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.

**Institute of Tumour Biology, Center of Experimental Medicine, University Medical Center Hamburg-Eppendorf, Hamburg 20246, Germany.*
‡Department of Molecular Oncology, John Wayne Cancer Institute, Santa Monica, California 90404, USA.
Correspondence to K.P.
e-mail:
pantel@uke.uni-hamburg.de
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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

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

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

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

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].

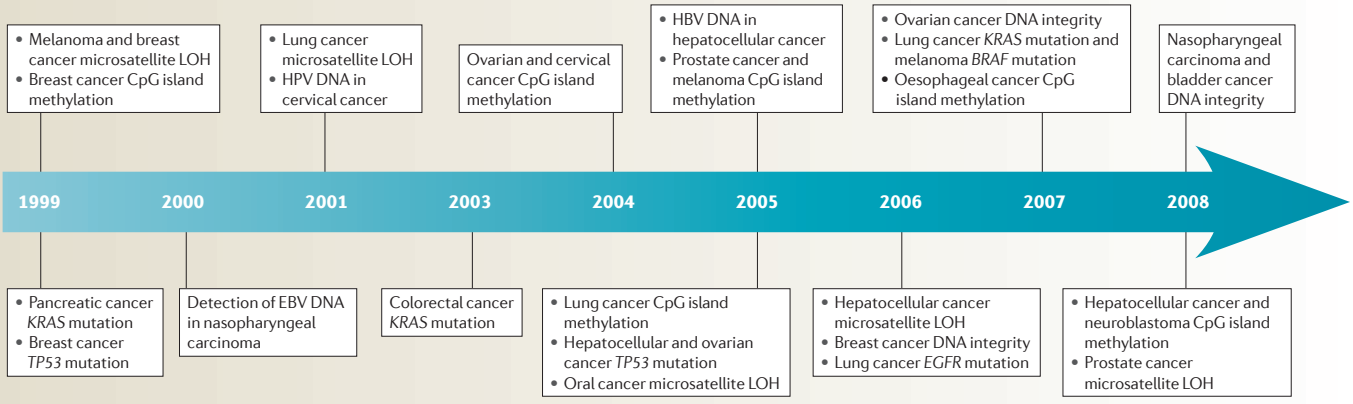
Tumour-specific LOH. Genetic alterations found in cfDNA frequently include LOH that is detected using PCR-based assays[13,18,33–38] (TABLE 1). Although similar plasma- and serum-based LOH detection methods have been used, a great variability in the detection of LOH in cfDNA has been reported. Despite 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.

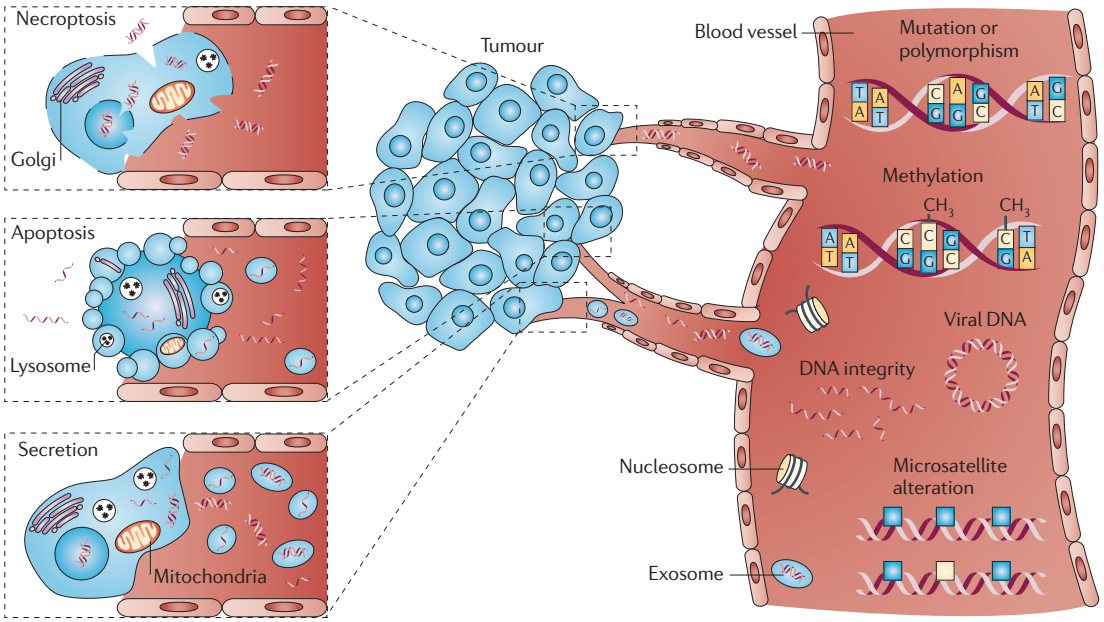


Figure 1 | **Cell-free nucleic acids in the blood.** Mutations, methylation, DNA integrity, microsatellite alterations and viral DNA can be detected in cell-free DNA (cfDNA) in blood. Tumour-related cfDNA, which circulates in the blood of cancer patients, is released by tumour cells in different forms 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 assays, and this might be applicable to personalized medicine, rather than diagnostic screens that can be used across a wide group of cancer patients.

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

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

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

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