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Cell-free nucleic acids as biomarkers in cancer patients

Heidi Schwarzenbach, Dave S. B. Hoon‡ and Klaus Pantel**

Abstract | DNA, mRNA and microRNA are released and circulate in the blood of cancer patients. Changes in the levels of circulating nucleic acids have been associated with tumour burden and malignant progression. In the past decade a wealth of information indicating the potential use of circulating nucleic acids for cancer screening, prognosis and monitoring of the efficacy of anticancer therapies has emerged. In this Review, we discuss these findings with a specific focus on the clinical utility of cell-free nucleic acids as blood biomarkers.

microRNAs
Small non-coding RNA molecules that modulate the activity of specific mRNA molecules by binding and inhibiting their translation into polypeptides.

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In 1948, Mandel and Métais[1] described the presence of cell-free nucleic acid (cfNA) in human blood for the first time. This attracted little attention in the scientific community and it was not until 1994 that the importance of cfNA was recognized as a result of the detection of mutated RAS gene fragments in the blood of cancer patients[2,3] (TIMELINE). In 1996, microsatellite alterations on cell-free DNA (cfDNA) were shown in cancer patients[4], and during the past decade increasing attention has been paid to cfNAs (such as DNA, mRNA and microRNAs (miRNAs)) that are present at high concentrations in the blood of cancer patients (FIG. 1). Indeed, their potential value as blood biomarkers was highlighted in a recent editorial in the journal *Science*[5].

Detecting cfNA in plasma or serum could serve as a 'liquid biopsy', which would be useful for numerous diagnostic applications and would avoid the need for tumour tissue biopsies. Use of such a liquid biopsy delivers the possibility of taking repeated blood samples, consequently allowing the changes in cfNA to be traced during the natural course of the disease or during cancer treatment. However, the levels of cfNA might also reflect physiological and pathological processes that are not tumour-specific[6]. cfNA yields are higher in patients with malignant lesions than in patients without tumours, but increased levels have also been quantified in patients with benign lesions, inflammatory diseases and tissue trauma[7]. The physiological events that lead to the increase of cfNA during cancer development and progression are still not well understood. However, analyses of circulating DNA allow the detection of tumour-related genetic and epigenetic alterations that are relevant to cancer development and progression. In addition, circulating miRNAs have recently been shown to be potential cancer biomarkers in blood.

This Review focuses on the clinical utility of cfNA, including genetic and epigenetic alterations that can be detected in cfDNA, as well as the quantification of nucleosomes and miRNAs, and discusses the relationship between cfNA and micrometastatic cells.

Biology of cfNA

The release of nucleic acids into the blood is thought to be related to the apoptosis and necrosis of cancer cells in the tumour microenvironment. Secretion has also been suggested as a potential source of cfDNA (FIG. 1). Necrotic and apoptotic cells are usually phagocytosed by macrophages or other scavenger cells[8]. Macrophages that engulf necrotic cells can release digested DNA into the tissue environment. *In vitro* cell culture experiments indicated that macrophages can be either activated or dying during the process of DNA release[8]. Fragments of cellular nucleic acids can also be actively released[9,10]. It has been estimated that for a patient with a tumour that weighs 100 g, which corresponds to 3×10^{10} tumour cells, up to 3.3% of tumour DNA may enter the blood every day[11]. On average, the size of this DNA varies between small fragments of 70 to 200 base pairs and large fragments of approximately 21 kilobases[12]. Tumour cells that circulate in the blood, and micrometastatic deposits that are present at distant sites, such as the bone marrow and liver, can also contribute to the release of cfNA[13,14].

Tumours usually represent a mixture of different cancer cell clones (which account for the genomic and epigenomic heterogeneity of tumours) and other normal cell types, such as haematopoietic and stromal cells. Thus, during tumour progression and turnover both tumour-derived and wild-type (normal) cfNA can be released into the blood. As such, the proportion of cfNA that originates from tumour cells varies owing to the state

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At a glance

- Increased levels of circulating nucleic acids (DNA, mRNA and microRNA (miRNA)) in the blood reflect pathological processes, including malignant and benign lesions, inflammatory diseases, stroke, trauma and sepsis. During these processes nucleic acids are shed into the blood by apoptotic and necrotic cells.
- In cancer patients, circulating DNA carries tumour-related genetic and epigenetic alterations that are relevant to cancer development, progression and resistance to therapy. These alterations include loss of heterozygosity (LOH) and mutations of tumour suppressor genes (such as *TP53*) and oncogenes (such as *KRAS* and *BRAF*).
- Additional genetic alterations that are detectable on circulating DNA and used as biomarkers in cancer include the integrity of non-coding genomic DNA repeat sequences (such as *ALU* and *LINE1*). Although still in their infancy, DNA integrity assays have the potential to become a universal blood biomarker for multiple cancers.
- Epigenetic alterations in genes (such as glutathione *S*-transferase P1 (*GSTP1* and septin 9 (*SEPT9*)) and adenomatous polyposis coli (*APC*)) that are relevant to tumorigenesis and the progression of solid tumours have been detected on circulating DNA in cancer patients, and their potential clinical utility is indicated by the launch of commercial tests for cancer screening.
- The detection of circulating nucleosomes in blood indicates that cell-free DNA (cfDNA) retains at least some features of the nuclear chromatin during the process of DNA release. Initial clinical studies have indicated that monitoring the abundance of nucleosomes has potential utility for monitoring the efficacy of therapy in cancer patients.
- Dying tumour cells also discharge miRNAs, which circulate stably in the blood. The pivotal functions of miRNAs in cancer development and progression may explain the promising results of pilot studies on cancer patients using miRNA blood tests for tumour detection and prognosis.
- The cellular source of tumour-derived circulating nucleic acids is still subject to debate. After complete removal of the primary tumour the detection of cfDNA may signal the presence of micrometastatic cells in distant organs, such as the bone marrow, which pose a risk of relapse.
- Metastatic and primary tumours from the same patient can vary at the genomic, epigenomic and transcriptomic levels. Minimally invasive blood analyses of cell-free nucleic acid allow repetitive real-time monitoring of these events and will, therefore, gain clinical utility in the determination of prognosis and treatment efficacy.

and size of the tumour. The amount of cfNA is also influenced by clearance, degradation and other physiological filtering events of the blood and lymphatic circulation. Nucleic acids are cleared from the blood by the liver and kidney and they have a variable half-life in the circulation ranging from 15 minutes to several hours[7]. Assuming an exponential decay model and plotting the natural logarithm of cfDNA concentration against time, serial DNA measurements have shown that some forms of cfNA might survive longer than others. When purified DNA was injected into the blood of mice, double-stranded DNA remained in the circulation longer than single-stranded DNA[15]. Moreover, viral DNA as a closed ring may survive longer than linear DNA[15]. However, regardless of its size or configuration, cfDNA is cleared from the circulation rapidly and efficiently[16]. miRNAs seem to be highly stable, but their clearance rate from the blood has not yet been well studied in cancer patients owing to the novelty of this area of research. The nuclease activity in blood may be one of the important factors for the turnover of cfNA. However, this area of cfNA physiology remains unclear and needs further examination.

Circulating cfDNA

DNA content. In patients with tumours of different histopathological types, increased levels of total cfDNA, which consists of epigenomic and genomic, as well as mitochondrial and viral DNA, have been assessed by different fluorescence-based methods (such as, PicoGreen staining and ultraviolet (UV) spectrometry) or quantitative PCR (such as, SYBR Green and TaqMan). Although cancer patients have higher cfDNA levels than healthy control donors, the concentrations of overall cfDNA vary considerably in plasma or serum samples in both groups[17–19]. A range of between 0 and >1,000 ng per ml of blood, with an average of 180 ng per ml cfDNA, has been measured[20–23]. By comparison, healthy subjects have concentrations between 0 and 100 ng per ml cfDNA of blood, with an average of 30 ng per ml cfDNA[7]. However, it is difficult to draw conclusions from these studies, as the size of the investigated patient cohort is often small and the techniques used to quantify cfDNA vary. A large prospective study assessed the value of plasma DNA levels as indicators for the development of neoplastic or pulmonary disease. The concentration of plasma DNA varied considerably between the European Prospective Investigation into Cancer and Nutrition (EPIC) centres that were involved in the study. This variation was proposed to be due to the type of population recruited and/or the treatment of the samples[24]. However, the quantification of cfDNA concentrations alone does not seem to be useful in a diagnostic setting owing to the overlapping DNA concentrations that are found in healthy individuals with those in patients with benign and malignant disease. The assessment of cfDNA concentration might prove to be useful in combination with other blood tumour biomarkers. Following surgery, the levels of cfDNA in cancer patients with localized disease can decrease to levels that are observed in healthy individuals[25]. However, when the cfDNA level remains high, it might indicate the presence of residual tumour cells[17]. Further studies are needed for the repeat assessment of quantitative cfNA in large cohorts of patients with well-defined clinical parameters. Such investigations will be crucial if we are to use cfDNA as a prognostic biomarker, as will the isolation and processing of cfNA to defined standards (discussed below).

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cfDNA is composed of both genomic DNA (gDNA) and mitochondrial DNA (mtDNA). Interestingly, the levels of cell-free mtDNA and gDNA do not correlate in some tumour types[26,27], indicating the different nature of circulating mtDNA and gDNA. In contrast to two copies of gDNA, a single cell contains up to several hundred copies of mtDNA. Whereas gDNA usually circulates in a cell-free form, circulating mtDNA in plasma exists in both particle-associated and non-particle-associated forms[28]. Diverging results have been reported regarding whether cell-free mtDNA levels are increased and clinically relevant in cancer patients.

The cfDNA can also include both coding and non-coding gDNA that can be used to examine microsatellite instability, loss of heterozygosity (LOH), mutations, polymorphisms, methylation and integrity (size). In recent years, considerable attention has been paid to non-coding DNA, particularly repetitive sequences, such as *ALU* (which is a short interspersed nucleic element (SINE)) and as long interspersed nucleotide elements such as *LINE1* (REFS 29–31) (discussed below). *ALU* and *LINE1* are distributed throughout the genome and are known to be less methylated in cancer cells compared with normal cells[32].

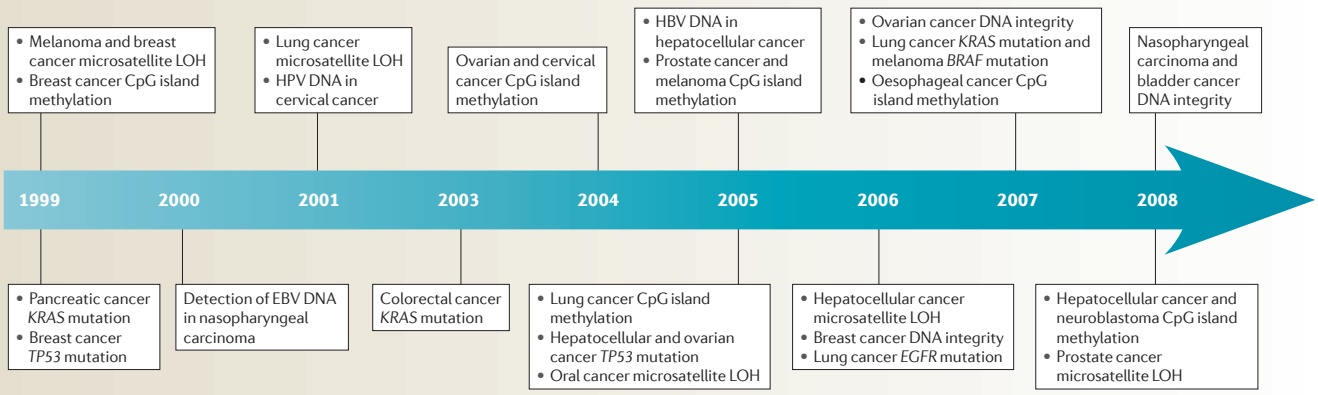
Tumour-specific LOH. Genetic alterations found in cfDNA frequently include LOH that is detected using PCR-based assays[13,18,33–38] (TABLE 1). Although similar plasma- and serum-based LOH detection methods have been used, a great variability in the detection of LOH in cfDNA has been reported. Despite the concordance between tumour-related LOH that is present in cfDNA in blood and LOH that is found in DNA isolated from matched primary tumours, discrepancies have also been found[7]. These contradictory LOH data that have been derived from blood and tumour tissue and the low incidence of LOH in cfDNA have partly been explained by technical problems and the dilution of tumour-associated cfDNA in blood by DNA released from normal cells[11,39–41]. Moreover, the abnormal proliferation of benign cells, owing to inflammation or tissue repair processes, for example, leads to an increase in apoptotic cell death, the accumulation of small, fragmented DNA in blood and the masking of LOH[42].

Alternative approaches, such as the detection of tumour-specific deletions are needed to better address the inherent problems of LOH analyses.

Tumour-specific gene mutations. The analysis of cfDNA for specific gene mutations, such as those in *KRAS* and *TP53*, is desirable because these genes have a high mutation frequency in many tumour types and contribute to tumour progression[43]. Additionally, clinically relevant mutations in *BRAF*, epidermal growth factor receptor (*EGFR*) and adenomatous polyposis coli (*APC*) have now been studied in cfDNA. Several therapeutic agents in clinical trials target the KRAS, BRAF, EGFR or p53 pathways[44,45], and require the identification of the mutation status of the patient's tumour to predict response to treatment. In this regard, cfDNA provides a unique opportunity to repeatedly monitor patients during treatment. In particular, in stage IV cancer patients, biopsies are not possible or repeat sampling of primary tumour and metastatic samples is not practical or ethical.

The major problem with this approach has been assay specificity and sensitivity. Assays targeting cfDNA mutations require that the mutation in the tumour occurs frequently at a specific genomic site. A major drawback of cfDNA assays is the low frequency of some of the mutations that occur in tumours. In general, wild-type sequences often interfere with cfDNA mutation assays. This is due to the low level of cfDNA mutations and the dilution effect of DNA fragments and wild-type DNA in circulation. In PCR-based assays technological design can significantly limit the assay sensitivity and specificity. An example is the *KRAS* mutation tissue assay that can frequently detect mutations in tumour tissues, such as the pancreas, colon and lung;

Timeline | **Detection of various forms of cfDNA in patients with different types of cancer**



The development of the detection of genetic and epigenetic alterations, as well as the measurement of DNA integrity and viral DNA, in blood from patients with different tumour types over the past decade is shown. We show only significant, prognostic findings from >40 patients with serum, plasma or bodily fluid detection of cell-free DNA (cfDNA) from individual cancers. This timeline is not meant to be comprehensive and is based on our own personal view of what have been important clinical translational events. EBV, Epstein–Barr virus; EGFR, epidermal growth factor receptor; HBV, hepatitis B virus; HPV, human papilloma virus; LOH, loss of heterozygosity.

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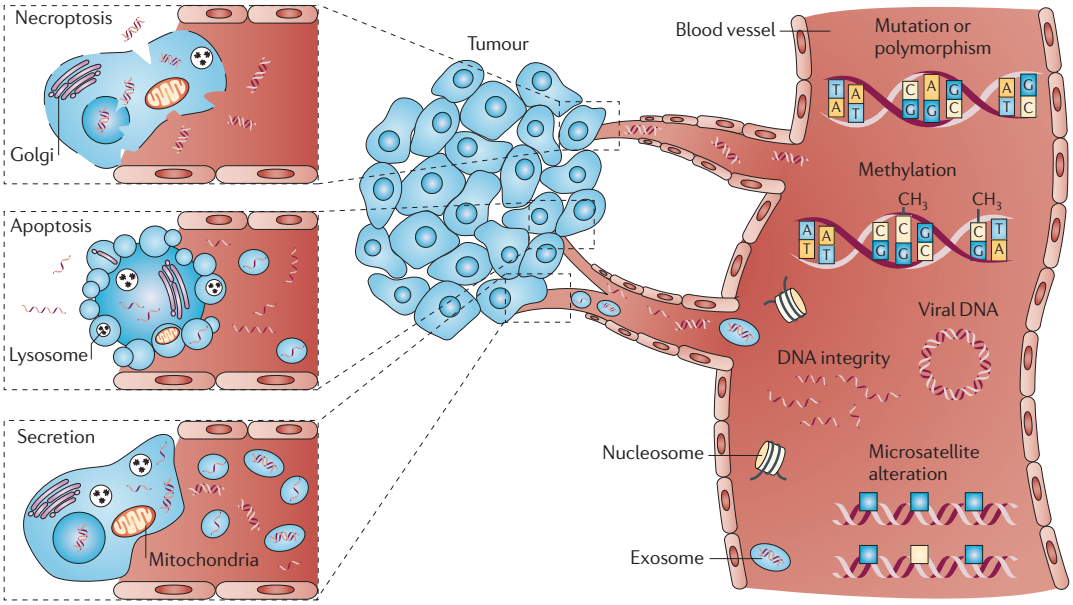

Figure 1 | **Cell-free nucleic acids in the blood.** Mutations, methylation, DNA integrity, microsatellite alterations and viral DNA can be detected in cell-free DNA (cfDNA) in blood. Tumour-related cfDNA, which circulates in the blood of cancer patients, is released by tumour cells in different forms and at different levels. DNA can be shed as both single-stranded and double-stranded DNA. The release of DNA from tumour cells can be through various cell physiological events such as apoptosis, necrosis and secretion. The physiology and rate of release is still not well understood; tumour burden and tumour cell proliferation rate may have a substantial role in these events. Individual tumour types can release more than one form of cfDNA.

however, cfDNA mutation assays using blood samples have not yet been concordantly successful[46–48]. New approaches are needed, such as cfDNA sequencing. The *BRAF* mutation V600E, which is present in >70% of metastatic melanomas, can be detected in cfDNA and has been shown to be useful in monitoring patients with melanoma who are receiving therapy[49]. This mutation has been detected in different stages of melanoma (according to the American Joint Committee on Cancer (AJCC) Cancer Staging Manual) using a quantitative real-time clamp PCR assay, with the highest levels found in the more advanced stages[49]. This is one of the first major studies to demonstrate that cfDNA mutation assays have the sensitivity to monitor patient responses before and after treatment. The utility of a cfDNA *BRAF* mutation assay has gained more importance, as new anti-BRAF drugs, such as PLX4032 (Roche)[50] and GSK2118436 (GlaxoSmithKline)[51], have shown substantial responses in patients in early clinical trials. *EGFR* mutations that occur in a specific subset of patients with lung cancers[52–54] make these tumours sensitive to EGFR-targeted therapies; however, the detection of EGFR mutations in cfDNA has not been well developed owing to issues with sensitivity and specificity. Patients whose tumours have a specific gene mutation would be strong candidates for monitoring of their cfDNA in blood for the respective specific mutation. However, sensitivity, specificity and validation need to be carried out in multicentre settings to determine true clinical utility. Alternatively, cfDNA assays might be more appropriate when used with other biomarker assays, and this might be applicable to personalized medicine, rather than diagnostic screens that can be used across a wide group of cancer patients.

Quantitative real-time clamp PCR assay
A technique that uses a peptide nucleic acid clamp and locked nucleic acid probes, which are DNA synthetic analogues that hybridize to complementary DNA and are highly sensitive and specific for recognizing single base pair mismatches.

DNA integrity. Another assay that is applicable to cfDNA that has gained interest in recent years is the integrity of non-coding gDNA, such as the repeat sequences of *ALU* and *LINE1*. The *ALU* and *LINE1* sequences have been referred to as 'junk DNA'; however, in recent years evidence has indicated their importance in various physiological events, such as DNA repair, transcription, epigenetics and transposon-based activity[55,56]. Approximately 17–18% of the human genome consists of *LINE1*. In normal cells *LINE1* sequences are heavily methylated, restricting the activities of these retrotransposon elements and thus preventing genomic instability. *LINE1* sequences are moderately CpG-rich, and most methylated CpGs are located in the 5′ region of the sequence that can function as an internal promoter[23]. These forms of DNA can be detected as cfDNA of different sizes, but also as methylated and unmethylated DNA. Studies on these types of cfDNA are still in their infancy; however, recent studies have shown potential prognostic and diagnostic utility[23,29–31]. The assays are based on the observation that common DNA repeat sequences are preferentially released by tumour cells that are undergoing non-apoptotic or necrotic cell death, and these fragments can be between 200 bp and 400 bp in size. The *ALU* and *LINE1* sequences are well interspersed throughout the genome on all chromosomes, so although specificity

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for an individual cancer type is lost in these assays, sensitivity is enhanced. Using a PCR assay, the integrity of cfDNA *ALU* sequences in blood has been shown to be sensitive for the assessment of the early stages of breast cancer progression, including micrometastasis[30]. DNA integrity cfDNA assays have also been used in testicular, prostate, nasopharyngeal and ovarian cancer[31,57–59]. These assays are still in their infancy and address an important challenge of whether a 'universal' blood biomarker for multiple cancers can be of clinical utility. Further validation of these assays will also determine their clinical utility in specific cancers.

Table 1 | **Detection of cfDNA and its alterations in patients with different tumour types***

Cancer	cfDNA	Diagnostic	Prognostic	Refs
Bladder	DNA integrity	✓	✓	123
	Methylation	✓		124
	Microsatellite alterations	✓		125
Breast	Methylation	✓	✓	126–130
	Microsatellite alterations	✓	✓	33–35
	DNA integrity		✓	30,131
	Mutation		✓	34
	Mitochondrial	✓		132
Cervical	Methylation	✓	✓	133,134
	Viral DNA	✓		135
Colorectal	Mutation	✓	✓	47,136–139
	DNA integrity	✓		31
	Methylation	✓	✓	136,140–143
Hepatocellular carcinoma	Methylation	✓	✓	144–146
	Microsatellite alterations		✓	147
	Mutation	✓	✓	148,149
	DNA integrity	✓	✓	29
	Viral DNA	✓		150
Lung	Mutation		✓	48,53,151,152
	Methylation	✓	✓	153–157
	Microsatellite alterations	✓	✓	36,37
Non-Hodgkin's lymphoma	Mutation		✓	158
	Viral DNA	✓	✓	159–161
	Methylation	✓		162
	DNA integrity	✓		162
Melanoma	Mutation	✓	✓	49,163,164
	Methylation		✓	111,115
	Microsatellite alterations	✓	✓	165–168
Ovarian	Methylation	✓	✓	169,170
	DNA integrity	✓		59
	Mutation		✓	171
	Mitochondrial	✓		172
Pancreatic	Methylation	✓		173,174
	DNA integrity	✓		31
	Mutation	✓	✓	46
Prostate	Methylation	✓	✓	38,175–179
	Microsatellite alterations	✓		13,38
	DNA integrity	✓	✓	180
	Mitochondrial		✓	26,181

*This table represents different forms of cell-free nucleic acid (cfNA) that have been detected in patients with the most prevalent cancers in both males and females[182]. This table is not meant to be comprehensive and is based on our own view of studies that offer substantial clinical insight. cfDNA, cell-free DNA.

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Epigenetic alterations. Epigenetic alterations can have a substantial effect on tumorigenesis and progression (BOX 1). Several studies have revealed the presence of methylated DNA in the serum or plasma of patients with various types of malignancy (TABLE 1). The detection of methylated cfDNA represents one of the most promising approaches for risk assessment in cancer patients.

Assays for the detection of promoter hypermethylation may have a higher sensitivity than microsatellite analyses, and can have advantages over mutation analyses. In general, aberrant DNA methylation, which seems to be common in cancer, occurs at specific CpG dinucleotides[60]. The acquired hypermethylation of a specific gene can be detected by sodium bisulphite treatment of DNA, which converts unmethylated (but not methylated) cytosines to uracil. The modified DNA is analysed using either methylation-specific PCR, with primers that are specific for methylated and unmethylated DNA, or DNA sequencing[61]. Nevertheless, to improve the assay conditions and the clinical relevance, the selection of appropriate tumour-related genes from a long list of candidate genes that are known to be methylated in neoplasia is essential. Although epigenetic alterations are not unique for a single tumour entity, there are particular tumour suppressor genes that are frequently methylated and downregulated in certain tumours[62,63]. For example, important epigenetic events in carcinogenesis include the hypermethylation of the promoter region of the genes pi-class glutathione *S*-transferase P1 (*GSTP1*) and *APC*, which are the most common somatic genome abnormalities in prostate and colorectal cancer, respectively[62,63]. Other important methylated genes that have shown prognostic utility using cfDNA assays in significant numbers of patients include RAS association domain family 1A (*RASSF1A*), retinoic acid receptor-β (*RARB*), septin 9 (*SEPT9*), oestrogen receptor-α (*ESR1*) and cyclin-dependent kinase inhibitor 2A (*CDKN2A*) (TABLE 1). The first commercial real-time PCR plasma test for the detection of early colorectal cancer (developed by Epigenomics AG and Abbott Molecular) is for the detection of *SEPT9*. This biomarker is still undergoing validation, but it demonstrates the potential diagnostic screening utility of methylated tumour-related cfDNA to differentiate cancer patients from healthy individuals and to identify the tumour type.

It is also possible to detect tumour-related alterations in histone modifications in the blood. By monitoring changes in the circulating histones and DNA methylation pattern, the antitumour effects of histone deacetylase and histone methyltransferase inhibitors may be evaluated and consequently allow a better screening of cancer patients[64,65].

Circulating nucleosomes. Circulating gDNA that is derived from tumours seems to predominantly exist as mononucleosomes and oligonucleosomes, or it is bound to the surface of blood cells by proteins that harbour specific nucleic acid-binding properties[66]. A nucleosome consists of a histone octamer core wrapped twice by a 200 bp-long DNA strand. Under physiological conditions these complexes are packed in apoptotic particles and engulfed by macrophages[67]. However, an excess of apoptotic cell death, as occurs in large and rapidly proliferating tumours or after chemotherapy treatment, can lead to a saturation of apoptotic cell engulfment and thus increased nucleosome levels in the blood[68]. The detection of circulating nucleosomes that are associated with cfDNA suggests that DNA in blood retains at least some features of the nuclear chromatin during the process of release.

Enzyme-linked immunosorbent assays (ELISAs) have been developed to quantify circulating nucleosomes. As increased concentrations are found in both benign and malignant tumours, high nucleosome levels in blood are not indicators of malignant disease[69]. However, the observed changes in apoptosis-related deregulation of proteolytic activities along with the increased serum levels of nucleosomes have been linked to breast cancer progression[70]. As typical cell-death products, the quantification of circulating nucleosomes seems to be valuable for monitoring the efficacy of cytotoxic cancer therapies[71]. For example, platinum-based chemotherapy induces caspase-dependent apoptosis of tumour cells and an increase in circulating nucleosomes in the blood of patients with ovarian cancer[17]. Moreover, the outcome of therapy can be indicated by nucleosome levels during the first week of chemotherapy and radiotherapy in patients with lung, pancreatic and colorectal cancer, as well as in patients with haematological malignancies[71].

Viral DNA. Viral cfDNA can also be detected in some tumour types. Viruses, such as human papillomavirus (HPV), hepatitis B virus (HBV) and Epstein–Barr virus (EBV), are aetiological factors in various malignancies, such as nasopharyngeal, cervical, head and neck, and hepatocellular cancer and lymphoma[72–75]. Their specific DNA may have the potential to be used as molecular biomarkers for neoplastic disease. Associations between circulating viral DNA and disease have been reported for EBV with Hodgkin's disease, Burkitt's lymphoma and nasopharyngeal carcinoma; for HBV with some forms of hepatic cell carcinoma; and for HPV with head and neck, cervical and hepatocellular cancers (TABLE 1). The clinical utility of EBV cfDNA in diagnosis and prognosis of nasopharyngeal carcinoma has been demonstrated in multiple studies with large cohorts of patients[76–80], and the use of this cfDNA has became one of the leading cfDNA blood tests for the assessment of nasopharyngeal carcinoma progression in Hong Kong, Taiwan and China, where this cancer is highly prevalent[77,78,81]. The limitation

Box 1 | **Epigenetics**

Epigenetic changes can include the methylation of gene promoter regions and histone modifications. In chromosomal regions where tumour-associated genes reside, epigenetic modifications may affect important regulatory mechanisms that normally limit malignant transformation[60]. Inactivation of tumour suppressor genes by promoter hypermethylation is thought to have a crucial role in this process[117]. DNA methylation of the cytosine base in CpG dinucleotides, which are found as isolated or clustered CpG islands, induces gene repression by inhibiting the access of transcription factors to their binding sites, and by recruiting methyl-CpG-binding proteins (MBDs) to methylated DNA together with histone-modifying enzymes[118]. Epigenetic modifications also alter the packing of nucleosomes that are implicated in transcriptional regulation[119,120].

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of most viral cfNA assays is that benign viral infections that are caused by the same viruses can complicate the interpretation of results, particularly in diagnostic screening. Establishing clinically meaningful cut-off levels is important to move these screens into the clinic.

Genometastasis. The genometastasis hypothesis describes the horizontal transfer of cell-free tumour DNA to other cells that results in transformation. If true, metastases could develop in distant organs as a result of a transfection-like uptake of dominant oncogenes that are released from the primary tumour[82]. García-Olmo *et al.*[83] showed that plasma isolated from patients with colon cancer is able to transform NIH-3T3 cells and that these cells can form carcinomas when injected into non-obese diabetic-severe combined immunodeficient mice[83]. Whether this biological function of circulating DNA has relevance in human blood is an aspect to be considered in the future.

cfDNA assay issues

One of the problems in evaluating cfNA is the standardization of assays, such as isolation technologies, standards, assay conditions, and specificity and sensitivity[7]. It remains controversial whether plasma or serum is the optimal sampling specimen. The diversity of protocols and reagents currently in use impedes the comparison of data from different laboratories.

The pre-analytical phases of cfDNA analysis such as blood collection, processing (plasma and serum), storage, baseline of patients, diurnal variations and accurate clinical conditions need to be better defined before comparisons and clinical utility can be validated[84]. A major technical issue that hampers consistency in all the cfDNA assays is the efficacy of the extraction procedures, with only small amounts of DNA obtained from plasma and serum. Another key issue is quantification before assessment on specific assay platforms. Improvement is needed in these aspects for cfDNA analysis to be more robust, consistent, comparative and informative. Extraction of cfDNA can be carried out in accordance with many methods; for example, commercial kits, company in-house procedures or individual laboratory protocols. To date no approach has been truly developed that is consistent, robust, reproducible, accurate, and validated on a large-scale patient and normal donor population. If these issues were solved a better universal standardization for the comparison of results would be provided and the clinical utility of the assays could be addressed. The development of a direct DNA assay without extraction would override many of these problems[30]. As new approaches in the assessment of cfDNA, such as next-generation sequencing, are being developed, the issue of extraction of DNA will continue to complicate cfDNA biomarker assay development and regulatory group approval.

The other major issue for cfDNA assessment is that after DNA extraction, different platform assays are used for analysis. This can vary owing to the type of cfDNA being analysed, assay sensitivity and specificity, and analytical approach. These variables are important and need to be standardized for consensus analysis and reporting. The development of PCR-based assay standardization is needed in order to report clinical and prognostic biomarker results that are similar to those outlined in the recent minimal information for the publication of quantitative real-time PCR guidelines[85]. However, this may take time to reach an international consensus, as has been apparent with the standardization of other cancer blood biomarkers. Unfortunately, the rate of approval of new cancer blood biomarkers over the past decade has been very slow. New regulatory guidelines, such as those listed for tumour biomarkers in clinical practice by the National Academy of Clinical Biochemistry (NACB USA)[84], should help to resolve some of these issues. The NACB website provides up-to-date informative detailed guidelines with references of pre-analytical and post-analytical phases, assay validation, internal quality controls, proficiency and requirements for minimizing the risk of method-related errors for biomarkers. Nevertheless, as with other types of biomarkers, new regulatory guidelines mean that developing cfNA biomarkers will be more time-consuming and costly.

Circulating cfRNA

mRNA content. Besides the quantification of cfDNA, circulating gene transcripts are also detectable in the serum and plasma of cancer patients. It is known that RNA released into the circulation is surprisingly stable in spite of the fact that increased amounts of RNases circulate in the blood of cancer patients. This implies that RNA may be protected from degradation by its packaging into exosomes[86], such as microparticles, microvesicles or multivesicles, which are shed from cellular surfaces into the bloodstream[87]. The detection and identification of RNA can be carried out using microarray technologies or reverse transcription quantitative real-time PCR[88].

Serum thyroglobulin levels are a specific and sensitive tumour marker for the detection of persistent or recurrent thyroid cancer. Levels of thyroglobulin change during thyroid hormone-suppressive therapy, as well as after stimulation with thyroid-stimulating hormone, and the levels correlate well with disease progression. The measurement of mRNA levels of thyroid-specific transcripts might be useful in the early detection of tumour relapse[89]. However, another study has shown that the detection of circulating thyroglobulin mRNA one year after thyroidectomy might be of no use in the prediction of early and midterm local and distant recurrences of this disease[90].

In patients with breast cancer, levels of *CCND1* mRNA (encoding cyclin D1) identified patients with poor overall survival in good-prognosis groups and patients who were non-responsive to tamoxifen[91]. Nasopharyngeal carcinoma has been associated with disturbances in the integrity of cell-free circulating RNA, suggesting that the measurement of plasma RNA integrity may be a useful biomarker for the diagnosis and monitoring of malignant diseases[92]. Several groups have tried to detect human telomerase reverse transcriptase (*TERT*) mRNA in plasma, and have not found any association between the presence of this mRNA and clinicopathological parameters[7].

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By contrast, Miura *et al.*[93] measured *TERT* mRNA together with *EGFR* mRNA levels in serum from patients with lung cancer and showed that *TERT* concentration correlated with tumour size, the presence of metastasis, disease recurrence and smoking. An increase in the concentration of *EGFR* mRNA correlated with advanced clinical stages, and decreased levels of *EGFR* and *TERT* were evident after surgery[93]. These findings show that although the use of mRNA has to be further assessed in large clinical trials, these data seem promising.

miRNA content. Currently, expression microarrays that cover >900 mature human miRNA sequences listed in the miRNA database (miRBase) allow the screening of deregulated transcript levels of miRNAs in different tumour tissues (BOX 2). Subsequently, the aberrant expression levels of miRNAs deduced from the array data can be examined by quantitative real-time PCR in single miRNA assays. The application of these techniques has shown conflicting quantitative data on the upregulation of circulating miRNAs from the same tumour type in different studies[94].

These discrepancies might mainly be due to the lack of an established endogenous miRNA control to normalize miRNA amounts. Indeed, a recent study[95] indicated the need for such a control. *mir-16* or the small nucleolar RNA *RNU62* are frequently used as reference genes[96], but others have argued that all tested miRNAs should have established mean expression levels to reduce the technical variation in the miRNA isolation and to more accurately assess the biological changes[97]. However, this approach is only applicable if the control miRNAs are well studied in relevant defined populations. The expression profile of blood miRNAs may change with respect to the established risk factors of the cancer patients and whether the blood samples were drawn before or after treatment, surgery or chemotherapy[94]. Therefore, for each study, the candidate reference miRNAs should be rigorously validated, as even frequently used reference miRNAs are variable under different physiological conditions or patient and donor demographics. This area is in need of universal standards to allow better comparisons and validations of specific blood miRNAs. This will continue to be a problem if the extraction of miRNAs from blood is variable from one sample to another. However, it has recently been shown that a direct miRNA assay of serum without extraction may simplify the problem and improve overall assay comparison[98]. The crucial problem is the extraction of small amounts of miRNA from plasma or serum, which is highly variable among different published papers. Because of the small size of the miRNAs and their attachment to lipids and proteins, efficient and reproducible extraction remains an inherent problem.

Nevertheless, on the basis of their biological role and involvement in transforming cells, circulating miRNAs may have potential as diagnostic, prognostic and predictive biomarkers and may also be considered as potential future therapeutic targets[94]. Although the analysis of circulating miRNAs has just begun, there are indications that circulating miRNAs may become promising biomarkers, particularly because of the strong link between their deregulation and cancer development and progression.

In 2008, the presence of miRNAs in serum was first described for patients with diffuse large B cell lymphoma[99]. miRNA expression profiles have since been shown to have signatures that are related to tumour classification, diagnosis and disease progression[95,100–102] — patients with breast cancer with advanced stage disease had significantly more miR-34a in their blood than patients at early tumour stages, and changes in miR-10b, miR-34a and miR-155 serum levels correlated with the presence of metastases[101]. Recently, an assay for circulating miR-21 was shown to be useful in the detection of early stage breast cancer progression[103], and in non-small-cell lung cancer (NSCLC) serum miRNA levels were found to be altered more than fivefold between patients with longer and shorter survival[104].

The developmental lineage and differentiation state of various tumour types might be reflected by the miRNA signature[105]. For example, detection of miR-92 in plasma could differentiate patients with colorectal cancer from patients with gastric cancer[102]. During liver development the expression of particular miRNAs has been reported to change dynamically, and one of these miRNAs, miR-500, is an oncofetal miRNA that is relevant to the diagnosis of human hepatocellular carcinoma[106].

In summary, the findings discussed above highlight the potential clinical utility of circulating miRNA profiling in cancer diagnosis. Considering the clinical relevance of miRNAs in cancer tissues, this field will inevitably grow.

Box 2 | **Characteristics of miRNAs**

MicroRNAS (miRNAs) are a class of naturally occurring small non-coding RNA molecules. Mature miRNAs consist of 19 to 25 nucleotides and are derived from hairpin precursor molecules of 70–100 nucleotides. As 50% of human miRNAs are localized in fragile chromosomal regions, which may exhibit DNA amplifications, deletions or translocations during tumour development, their expression is frequently deregulated in cancer[121]. Therefore, miRNAs have important roles in the regulation of gene expression in cancer[122]. To date, studies on solid cancers (ovarian, lung, breast and colorectal cancer, for example) report that miRNAs are involved in the regulation of different cellular processes, such as apoptosis, cell proliferation, epithelial to mesenchymal transition and metastasis. In blood, miRNAs seem to be highly stable, because most of them are included in apoptotic bodies, microvesicles or exosomes and can withstand known mRNA degradation factors[94,103].

cfDNA and micrometastatic cells

Besides numerous studies on the clinical utility of circulating tumour cells (CTCs) in blood, disseminated tumour cells (DTCs) in the bone marrow and cfNA in the blood of cancer patients[107–109], the investigation into combined analyses of circulating nucleic acids with CTCs or DTCs has just begun. The observed correlations of cfDNA with CTCs and DTCs suggest that cfDNA may be derived not only from the primary tumour but also from micrometastatic cells[13,14,110–114].

In a study of primary head and neck squamous cell carcinoma, microsatellite alterations in serum DNA have been reported to predict distant metastasis. In this report it was also shown that CTCs may contribute to the presence of cfDNA that is detected by microsatellite analysis[110]. In prostate cancer, the presence of CTCs significantly correlated with an increase in the detection of LOH of dematin, *CDKN2A* and *BRCA1* in cfDNA[13].

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The biological relevance of LOH in these regions might, therefore, contribute to a better understanding of the early steps of the metastatic cascade in this carcinoma type. Interestingly, in patients with prostate cancer who had tumour cells in their bone marrow, the frequency of LOH that was detected using cfDNA from the bone marrow plasma increased compared with patients who did not have DTCs in their bone marrow. These data suggest that tumour-specific cfDNA is also present in the bone marrow and indicate a possible relationship with bone marrow micrometastasis[14]. In breast cancer, there was no significant correlation between the presence of DTCs in the bone marrow and LOH of *CDKN2A*, *PTEN*, *BRCA1*, *BRCA2* and E-cadherin (*CDH1*) in cfDNA from blood samples[33]. Presumably, this lack of concordance is caused by the restricted set of microsatellite markers used. However, patients with DTC-positive bone marrow had higher DNA yields in their blood than patients with DTC-negative bone marrow[33], indicating that tumour cfDNA may at least partly stem from DTCs.

An association between CTCs and serum tumour-related methylated DNA has also been observed. Patients with melanoma who had CTCs and methylated *RASSF1A* and *RARB* in their blood showed a significantly poorer response to chemotherapy and a shorter time to progression, as well as poorer overall survival[111]. These findings indicate that a combined assessment of methylated cfDNA and CTCs in blood may be a useful determinant of disease status and the efficacy of systemic therapy in patients with melanoma. In patients with breast cancer, the detection of large amounts of methylated cfDNA correlated with the presence of CTCs in the blood[114]. Based on an association of cell-free, methylated *APC*, *RASSF1A* and *ESR1* molecules with CTCs, it has been suggested that CTCs are a potential source of circulating tumour-specific DNA, and that high numbers of CTCs and methylated cfDNA molecules are both a phenotypic feature of more aggressive breast tumour biology[114]. In this regard, the association of cell-free, methylated *APC* and *GSTP1* molecules with CTCs in the blood of patients with breast cancer correlated with a more aggressive tumour phenotype and an advanced disease stage[112].

Although the findings discussed above are still preliminary, they emphasize that cfDNA may also stem from CTCs that have undergone cell death when in the circulatory system.

Conclusion and perspectives

Carcinogenesis and tumour progression are complex and progressive processes that are associated with numerous genetic and epigenetic alterations, some of which can also be detected as cfNA in plasma and serum. Although there are cancer protein blood biomarkers that have been approved by the American Society of Clinical Oncology, their number and clinical use are limited. More studies are needed in large cohorts of cancer patients with well-defined clinical staging and outcomes. The cfNAs might be excellent blood cancer biomarkers, as they may be more informative, specific and accurate than protein biomarkers. Currently, efficient management of cancer patients relies on early diagnosis, precise tumour staging and monitoring of treatment. Histological evaluation of tumour tissues obtained from biopsies, as well as blood samples, are the 'gold standard' of diagnosis, but most studies usually carry out these evaluations once only. We now know that metastatic and primary tumours from the same patient can vary at the genomic, epigenomic and transcriptomic levels, thus assays that allow the repetitive monitoring of these events using blood samples would be more efficient in assessing cancer progression in patients from whom tumour tissue is not available[11,111,115,116]. Minimally invasive blood analyses of cfNA may have the potential to complement or replace the existing cancer tissue and blood biomarkers in the future.

One crucial factor in the continued development of cfNA biomarkers is addressing technical issues such as cfNA extraction (as described above) and rigorously following the guidelines of the NACB USA. This will be a major task that will require cooperation among the leading groups in the world in this field to obtain a consensus on assays and reporting results. Our recommendation is to develop a task force with expertise in cancer cfNA. As many of the new approved targeted therapies are focused on DNA aberrations, such as mutations in the *KRAS*, *BRAF* and *EGFR* genes, the investment by pharmaceutical and biotechnology companies into specific cfNA assays is likely to be highly important, particularly in monitoring drug responses. As the individual genomic profiles of a patient's tumour become more readily available, the use of cfNA assays can be better exploited for personalized medicine and for monitoring treatment efficacy.

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Competing interests statement
The authors declare no competing financial interests.

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