

Guiding treatment decisions

One important application of cfDNA analysis is to guide treatment decisions, particularly relating to the use of targeted therapies. For example, the analysis of *EGFR* (coding for epidermal growth factor receptor) mutations using liquid biopsy has been widely applied to guide the use of EGFR tyrosine kinase inhibitors. In this regard, a number of studies have demonstrated that cancer-associated driver mutations can be identified in the cfDNA of patients with HCC using targeted massively parallel sequencing.[74–76] The mutational profile of cfDNA was further shown to reflect the status in the corresponding tumour tissues.[77] Currently, mutational analysis in patients with HCC is not routinely used to guide treatment decisions for 2 reasons. First, there are only 6 approved systemic therapies for HCC, including sorafenib and lenvatinib as first-line treatments for advanced HCC and regorafenib, cabozantinib, nivolumab and pembrolizumab as second-line treatments for patients who were previously treated with sorafenib or lenvatinib. Moreover, the responsiveness to these 2 approved agents has not been linked to particular mutations as in the case of EGFR tyrosine kinase inhibitors in patients with lung cancer.

However, the potential of mutational profiling of cfDNA for HCC management cannot be underestimated. One key reason that most clinical trials on the use of targeted therapies in HCC have failed to demonstrate notable results or meet their primary endpoints is the heterogeneity of patients with HCC in terms of aetiology and molecular pathomechanisms.[78] Liquid biopsy provides a non-invasive approach for the large-scale assessment of mutational profiles in patients with advanced HCC. This could be useful for identifying subgroups of patients who will respond to a therapeutic agent and those who are more likely to develop severe adverse drug reactions.

Recently, tumour mutational burden, which refers to the frequency of mutations in a cancer genome, has been shown to predict clinical response to immunotherapies in patients suffering from a number of cancers.[79] Furthermore, the measurement of tumour mutational burden in cfDNA has been shown to be useful in predicting the response to the anti-PD-L1 immunotherapy atezolizumab in patients with non-small cell lung cancer regardless of PD-L1 biomarker expression as assessed by immunohistochemistry analysis of tissue biopsy.[80] It is worth exploring whether the measurement of tumour mutational burden in cfDNA is useful for predicting the response to immunotherapy in patients with HCC.

Monitoring and prognostication

Cancer-derived CNAs, mutations and methylation changes can be used for the monitoring of disease progression of HCC after treatment. The presence of these cancer-associated changes after treatment with curative-intent is indicative of the presence of residual tumour cells in the body. In a proof-of-principle study involving 4 patients, Cai *et al.* sequenced 574 known cancer-associated genes in resected HCC tumour tissues and identified 204–253 mutations.[4] They showed that more than 98% of these mutations were detectable in the cfDNA of the corresponding patients using capture sequencing and the concentrations of these mutations correlated positively with the clinical status of the patients. Garcia-Fernandez *et al.* also demonstrated that the detection of p53 mutations in the cfDNA of patients with HCC who underwent liver transplantation was associated with tumour recurrence.[81]

In the study by Xu *et al.*, cfDNA methylation based on 10 methylation markers increased progressively from stage I to stage IV HCC, and dropped after tumour resection. A significantly higher score was observed in patients with disease progression compared with those in clinical remission, suggesting that this method can potentially be used for monitoring patient progression and detecting residual disease.[68]

Liver diseases other than HCC

The applications of cfDNA extended beyond the management of cancers to other non-malignant conditions. Schutz *et al.* carried out a prospective study to evaluate whether the measurement of graft-derived DNA levels correlated with rejection and graft damage in patients receiving liver transplantation.[82] They developed a series of digital PCR systems targeting the different alleles at common single nucleotide polymorphisms (SNPs). The concentration of the liver-derived DNA was determined by targeting the SNP alleles that are present in the donor's liver but not present in the transplant recipient. They showed that, in 31 plasma samples taken from 17 patients during biopsy-proven acute rejection episodes, the median percentage of liver-derived DNA was substantially higher (29.6% *vs.* 3.3%) compared with the 282 samples collected from 88 patients during stable periods. In the multivariate logistic regression analysis, they further showed that the prediction value of graft-derived DNA concentration was independent of the conventional liver function tests.[82] Although the analysis of SNP alleles can provide very accurate measurement of DNA released from the grafted liver, a panel of multiple assays would be required to cater for the difference in genetic variations of different donor-recipient pairs. A universal marker independent of genetic polymorphisms would be of practical advantage. By comparing the methylation profiles of the liver and other tissues, Gai *et al.* developed a digital PCR assay targeting liver-specific methylation markers.[83] In addition to the monitoring of graft damage in liver transplant recipients, they further demonstrated that the measurement of liver-derived DNA was useful for indicating the presence of liver metastasis in patients with colorectal cancer.[83] Similarly, Lehmann-Werman *et al.* demonstrated the feasibility of using liver-specific methylation markers to monitor liver damage during liver transplantation.[84] They found that the concentration of circulating DNA of liver origin increased during rejection and could indeed be observed prior to clinically detectable rejection.[84]

Biology of cfDNA

Beyond clinical applications, much research effort has focussed on determining the biological characteristics of cfDNA in patients with cancer, in particular the size distribution. In this regard, Chan *et al.* used a series of PCRs with different amplicon size to show that over 90% of cancer-derived DNA in patients with nasopharyngeal cancer were below 180 base pairs.[85] Subsequent studies using massively parallel sequencing of both ends of plasma DNA molecules (paired-end sequencing) further improved the size measurement of cfDNA to single nucleotide resolution.[43,86] When both ends of a plasma DNA molecule are sequenced, the size of the molecule can be accurately deduced from the genomic coordinates of the nucleotides of the two ends after the sequenced reads are aligned to a reference human genome. In healthy individuals and those with different disease and physiological conditions, including cancer, pregnancy and autoimmune disease, the most consistent observation in the size distribution of cfDNA is the presence of a peak at 166 base pairs.[43,86] The size distribution of DNA derived from cancer cells was shorter compared with DNA derived from non-malignant cells.[43] Intriguingly, a parallel phenomenon can also be observed in the cfDNA of pregnant women, where foetally derived DNA is shorter than maternally derived DNA. These characteristic size patterns were suggested to be related to the nucleosomal packing of DNA and the interaction with histone proteins. The study of such fragmentation patterns can be referred to as "fragmentomics".

Snyder *et al.* reasoned that there was a relationship between nucleosome structure and gene expression.[87] They then built a genome-wide nucleosome profile using frequencies of plasma DNA molecules from different parts of the genome. As gene expression profiles were different from one tissue type to another, Snyder *et al.* were able to use their plasma-derived nucleosome profile to deduce the contribution of different tissues to cfDNA in plasma.[87] They further showed that this approach could be used to provide anatomical information regarding tumours in patients with late-stage cancer. For example, the plasma DNA nucleosome profile from a patient diagnosed with a stage IV HCC showed the highest correlation with the gene expression profile of an HCC cell line (HepG2).[87]

Chan *et al.* further reported that the fragmentation of cfDNA is non-random and there were genomic regions which were over-represented in the ends of cfDNA. They called such entities "cfDNA preferred ends".[88] They showed that the genomic location of the preferred end actually reflected the nucleosome pattern in which a particular cfDNA fragment originated from. For example, the fraction of cfDNA fragments possessing placental DNA preferred ends correlated positively with the foetal DNA fraction in the plasma DNA of pregnant women. Recently, Jiang *et al.* identified a set of preferred end sites which appeared to be preferentially associated with HCC. Through the use of such HCC-associated preferred end sites, the robustness of detecting HCC-associated mutations in plasma DNA could potentially be enhanced.[89] The principle of such a newly emerging technology is illustrated in Fig. 4.

Key point

Proof of concept studies have been performed for the detection of liver-derived circulating nucleic acids in non-malignant liver diseases.

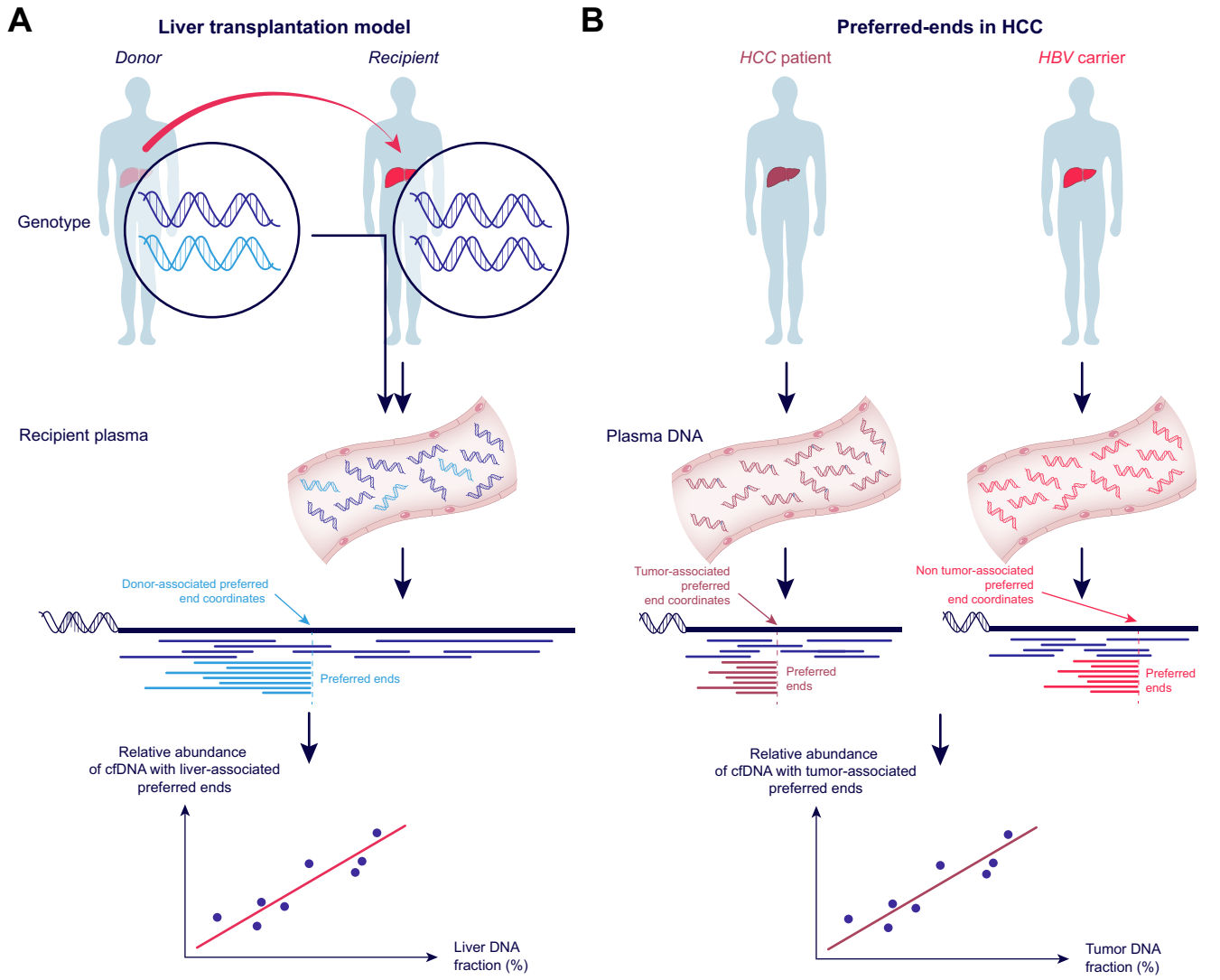

Fig. 4. Schematic illustration of the principle of identifying cell-free DNA end signatures. (A) Through using the liver-specific alleles, the liver DNA molecules in the plasma of a liver transplant recipient can be identified. With ultra-deep sequencing, the genomic locations showing a significant overrepresentation of liver DNA fragment ends (light blue) can be determined by comparing with the Poisson distribution, being termed donor-associated preferred end coordinates. In a similar way, the recipient-associated preferred end coordinates can be defined. The relative abundance of cfDNA with liver-associated ends correlated with liver DNA fraction. (B) A genome-wide scanning strategy can be used to identify genomic locations showing a significant increase of plasma DNA ends compared with that expected for a Poisson distribution in plasma of patients with HCC and chronic hepatitis B virus carriers, respectively, being termed tumour-associated preferred end coordinates and non-tumour-associated preferred end coordinates. The relative abundance of cfDNA with tumour-associated preferred ends correlates with tumour DNA fraction. cfDNA, cell-free DNA; HCC, hepatocellular carcinoma.

Conclusion

Cancer-associated aberrations, including single nucleotide mutations, CNAs and aberrant methylation are increasingly reported in the cfDNA of patients with HCC. Initial exploratory studies have reported that a reasonable sensitivity and specificity for the detection of HCC can be achieved using multiple markers or a combination of genetic and epigenetic approaches.[4,17,19,68] As most of the existing reports are retrospective case-control studies, an important consideration would be the choice of controls. In areas with a high incidence of HCC, for example Southeast Asia, the at-risk group would be those with chronic hepatitis B/C infection and/or cirrhosis. Therefore, the specificity of these tests in patients with chronic hepatitis and/or cirrhosis would be most relevant. Furthermore, whether the application of these tests can lead to improved clinical outcomes needs to be further evaluated. Recently, a large-scale prospective trial on nasopharyngeal cancer screening has demonstrated that cfDNA analysis could be used to identify early asymptomatic cancer, resulting in improved survival.[27] This kind of study would be useful to define the role of liquid biopsy in the surveillance of HCC. Additionally, as HCC is a relatively heterogeneous disease with a wide variety of aetiologies and carcinogenic driver events, the profiling of mutations using cfDNA analysis could also be useful for better characterisation of patients. This is important for optimising the treatment of patients and for evaluating the efficacy of different therapeutic options in drug trials.

Financial support

This work was supported by the Research Grants Council of the Hong Kong SAR Government under the Theme-based research scheme T12-401/16-W and T12-403/15-N, as well as the Collaborative Research Agreement from Grail.

Conflict of interest

P.J., K.C.A.C. and Y.M.D.L. hold equities in Grail. P.J., K.C.A.C., and Y.M.D.L. are consultants to Grail. K.C.A.C., and Y.M.D.L. receive research funding from Grail. Y.M.D.L. is a scientific cofounder of and serves on the scientific advisory board of Grail. K.C.A.C. and Y.M.D.L. are co-founders and shareholders of Take2 Health and serve on the Board. Y.M.D.L. is a consultant to Decheng Capital. P.J., K.C.A.C. and Y.M.D.L. have filed patent applications regarding cell-free nucleic acids analysis. Patent royalties are received from Grail, Illumina, Sequenom, DRA, Xcelom and Take2 Health. Please refer to the accompanying ICMJE disclosure forms for further details.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2019.04.003.

References

Author names in bold designate shared co-first authorship

[1] Corcoran RB, Chabner BA. Application of cell-free DNA analysis to cancer treatment. N Engl J Med 2018;379:1754–1765. https://doi.org/10.1056/NEJMra1706174.

[2] Yu J, Xiao W, Dong S, Liang H, Zhang Z, Zhang B, et al. Effect of surgical liver resection on circulating tumor cells in patients with hepatocellular carcinoma. BMC Cancer 2018;18:835. https://doi.org/10.1186/s12885-018-4744-4.

[3] **Yung TKF**, **Chan KCA**, Mok TSK, Tong J, To KF, Lo YMD. Single-molecule detection of epidermal growth factor receptor mutations in plasma by microfluidics digital PCR in non-small cell lung cancer patients. Clin Cancer Res 2009;15:2076–2084. https://doi.org/10.1158/1078-0432.CCR-08-2622.

[4] Cai ZX, Chen G, Zeng YY, Dong XQ, Lin MJ, Huang XH, et al. Circulating tumor DNA profiling reveals clonal evolution and real-time disease progression in advanced hepatocellular carcinoma. Int J Cancer 2017. https://doi.org/10.1002/ijc.30798.

[5] Mandel P, Metais P. Les acides nucléiques du plasma sanguin chez l'homme. C R Seances Soc Biol Fil 1948;142:241–243.

[6] Thierry AR, El Messaoudi S, Gahan PB, Anker P, Stroun M. Origins, structures, and functions of circulating DNA in oncology. Cancer Metastasis Rev 2016;35:347–376. https://doi.org/10.1007/s10555-016-9629-x.

[7] Leon SA, Shapiro B, Sklaroff DM, Yaros MJ. Free DNA in the serum of cancer patients and the effect of therapy. Cancer Res 1977;37:646–650.

[8] Vasioukhin V, Anker P, Maurice P, Lyautey J, Lederrey C, Stroun M. Point mutations of the N-ras gene in the blood plasma DNA of patients with myelodysplastic syndrome or acute myelogenous leukaemia. Br J Haematol 1994;86:774–779. https://doi.org/10.1111/j.1365-2141.1994.tb04828.x.

[9] Sorenson GD, Pribish DM, Valone FH, Memoli VA, Bzik DJ, Yao SL. Soluble normal and mutated DNA sequences from single-copy genes in human blood. Cancer Epidemiol Biomarkers Prev 1994;3:67–71. https://doi.org/10.1016/j.scriptamat.2005.11.022.

[10] Chen XQ, Stroun M, Magnenat JL, Nicod LP, Kurt AM, Lyautey J, et al. Microsatellite alterations in plasma DNA of small cell lung cancer patients. Nat Med 1996;2:1033–1035. https://doi.org/10.1038/nm0996-1033.

[11] Nawroz H, Koch W, Anker P, Stroun M, Sidransky D. Microsatellite alterations in serum DNA of head and neck cancer patients. Nat Med 1996;2:1035–1037. https://doi.org/10.1038/nm0996-1035.

[12] Lo YMD, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, et al. Presence of fetal DNA in maternal plasma and serum. Lancet 1997;350:485–487. https://doi.org/10.1016/S0140-6736(97)02174-0.

[13] Chiu RWK, Chan KCA, Gao Y, Lau VYM, Zheng W, Leung TY, et al. Noninvasive prenatal diagnosis of fetal chromosomal aneuploidy by massively parallel genomic sequencing of DNA in maternal plasma. Proc Natl Acad Sci U S A 2008;105:20458–20463. https://doi.org/10.1073/pnas.0810641105.

[14] Fan HC, Blumenfeld YJ, Chitkara U, Hudgins L, Quake SR. Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood. Proc Natl Acad Sci 2008;105:16266–16271. https://doi.org/10.1073/pnas.0808319105.

[15] Allyse M, Minear MA, Berson E, Sridhar S, Rote M, Hung A, et al. Non-invasive prenatal testing: a review of international implementation and challenges. Int J Womens Health 2015;7:113–126. https://doi.org/10.2147/IJWH.S67124.

[16] Chandrasekharan S, Minear MA, Hung A, Allyse M. Noninvasive prenatal testing goes global. Sci Transl Med 2014;6:231fs15. https://doi.org/10.1126/scitranslmed.3008704.

[17] Chan KCA, Jiang P, Zheng YWL, Liao GJWW, 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;59:211–224. https://doi.org/10.1373/clinchem.2012.196014.

[18] Dawson S-JJ, Tsui DWY, Murtaza M, Biggs H, Rueda OM, Chin S-FF, et al. Analysis of circulating tumor DNA to monitor metastatic breast cancer. N Engl J Med 2013;368:1199–1209. https://doi.org/10.1056/NEJMoa1213261.

[19] 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;359:926–930. https://doi.org/10.1126/science.aar3247.

[20] Murtaza M, Dawson SJ, Tsui DWY, Gale D, Forshew T, Piskorz AM, et al. Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. Nature 2013;497:108–112. https://doi.org/10.1038/nature12065.

[21] Heitzer E, Ulz P, Geigl JB, Speicher MR. Non-invasive detection of genome-wide somatic copy number alterations by liquid biopsies. Mol Oncol 2016;10:494–502. https://doi.org/10.1016/j.molonc.2015.12.004.

[22] **Chan KCA**, **Jiang P**, **Chan CWM**, Sun K, Wong J, Hui EP, et al. Noninvasive detection of cancer-associated genome-wide hypomethylation and copy number aberrations by plasma DNA bisulfite sequencing. Proc Natl Acad Sci U S A 2013;110:18761–18768. https://doi.org/10.1073/pnas.1313995110.

[23] 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;4:162ra154. https://doi.org/10.1126/scitranslmed.3004742.

[24] Wen L, Li J, Guo H, Liu X, Zheng S, Zhang D, et al. Genome-scale detection of hypermethylated CpG islands in circulating cell-free DNA of hepatocellular carcinoma patients. Cell Res 2015;25:1250–1264. https://doi.org/10.1038/cr.2015.126.

[25] Tsui NBY, Jiang P, Wong YF, Leung TY, Chan KCA, Chiu RWK, et al. Maternal plasma RNA sequencing for genome-wide transcriptomic profiling and identification of pregnancy-associated transcripts. Clin Chem 2014;60:954–962. https://doi.org/10.1373/clinchem.2014.221648.

[26] Koh W, Pan W, Gawad C, Fan HC, Kerchner GA, Wyss-Coray T, et al. Noninvasive in vivo monitoring of tissue-specific global gene expression in humans. Proc Natl Acad Sci 2014;111:7361–7366. https://doi.org/10.1073/pnas.1405528111.

[27] Chan KCA, Woo JKS, King A, Zee BCY, Lam WKJ, Chan SL, et al. Analysis of plasma Epstein-Barr virus DNA to screen for nasopharyngeal cancer. N Engl J Med 2017;377:513–522. https://doi.org/10.1056/NEJMoa1701717.

[28] Lam WKJ, Jiang P, Chan KCA, Cheng SH, Zhang H, Peng W, et al. Sequencing-based counting and size profiling of plasma Epstein-Barr virus DNA enhance population screening of nasopharyngeal carcinoma.

Proc Natl Acad Sci 2018;115:E5115–E5124. https://doi.org/10.1073/pnas.1804184115.
[29] Guichard C, Amaddeo G, Imbeaud S, Ladeiro Y, Pelletier L, Maad I Ben, et al. Integrated analysis of somatic mutations and focal copy-number changes identifies key genes and pathways in hepatocellular carcinoma. Nat Genet 2012;44:694–698. https://doi.org/10.1038/ng.2256.
[30] Kan Z, Zheng H, Liu X, Li S, Barber TD, Gong Z, et al. Whole-genome sequencing identifies recurrent mutations in hepatocellular carcinoma. Genome Res 2013;23:1422–1433. https://doi.org/10.1101/gr.154492.113.
[31] Ahn SM, Jang SJ, Shim JH, Kim D, Hong SM, Sung CO, et al. Genomic portrait of resectable hepatocellular carcinomas: Implications of RB1 and FGF19 aberrations for patient stratification. Hepatology 2014;60:1972–1982. https://doi.org/10.1002/hep.27198.
[32] Schulze K, Imbeaud S, Letouzé E, Alexandrov LB, Calderaro J, Rebouissou S, et al. Exome sequencing of hepatocellular carcinomas identifies new mutational signatures and potential therapeutic targets. Nat Genet 2015;47:505–511. https://doi.org/10.1038/ng.3252.
[33] Fujimoto A, Furuta M, Totoki Y, Tsunoda T, Kato M, Shiraishi Y, et al. Whole-genome mutational landscape and characterization of noncoding and structural mutations in liver cancer. Nat Genet 2016;48:500–509. https://doi.org/10.1038/ng.3547.
[34] Ally A, Balasundaram M, Carlsen R, Chuah E, Clarke A, Dhalla N, et al. Comprehensive and Integrative Genomic Characterization of Hepatocellular Carcinoma 1327–1341.e23. Cell 2017;169. https://doi.org/10.1016/j.cell.2017.05.046.
[35] Totoki Y, Tatsuno K, Covington KR, Ueda H, Creighton CJ, Kato M, et al. Trans-ancestry mutational landscape of hepatocellular carcinoma genomes. Nat Genet 2014;46:1267–1273. https://doi.org/10.1038/ng.3126.
[36] Kinde I, Wu J, Papadopoulos N, Kinzler KW, Vogelstein B. Detection and quantification of rare mutations with massively parallel sequencing. Proc Natl Acad Sci U S A 2011;108:9530–9535. https://doi.org/10.1073/pnas.1105422108.
[37] Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies 224ra24–224ra24. Sci Transl Med 2014;6. https://doi.org/10.1126/scitranslmed.3007094.
[38] Schmitt MW, Kennedy SR, Salk JJ, Fox EJ, Hiatt JB, Loeb LA. Detection of ultra-rare mutations by next-generation sequencing. Proc Natl Acad Sci U S A 2012;109:14508–14513. https://doi.org/10.1073/pnas.1208715109.
[39] Chiu RWK, Akolekar R, Zheng YWL, Leung TY, Sun H, Chan KCA, et al. Non-invasive prenatal assessment of trisomy 21 by multiplexed maternal plasma DNA sequencing: Large scale validity study. BMJ 2011;342:217. https://doi.org/10.1136/bmj.c7401.
[40] Palomaki GE, Kloza EM, Lambert-Messerlian GM, Haddow JE, Neveux LM, Ehrich M, et al. DNA sequencing of maternal plasma to detect Down syndrome: an international clinical validation study. Genet Med 2011;13:913–920. https://doi.org/10.1097/GIM.0b013e3182368a0e.
[41] Palomaki GE, Kloza EM. Prenatal cell-free DNA screening test failures: a systematic review of failure rates, risks of Down syndrome, and impact of repeat testing. Genet Med 2018;20:1312–1323. https://doi.org/10.1038/gim.2018.22.
[42] Heitzer E, Ulz P, Belic J, Gutschi S, Quehenberger F, Fischereder K, et al. Tumor-associated copy number changes in the circulation of patients with prostate cancer identified through whole-genome sequencing. Genome Med 2013;5:30. https://doi.org/10.1186/gm434.
[43] **Jiang P**, **Chan CWM**, **Chan KCA**, Cheng SH, Wong J, Wong VW-S, et al. Lengthening and shortening of plasma DNA in hepatocellular carcinoma patients. Proc Natl Acad Sci 2015;112:E1317–E1325. https://doi.org/10.1073/pnas.1500076112.
[44] Allis CD, Jenuwein T. The molecular hallmarks of epigenetic control. Nat Rev Genet 2016;17:487–500. https://doi.org/10.1038/nrg.2016.59.
[45] Nebbioso A, Tambaro FP, Dell'Aversana C, Altucci L. Cancer epigenetics: moving forward. PLoS Genet 2018;14. https://doi.org/10.1371/journal.pgen.1007362 e1007362.
[46] Dor Y, Cedar H. Principles of DNA methylation and their implications for biology and medicine. Lancet 2018;392:777–786. https://doi.org/10.1016/S0140-6736(18)31268-6.
[47] Zhong S, Tang MW, Yeo W, Liu C, Lo YMD, Johnson PJ. Silencing of GSTP1 gene by CpG island DNA hypermethylation in HBV-associated hepatocellular carcinomas. Clin Cancer Res 2002;8:1087–1092. https://doi.org/10.1158/1078-0432.ccr-06-1900.
[48] Yang B, House MG, Guo M, Herman JG, Clark DP. Promoter methylation profiles of tumor suppressor genes in intrahepatic and extrahepatic cholangiocarcinoma. Mod Pathol 2005;18:412–420. https://doi.org/10.1038/modpathol.3800287.
[49] Herman JG, Graff JR, Myöhänen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci U S A 1996;93:9821–9826. https://doi.org/10.1073/pnas.93.18.9821.
[50] Wong IH, Lo YM, Zhang J, Liew CT, Ng MH, Wong N, et al. Detection of aberrant p16 methylation in the plasma and serum of liver cancer patients. Cancer Res 1999;59:71–73.
[51] Wong IHN, Lo YMD, Yeo W, Lau WY, Johnson PJ. Frequent p15 promoter methylation in tumor and peripheral blood from hepatocellular carcinoma patients. Clin Cancer Res 2000;6:3516–3521.
[52] **Sun K**, **Jiang P**, **Chan KCA**, Wong J, Cheng YKY, Liang RHS, et al. Plasma DNA tissue mapping by genome-wide methylation sequencing for noninvasive prenatal, cancer, and transplantation assessments. Proc Natl Acad Sci 2015;112:E5503–E5512. https://doi.org/10.1073/pnas.1508736112.
[53] Kopreski MS, Benko FA, Kwak LW, Gocke CD. Detection of tumor messenger RNA in the serum of patients with malignant melanoma. Clin Cancer Res 1999;5:1961–1965. https://doi.org/10.1373/clinchem.2013.202663.
[54] Lo KW, Lo YMD, Leung SF, Tsang YS, Chan LYS, Johnson PJ, et al. Analysis of cell-free Epstein-Barr virus-associated RNA in the plasma of patients with nasopharyngeal carcinoma. Clin Chem 1999;45:1292–1294. https://doi.org/10.1016/S0016-5085(08)61896-X.
[55] Wang X, Kwak KJ, Yang Z, Zhang A, Zhang X, Sullivan R, et al. Extracellular mRNA detected by molecular beacons in tethered lipoplex nanoparticles for diagnosis of human hepatocellular carcinoma. PLoS ONE 2018;13. https://doi.org/10.1371/journal.pone.0198552 e0198552.
[56] Chan RWY, Wong J, Lai PBS, Lo YMD, Chiu RWK. The potential clinical utility of serial plasma albumin mRNA monitoring for the post-liver transplantation management. Clin Biochem 2013;46:1313–1319. https://doi.org/10.1016/j.clinbiochem.2013.04.022.
[57] Chim SSC, Shing TKF, Hung ECW, Leung TY, Lau TK, Chiu RWK, et al. Detection and characterization of placental microRNAs in maternal plasma. Clin Chem 2008;54:482–490. https://doi.org/10.1373/clinchem.2007.097972.
[58] Williams Z, Ben-Dov IZ, Elias R, Mihailovic A, Brown M, Rosenwaks Z, et al. Comprehensive profiling of circulating microRNA via small RNA sequencing of cDNA libraries reveals biomarker potential and limitations. Proc Natl Acad Sci 2013;110:4255–4260. https://doi.org/10.1073/pnas.1214046110.
[59] Greystoke A, Ayub M, Rothwell DG, Morris D, Burt D, Hodgkinson CL, et al. Development of a circulating miRNA assay to monitor tumor burden: From mouse to man. Mol Oncol 2016;10:282–291. https://doi.org/10.1016/j.molonc.2015.10.004.
[60] Elias KM, Fendler W, Stawiski K, Fiascone SJ, Vitonis AF, Berkowitz RS, et al. Diagnostic potential for a serum miRNA neural network for detection of ovarian cancer. Elife 2017;6:6742–6747. https://doi.org/10.7554/eLife. 28932.
[61] Akuta N, Kawamura Y, Suzuki F, Saitoh S, Arase Y, Fujiyama S, et al. Analysis of association between circulating miR-122 and histopathological features of nonalcoholic fatty liver disease in patients free of hepatocellular carcinoma. BMC Gastroenterol 2016;16:141. https://doi.org/10.1186/s12876-016-0557-6.
[62] Appourchaux K, Dokmak S, Resche-Rigon M, Treton X, Lapalus M, Gattolliat C-H, et al. MicroRNA-based diagnostic tools for advanced fibrosis and cirrhosis in patients with chronic hepatitis B and C. Sci Rep 2016;6:34935. https://doi.org/10.1038/srep34935.
[63] Ye D, Zhang T, Lou G, Xu W, Dong F, Chen G, et al. Plasma miR-17, miR-20a, miR-20b and miR-122 as potential biomarkers for diagnosis of NAFLD in type 2 diabetes mellitus patients. Life Sci 2018;208:201–207. https://doi.org/10.1016/j.lfs.2018.07.029.
[64] Liao W, Yang H, Xu H, Wang Y, Ge P, Ren J, et al. Noninvasive detection of tumor-associated mutations from circulating cell-free DNA in hepatocellular carcinoma patients by targeted deep sequencing. Oncotarget 2016;7. https://doi.org/10.18632/oncotarget.9629.
[65] Genovese G, Kähler AK, Handsaker RE, Lindberg J, Rose SA, Bakhoum SF, et al. Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. N Engl J Med 2014;371:2477–2487. https://doi.org/10.1056/NEJMoa1409405.
[66] Zhang YJ, Wu HC, Shen J, Ahsan H, Wei YT, Yang HI, et al. Predicting hepatocellular carcinoma by detection of aberrant promoter methylation in serum DNA. Clin Cancer Res 2007;13:2378–2384. https://doi.org/10.1158/1078-0432.CCR-06-1900.

[67] Chan KCA, Lai PBS, Mok TSK, Chan HLY, Ding C, Yeung SW, et al. Quantitative analysis of circulating methylated DNA as a biomarker for hepatocellular carcinoma. Clin Chem 2008;54:1528–1536. https://doi.org/10.1373/clinchem.2008.104653.
[68] Xu RH, Wei W, Krawczyk M, Wang W, Luo H, Flagg K, et al. Circulating tumour DNA methylation markers for diagnosis and prognosis of hepatocellular carcinoma. Nat Mater 2017;16:1155–1162. https://doi.org/10.1038/NMAT4997.
[69] Kang S, Li Q, Chen Q, Zhou Y, Park S, Lee G, et al. CancerLocator: non-invasive cancer diagnosis and tissue-of-origin prediction using methylation profiles of cell-free DNA. Genome Biol 2017;18:53. https://doi.org/10.1186/s13059-017-1191-5.
[70] Li W, Li Q, Kang S, Same M, Zhou Y, Sun C, et al. CancerDetector: ultrasensitive and non-invasive cancer detection at the resolution of individual reads using cell-free DNA methylation sequencing data e89–e89. Nucleic Acids Res 2018;46. https://doi.org/10.1093/nar/gky423.
[71] Guo S, Diep D, Plongthongkum N, Fung H-L, Zhang K, Zhang K. Identification of methylation haplotype blocks aids in deconvolution of heterogeneous tissue samples and tumor tissue-of-origin mapping from plasma DNA. Nat Genet 2017;49:635–642. https://doi.org/10.1038/ng.3805.
[72] Wu H-C, Yang H-I, Wang Q, Chen C-J, Santella RM. Plasma DNA methylation marker and hepatocellular carcinoma risk prediction model for the general population. Carcinogenesis 2017;38:1021–1028. https://doi.org/10.1093/carcin/bgx078.
[73] Huang Y-H, Liang K-H, Chien R-N, Hu T-H, Lin K-H, Hsu C-W, et al. A circulating microRNA signature capable of assessing the risk of hepatocellular carcinoma in cirrhotic patients. Sci Rep 2017;7:523. https://doi.org/10.1038/s41598-017-00631-9.
[74] Labgaa I, Villacorta-Martin C, D'avola D, Craig AJ, Von Felden J, Martins-Filho SN, et al. A pilot study of ultra-deep targeted sequencing of plasma DNA identifies driver mutations in hepatocellular carcinoma. Oncogene 2018. https://doi.org/10.1038/s41388-018-0206-3.
[75] Ikeda S, Tsigelny IF, Skjevik ÅA, Kono Y, Mendler M, Kuo A, et al. Next-generation sequencing of circulating tumor DNA reveals frequent alterations in advanced hepatocellular carcinoma. Oncologist 2018. https://doi.org/10.1634/theoncologist.2017-0479.
[76] Ho DWH, Chan LK, Chiu YT, Xu IMJ, Poon RTP, Cheung TT, et al. TSC1/2 mutations define a molecular subset of HCC with aggressive behaviour and treatment implication. Gut 2017. https://doi.org/10.1136/gutjnl-2016-312734.
[77] Ikeda S, Lim JS, Kurzrock R. Analysis of tissue and circulating tumor DNA by next-generation sequencing of hepatocellular carcinoma: implications for targeted therapeutics. Mol Cancer Ther 2018. https://doi.org/10.1158/1535-7163.MCT-17-0604.
[78] Chan SL, Wong AM, Lee K, Wong N, Chan AKC. Personalized therapy for hepatocellular carcinoma: where are we now? Cancer Treat Rev 2016. https://doi.org/10.1016/j.ctrv.2016.02.008.
[79] Goodman AM, Kato S, Bazhenova L, Patel SP, Frampton GM, Miller V, et al. Tumor mutational burden as an independent predictor of response to immunotherapy in diverse cancers. Mol Cancer Ther 2017;16:2598–2608. https://doi.org/10.1158/1535-7163.MCT-17-0386.
[80] Gandara DR, Paul SM, Kowanetz M, Schleifman E, Zou W, Li Y, et al. Blood-based tumor mutational burden as a predictor of clinical benefit in non-small-cell lung cancer patients treated with atezolizumab. Nat Med 2018;24:1441–1448. https://doi.org/10.1038/s41591-018-0134-3.
[81] Garcia-Fernandez N, Macher HC, Rubio A, Jimenez-Arriscado P, Bernal-Bellido C, Bellido-Diaz ML, et al. Detection of p53 mutations in circulating DNA of transplanted hepatocellular carcinoma patients as a biomarker of tumor recurrence. Adv Exp Med Biol 2016. https://doi.org/10.1007/978-3-319-42044-8_5.
[82] Schütz E, Fischer A, Beck J, Harden M, Koch M, Wuensch T, et al. Graft-derived cell-free DNA, a noninvasive early rejection and graft damage marker in liver transplantation: a prospective, observational, multicenter cohort study. PLoS Med 2017;14. https://doi.org/10.1371/journal.pmed.1002286 e1002286.
[83] Gai W, Ji L, Lam WKJ, Sun K, Jiang P, Chan AWH, et al. Liver- and colon-specific DNA methylation markers in plasma for investigation of colorectal cancers with or without liver metastases. Clin Chem 2018;64:1239–1249. https://doi.org/10.1373/clinchem.2018.290304.
[84] Lehmann-Werman R, Magenheim J, Moss J, Neiman D, Abraham O, Piyanzin S, et al. Monitoring liver damage using hepatocyte-specific methylation markers in cell-free circulating DNA. JCI Insight 2018;3. https://doi.org/10.1172/jci.insight.120687.
[85] Chan KCA, Zhang J, Chan ATC, Lei KIK, Leung SF, Chan LYS, et al. Molecular characterization of circulating EBV DNA in the plasma of nasopharyngeal carcinoma and lymphoma patients. Cancer Res 2003;63:2028–2032.
[86] Lo YMD, Chan KCA, Sun H, Chen EZ, Jiang P, Lun FMF, et al. Maternal plasma DNA sequencing reveals the genome-wide genetic and mutational profile of the fetus. Sci Transl Med 2010;2:61ra91. https://doi.org/10.1126/scitranslmed.3001720.
[87] Snyder MWW, Kircher M, Hill AJJ, Daza RMM, Shendure J. Cell-free DNA comprises an in vivo nucleosome footprint that informs its tissues-of-origin. Cell 2016;164:57–68. https://doi.org/10.1016/j.cell.2015.11.050.
[88] Chan KCA, Jiang P, Sun K, Cheng YKY, Tong YK, Cheng SH, et al. Second generation noninvasive fetal genome analysis reveals de novo mutations, single-base parental inheritance, and preferred DNA ends. Proc Natl Acad Sci 2016;113:E8159–E8168. https://doi.org/10.1073/pnas.1615800113.
[89] Jiang P, Sun K, Tong YK, Cheng SH, Cheng THT, Heung MMS, et al. Preferred end coordinates and somatic variants as signatures of circulating tumor DNA associated with hepatocellular carcinoma. Proc Natl Acad Sci U S A 2018;115:E10925–E10933. https://doi.org/10.1073/pnas.1814616115.