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Diagnostic potential of circulating nucleic acids for oncology

Carsten Goessl

Approximately a decade ago, the PCR-based detection of extracellular, tumor-derived circulating nucleic acids in the plasma and serum of cancer patients was introduced as a noninvasive tool for cancer detection. Although the test criteria, sensitivity and specificity, compare favorably with conventional diagnostic measures, until now the methodical ponderousness of circulating nucleic acids in plasma and serum analysis prevented it from becoming a clinical routine application. However, with rapid technical improvement towards automated high-throughput platforms, it is expected that the next 5 years will see circulating nucleic acids in plasma and serum analysis integrated into the initial diagnosis and follow-up monitoring of cancer patients. The hope is that the use of circulating nucleic acids in plasma and serum as a molecular tumor marker and potential profiling tool will finally translate into a longer survival and better quality of life for cancer patients.

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Novartis Pharma AG,
BU Oncology,
WSJ 27.2.055,
Lichtstr. 15,
4002 Basel, Switzerland
Tel.: +41 613 244 271
Fax: +41 613 242 034
carsten.goessl@pharma.novartis.com

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Noninvasive nucleic acid-based molecular diagnosis of cancer using body fluids has emerged rapidly over the last 12 years. The first of these reports dealt with the detection of urothelial cancer by DNA-based analysis of exfoliated bladder tumor cells in voided urine [1]. In selected cases it has been repeatedly demonstrated that molecular detection of tumor cells in body secretions can precede conventional diagnosis of primary and recurrent malignancy [2,3]. Consequently, molecular tumor detection methods have been ascribed a great potential to impact on future therapeutic management of cancer patients [4].

Beyond excreted body fluids such as urine and sputum, blood constitutes an attractive reservoir for nucleic acid-based detection of cancer [4]. Blood can be drawn in a minimally invasive manner (classified thereafter as noninvasive) and this enables repeated testing, a feature especially useful for longitudinal monitoring of responses to therapy and during follow-up of oncologic patients. Blood consists of two main fractions: a cellular and a plasma/serum fraction. Nucleic acid-based cancer detection analyzing the cellular blood fraction for circulating tumor cells has been extensively described elsewhere [5,6]. The focus of this review is the detection of cancer patients by analyzing their plasma/serum fraction for the existence of circulating extracellular nucleic acids (both DNA and RNA) derived from tumoral sources. Cell-free circulating nucleic acids in the plasma and serum (CNAPS) of cancer patients have been investigated extensively as evidenced by more than 200 articles on the subject. A recent review has summarized detailed results on colorectal, pancreatic, breast, lung and prostate cancer, and has concluded that detection and quantification of viral DNA in virus-associated malignancies such as nasopharyngeal carcinoma (associated with Epstein–Barr virus [EBV]) might be closest to broad clinical application [7–8].

This review is structured into four sections: first, the medical context for the detection of tumor-derived CNAPS; second, a brief description of the methodology used and the obstacles observed; third, a selection of the results obtained with different detection methods in various cancer entities; and finally, a 5-year outlook into the future, taking into account competing methods for noninvasive cancer detection in blood samples.

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CNAPS: from plant physiology to oncology

The existence of circulating, noncellular nucleic acids (both RNA and DNA) was first described more than 50 years ago [9]. These findings remained largely unnoticed by the oncological community until in the late 1970s, when elevated amounts of DNA were characterized in the serum of cancer patients [10]. Successful therapy was associated with a decrease in serum DNA quantities, however, benign diseases, especially autoimmune disorders, had also been found to be associated with increased amounts of circulating DNA [11]. As a consequence, the malignant versus benign origin of the increased DNA concentrations found in the plasma/serum of cancer patients remained unknown.

A group of Swiss plant physiologists associated with Philippe Anker and Maurice Stroun was the first to characterize malignant features of CNAPS in cancer patients [12]. This group had formerly investigated nucleic acid release from bacteria into plants [13], from frog auricles and from human lymphocytes [14]. With another group [15], they further established the malignant nature of CNAPS in cancer patients by demonstrating gene mutations matching those of their primary tumors [16–17]. Thus, as illustrated in FIGURE 1, translating the general biological phenomenon of DNA release from plant physiology to human oncology led to the establishment of a noninvasive molecular tumor detection method which has resulted in more than 200 articles on the subject to date [7].

The initial targeting of genomic tumor DNA in CNAPS analysis has now been expanded to circulating mitochondrial DNA, RNA and tumor-associated viral nucleic acids. The related use of CNAPS in nonmalignant indications [18], such as prenatal diagnosis, transplant rejection and infectious diseases, will not be covered by this review. CNAPS have been found to be particle-associated (see Methods). The use of quantitative detection methods for CNAPS has emerged rapidly since 1999 and has further increased the diagnostic utility of this method [19–23]. However, despite favorable test characteristics compared with conventional serum tumor markers and other molecular detection methods, so far CNAPS analysis has not left the research stage. Handling of the very small quantities of nucleic acids isolated from plasma or serum and dependence on comparably laborious PCR amplification processes are regarded as the main obstacles precluding this method from becoming clinical routine.

Genometastasis hypothesis

A horizontal transfer of circulating tumor DNA into tissues has been demonstrated in animal experiments and raises questions on the possible functional relevance of this so-called genometastasis, as opposed to conventional cellular metastatic spread [24]. Hypothetically, under the assumption that horizontal transfer of tumor DNA sequences has a transforming potential [25,26], stem cells in distant organ tissues would constitute possible targets for uptake.

The other plasma: cell-free tumor DNA in bone marrow supernatants

According to a recent report by Taback and coworkers, tumor DNA displaying tumor-specific genetic alterations is present in the plasma (supernatant) of bone marrow aspirates from patients with early breast cancer [27]. The sensitivity associated with this approach was determined to be superior to analysis of circulating tumor DNA in the blood serum of the same patients. Due to its invasive nature, bone marrow analysis is not the focus of this review, although the work by Taback clearly demonstrates that analysis of cell-free tumor DNA begins to enter fields considered to be the domain of RNA-based detection of micrometastatic tumor cells [6].

Methods

Isolation of nucleic acids

The first step in CNAPS analysis consists of DNA isolation from plasma or serum. Careful two-step centrifugation has been recommended to ensure complete cell removal [28]. Whereas higher absolute DNA concentrations have been observed in serum [29], the relative proportion of tumor DNA tends to be higher in plasma [30]. *In vitro* release of benign DNA from leukocytes during the coagulation process has been proffered as an explanation for this observation [30,31]. There is no agreement on the most efficient method of DNA isolation from plasma/serum. The commercially available DNA isolation kit from Qiagen (Venlo, The Netherlands) is widely used [31] and has compared favorably with manual isolation methods [32], although the superiority of that kit has been challenged by other authors [33]. Quantities of CNAPS have been determined using different methods yielding DNA concentrations of

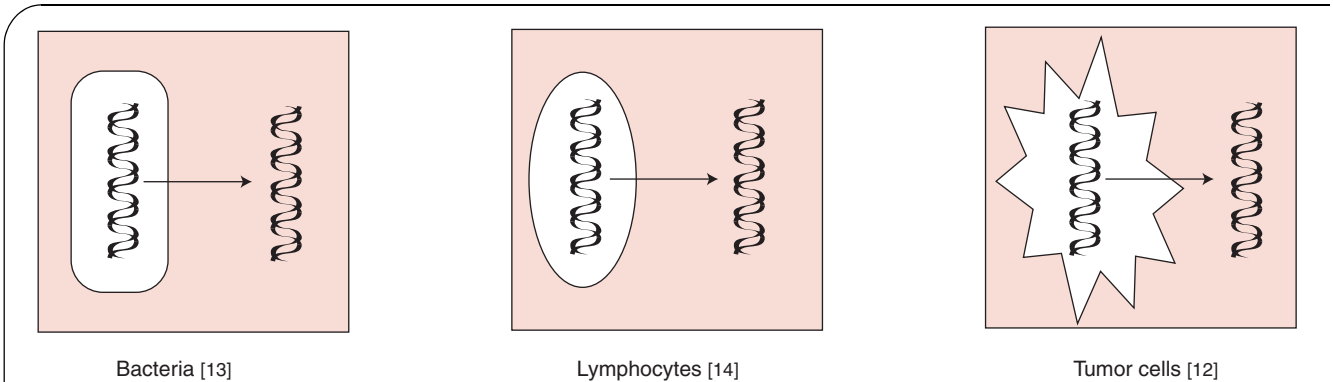

Figure 1. Cellular DNA release: from bacteria to use as a molecular tumor marker in humans.

2–30 ng/ml in healthy controls [11,16,34–36] and 20–200 ng/ml in cancer patients [16,34–36]. However, absolute DNA amounts in plasma/serum do not reliably differentiate between cancer patients and controls, especially not in those with autoimmune diseases [11]. Fournie and coworkers found a correlation between advanced tumor stage and absolute DNA concentrations in lung cancer patients [35], however, no such associations were found in various other tumor entities including lung cancer [31,34]. The relative proportion of tumor DNA within whole plasma/serum DNA is quite variable (3–93%) [36]. Colorectal cancer appears to be associated with comparatively low amounts of circulating tumor DNA as evidenced by the findings of Hibi and coworkers [37]. Conversely, in many other tumor types, circulating tumor DNA appears to be greatly enriched in the plasma/serum fraction [16,38]. The reasons for this phenomenon have been attributed to either increased release from malignant sources and/or reduced clearance from the circulation, the latter most likely attributable to its nucleoprotein structure [31,36,39]. The main bulk of DNA fragments in plasma/serum of cancer patients was found to be shorter than 200 bp [36]. Botezatu and coworkers reported on short mutated *k-ras* sequences secreted into the urine of cancer patients, indicating that at least short CNAPS might undergo renal filtration [40]. If confirmed in a larger series of different tumor entities, urine may emerge as a second source for universal molecular diagnosis of malignancy based upon cell-free methodology [18,41].

Recently, cell-free circulating tumor RNA also has been successfully isolated from plasma/serum of cancer patients [42–48]. Given the significant RNAse activity of whole blood, this finding was unexpected. However, as demonstrated by Hasselmann and coworkers, circulating tumor RNA is embedded in apoptotic bodies and thus, as with circulating DNA, is protected against degradation. Although the exact mechanism of tumor DNA and RNA release into the circulation remains unknown, there is accumulating evidence that most tumor-derived CNAPS are from cancer cells undergoing apoptosis [31,36,39].

Circulating tumor DNA: target selection for molecular diagnosis

DNA mutations, microsatellite alterations and epigenetic DNA alterations manifested by gene promoter hypermethylation constitute the three main targets for analyzing circulating tumor DNA in cancer patients. The rationale behind this approach is the expected occurrence of the same alterations in primary tumor tissue. However, most likely due to the existence of heterogeneous tumor clones (and/or undetected micrometastatic spread), alterations found in a small tumor sample do not always match those found in plasma/serum DNA [50–51]. Only the main features of the three most commonly used detection methods for circulating tumor DNA in plasma/serum of cancer patients will be presented.

DNA mutation analysis

Most investigators choose *ras* mutations when analyzing circulating tumor DNA. The existence of *ras* mutations in solid malignancies is especially common in cancers of the pancreas and gastrointestinal tract, where they occur in 90 and 50% of the primary tumors, respectively [7,52]. Since these mutations are clustered in codon 12 of the *k-ras* gene, PCR-based methods have been developed which enrich for common mutation variants in plasma/serum DNA [36,52]. However, the finding that *k-ras* mutations can occur in benign conditions and thus appear to be not entirely tumor-specific, confers some concern about their usefulness as molecular tumor markers. In addition, Ramirez and coworkers found that *k-ras* mutations in primary non-small cell lung carcinomas only rarely matched the consistent mutation pattern (i.e., TGT) in corresponding serum [53]. Prospective, longitudinal studies are needed to eliminate specificity concerns associated with this molecular target [54]. Mutations of the tumor suppressor gene *p53* have been investigated in plasma/serum of patients suffering from hepatocellular, breast and colorectal carcinoma [31], although the methodical workload associated with this approach does not favor routine clinical application.

Recently, mitochondrial DNA mutations have been exploited as a target for analyzing plasma/serum DNA from cancer patients. Mitochondrial DNA is present in approximately 200–10,000 copies per cell and should therefore provide a better sensitivity for detecting critically low quantities of target DNA [31,55]. However, although three out of three prostate cancer patients with mitochondrial DNA mutations in their primary tumor exhibited the same mutations in serum [56], the detection rate in serum from ovarian cancer patients was 0%, perhaps due to the fact that no mutation enrichment method was applied [57]. However, even when using mutation enrichment PCR, the detection rate of tumor-derived mitochondrial DNA in colorectal cancer patients was disappointingly low (14% in primary tumors harboring target mitochondrial mutations) [58]. As mutations of genomic DNA (*k-ras*) can be detected in plasma/serum of more than 80% of colorectal cancer patients harboring the same mutations in the primary tumor [59], the high copy number of mitochondrial DNA does not compensate for decreased release efficacy and/or increased breakdown in plasma/serum of cancer patients.

Results from different tumor entities with various disease stages are deemed necessary to better define the overall diagnostic sensitivity of this approach, irrespective of the problems associated with sequencing the whole or parts of the 16.5 kb mitochondrial genome.

Microsatellite analysis

Generally, microsatellite analysis is easy to perform, however, it requires tumor DNA within DNA originating from benign sources at a ratio of 0.5–5% when targeting microsatellite instability (MIN) and more than 10–20% when targeting loss of heterozygosity (LOH) [16]. Since some tumor entities, such as bladder cancer, are not associated with consistent alterations at characteristic chromosomal locations, a panel of 17 markers was used by von Knobloch and coworkers for detection of tumor DNA in serum [60]. Despite the possibility of using multiplex PCR to reduce the workload, this level of complexity

Table 1. DNA alterations in plasma/serum of cancer patients (at diagnosis, all stages if not otherwise indicated).

Alteration	Neoplasm	Patients (n)	Markers (n)	Alterations in serum/plasma (%)	Specificity (%)	Ref.
Microsatellite alterations						
LOH, MIN	Non-small cell bronchial cancer	21	3	71	100	[16]
LOH, MIN	Lung cancer	34	6	85	100	[51]
LOH, MIN	Neck/head tumors	21	12	29	100	[17]
LOH, MIN	Colorectal cancer	44	8	0 (!)	nd	[37]
LOH, MIN	Colorectal cancer	27	9	59	nd	[78]
LOH, MIN	Clear-cell renal cancer	40	4	65	100	[38]
LOH, MIN	Renal cell cancer	40	20	87	85 (10 markers)	[79]
LOH, MIN	Melanoma	40	10	58	100	[80]
LOH, MIN	Breast cancer	21	7	48	100	[61]
LOH, MIN	Breast cancer	57	2	30	100	[81]
LOH, MIN	Bladder cancer	39	17	85	100	[60]
LOH (allelic imbalance)	Ovarian cancer	54	8	93	100	[64]
Gene mutations						
N-ras	Hematologic neoplasms	10	1	50	Nd	[82]
Ig chain DNA	B-cell leukemia	110	1	86	100	[83]
k-ras	Colorectal cancer[§]	8 (adenoma: 62)	1	63 (adenoma: 35)	86[§§]	[54]
k-ras	Colorectal cancer	14	1	50	100	[59]
k-ras	Colorectal cancer	69 (adenoma: 9)	1	41 (adenoma: 44)	100	[52]
k-ras	Pancreatic carcinoma	21	1	81	100	[76]
p53	Hepatocellular cancer	20	1	30	100	[84]
Gene promoter hypermethylation[§§§]						
p16, *MGMT*, *GSTP1*, DAP kinase	Non-small cell bronchial carcinoma	22	4	52	Nd	[85]
p16 (Quant)	Hepatocellular carcinoma	22	1	59	100	[23]
p16	Breast cancer	43	1	14	100	[86]
p16, *MGMT*, *GSTP1*, DAP kinase	Neck/head tumors	50	4	42	100	[87]
APC (Quant)	Esophageal cancer	84	1	18	100	[20]
APC (Quant)	Lung cancer	89	1	47	100	[21]
GSTP1	Prostate cancer	32	1	72	100	[88]
GSTP1 (Quant)	Prostate cancer (early stage)	69	1	32	100	[22]

Table 1. DNA alterations in plasma/serum of cancer patients (at diagnosis, all stages if not otherwise indicated) (cont.).

Alteration	Neoplasm	Patients (n)	Markers (n)	Alterations in serum/plasma (%)	Specificity (%)	Ref.
Mitochondrial DNA mutations						
	Prostate cancer (early stage)	3	OML	100	Nd	[56]
	Ovarian cancer	14	Seq	0	Nd	[57]
	Colorectal cancer	77 (7[§§§§])	OML[§§§§]	1 (14[§§§§])	Nd	[58]
	Hepatocellular carcinoma	10[§§§§]	OML	80[§§§§]	Nd	[89]

[§]Prospective analysis: plasma DNA samples were taken before colonoscopy and pathohistological confirmation (screening situation).
[§§]Control group consisted mainly of symptomatic or high-risk patients. [§§§]Review: [66]. [§§§§]Only patients with confirmed mitochondrial mutations in their primary tumor.
APC: Adenomatous polyposis coli gene; DAP: Death-associated protein; GSTP1: Glutathione-*S*-transferase P1 gene; Ig: Immunoglobulin; LOH: Loss of heterozygosity; MGMT: O6-methylguanine-DNA methyltransferase gene; MIN: Microsatellite instability; Nd: Not determined; OML: Oligonucleotide mismatch ligation assay (mutation enriching PCR technique); Quant: Quantitative determination using real-time methylation-specific PCR; Seq: Determination by sequencing serum DNA.

raises concerns regarding the practicality of broad clinical application in CNAPS analysis. In addition, microsatellite analysis using small DNA concentrations, such as those isolated from plasma/serum, is prone to technical artifacts [61–62]. Longer alleles (>200 bp) are more likely to display false-positive LOH than their corresponding shorter counterparts [63]. In CNAPS analysis, the underlying difficulty to amplify longer microsatellite sequences can be ascribed to the highly fragmented nature of plasma/serum DNA [36]. Although true-positive LOH findings in plasma/serum DNA can usually be confirmed by comparison with findings in primary tumor tissue and by use of additional neighboring markers [38], specificity concerns, amongst others, are prohibiting broader routine clinical use of this method. According to Chang and coworkers, allelic imbalance in plasma/serum DNA can be analyzed using digital single-nucleotide polymorphism analysis without the potential risk of preferential amplification of shorter microsatellite alleles [64]. This method is, however, associated with additional workload, leaving its suitability for routine CNAPS diagnosis doubtful.

Gene promoter hypermethylation analysis

Gene promoter hypermethylation has become an attractive target for molecular cancer detection as promoter hypermethylation often constitutes an early neoplastic change, occurring even in premalignant or morphologically yet benign lesions [65]. A comprehensive review on the use of gene promoter hypermethylation analysis to detect exfoliated tumor cells in secreted body fluids as well as circulating tumor DNA in blood fractions from cancer patients was recently published by Laird [66]. Detection of tumor-associated gene promoter hypermethylation in plasma/serum DNA by methylation specific PCR (MSP) [67] significantly enhances the analytical sensitivity compared with microsatellite analysis. The methodical sensitivity of such assays equals approximately 1×10^4 [67] to 1×10^5 [68]. Since three methylated sites are usually targeted by each of the corresponding two MSP-primers, false-positive results due to technical artifacts are not considered to be of concern. Age-related gene promoter hypermethylation (e.g., of *APC* and *p16* in normal gastric mucosa) is a well-known event with possible implications for analysis of plasma/serum DNA [20]. Fortunately, because normal tissues affected by gene promoter hypermethylation apparently do not secrete DNA into the blood circulation, this problem appears to be theoretical in nature as *p16* and *APC* gene promoter hypermethylation has not yet been observed in the serum/plasma of controls [20,66]. To further protect against false-positive scoring, suitable cut-off levels can be defined when using quantitative MSP for tissue and/or plasma/serum samples [20–23]. Quantification of promoter hypermethylation provides a tool to distinguish low-level hypermethylation due to age-related processes and/or biological noise from high-level hypermethylation associated with neoplastic growth.

Circulating tumor RNA

The same rationale applies for analysis of circulating tumor cells as for analysis of circulating tumor RNA [5,6]. It is assumed that transcripts of epithelial origin do not normally occur in the blood, thus their existence is thought to indicate malignancy which has gained access to the circulation. This concept has been challenged by the observation of illegitimate transcription; the secretion – albeit at low levels – of epithelial cell transcripts by any cell including normal blood cells and/or endothelial cells. Certain transcripts used as tumor targets are more likely to be produced by normal nonepithelial cells (e.g., albumin and CK-19) than others whose occurrence is known to be more closely tumor-associated [69].

Circulating viral nucleic acids

In addition to tumor nucleic acids from endogenous sources, analysis of tumor-associated viral DNA has emerged rapidly as a molecular tool for initial diagnosis and follow-up monitoring in defined populations of cancer patients. Chan and coworkers found that patients with EBV-associated nasopharyngeal carcinoma had persistently elevated EBV-DNA levels in their plasma/serum, whereas patients after recovery from benign mononucleosis (caused by EBV) did not [19,70]. Quantitative determination of EBV-DNA also yielded prognostic information and decreasing levels were strongly correlated with favorable response to therapy [70]. Determination of viral DNA levels is

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