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LABORATORY–CLINIC INTERFACE

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Circulating DNA: a new diagnostic gold mine? ☆

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The recent discovery that cell-free DNA can be shed into the bloodstream as a result of tumour cell death has generated great interest. Numerous studies have demonstrated tumour-specific alterations in DNA recovered from plasma or serum of patients with various malignancies, a finding that has potential for molecular diagnosis and prognosis. The implication is that tumour-derived nucleic acids of human or viral origin can be retrieved from blood by a minimally invasive procedure, and used as a surrogate tumour marker to monitor the course of the disease or aid in early diagnosis. The present review will describe the main areas of ongoing investigation, with particular emphasis on technical issues and available data of clinical relevance.

Key words: Plasma DNA; cancer diagnosis; disease management; noninvasive approaches.

INTRODUCTION

In biomedical research as well as in other areas, the past decades have been marked by an impressive advancement of technology. In the case of molecular biology, the development of sensitive techniques for the detection of minute amounts of nucleic acids has provided powerful tools for molecular analysis. Among the numerous applications, molecular biology has proven particularly useful for the diagnosis of human disease based on the detection of genetic anomalies or foreign DNA sequences. However, the great sensitivity provided by current methods is not sufficient to overcome inherent limitations, such as the need for adequate testing material. Ideally, diagnostic specimens should be easily accessible by a minimally invasive procedure to limit patient inconvenience and risk. Although blood and urine have proven useful for numerous diagnostic applications, the molecular diagnosis of diseases such as cancer often require tumour biopsies gained by invasive methods. With the exception of haematological malignancies, cells recovered from blood have not yielded the necessary specificity for the routine use in disease management of patients with solid tumours. For these reasons, the finding of circulating cell-free DNA in the blood of healthy and diseased individuals has gained increasing attention during the last years. The present review will describe the recent developments in serum and plasma DNA research, with particular emphasis on current methodologies and potential diagnostic applications in cancer disease management.

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Cell-free DNA

The presence of extracellular nucleic acids in the human bloodstream was first described in 1948 by Mandel and Métais (1). Using a quantitative technique, the authors detected circulating RNA and DNA in the plasma of healthy and diseased individuals. Despite the innovative nature of this work, little attention was drawn to the findings until 1966, when Tan *et al*. (2) demonstrated the presence of

DNA in the serum and plasma of patients afflicted by systemic lupus erythematosus. Since DNA could also be detected in other diseases associated with tissue destruction (hepatitis, metastatic carcinoma, and miliary tuberculosis), the authors proposed that serum DNA might originate from endogenous tissue breakdown. Several years later, Leon *et al*. (3) reported the presence of DNA in the serum of cancer patients. For the first time, DNA amounts were quantitated using a sensitive radioimmunoassay based on anti-DNA antibodies obtained from lupus erythematosus patients. Compared to healthy controls, 50% of cancer patients presented elevated serum DNA levels. Furthermore, a partial correlation was noted between the persistence of elevated serum DNA levels and the lack of response to therapy. In the following years, three further reports described the presence of circulating DNA in patients with different malignancies (4–6).

First evidence supporting the tumour origin of plasma DNA in cancer patients was provided in the late eighties (7). The work relied on physical and biological properties of DNA that differ in normal tissues compared to tumours. However, the final proof that tumours can shed DNA into circulation came from two studies which detected oncogenic N-*ras* mutations in the plasma of patients with myelodysplastic syndrome (MDS) and acute myelogenous leukemia (AML) (8), and K-*ras* mutations in the plasma and serum of patients with pancreatic adenocarcinoma (9), respectively. Since the publication of these studies, numerous groups have demonstrated the presence of genetic and epigenetic alterations in the plasma and serum of cancer patients, although the sensitivity and specificity of the various methods utilized has shown great variability. This issue will be addressed in more detail later, as well as the additional research areas searching for diagnostic applications of circulating nucleic acids.

The origin of plasma and serum DNA

The early suggestion that circulating DNA is of endogenous origin is now widely accepted (2,6). However, the precise mechanism by which DNA is released into the bloodstream remains uncertain. Initial experiments indicated that DNA recovered from plasma consists of double-stranded fragments with sizes ranging from 21 kb to less than 0.5 kb (4,6). Later work showed that DNA circulates mainly in the form of mononucleosomes (10), a nucleo-protein structure that likely protects DNA from degradation. These physicochemical characteristics suggest that plasma DNA might originate from internucleosomal cleavage of chromatin, a major hallmark of the programmed cell death process known as apoptosis (11). The correlation of plasma DNA levels with known markers of cell death in lung cancer patients is in agreement with this notion (12), and similar conclusions were drawn after treatment of mice with agents that selectively induce necrosis or apoptosis of the liver (13). In the latter case, DNA fragments recovered from plasma were consistent with release from either apoptotic or necrotic cells. Moreover, the same DNA patterns were identified in the plasma of several cancer patients.

An alternative mechanism was suggested by an early report that described the release of DNA from activated lymphocytes (14). Although this possibility has not been examined in detail, it raises a second relevant question, namely which are the cells able to shed DNA into circulation. Obviously these are not limited to tumours and other diseased tissues, since cell-free DNA can be recovered from the plasma and serum of healthy individuals, albeit at low concentrations (3,12). The issue is further complicated by the fact that DNA amounts recovered from serum are several-fold higher than those in matched plasma samples (15–17). A recent report provides evidence for the haematopoietic origin of cell-free DNA (17) in healthy individuals. The authors quantitated Y-chromosome sequences in plasma and serum of patients receiving sex-mismatched bone marrow transplants, and found that circulating DNA was predominantly of donor origin. Concordantly, blood spiking experiments suggested that most cell-free DNA in serum samples is generated *in vitro* by lysis of white blood cells during the process of clotting (16). It was concluded that serum is not suited to monitor the concentration of cell-free DNA. A correlation between increased serum DNA levels and elevated leukocyte counts was also noted in leukemia patients (4).

With regard to the identity of cells shedding DNA into circulation, one study found little contribution of T-cells to the bulk of plasma DNA in cancer patients (13). Interestingly, the fraction of plasma DNA contributed by tumours was variable (3–93% of total circulating DNA). This further undermines the utility of monitoring cell-free DNA concentrations for diagnostic purposes. Comparable observations were made in women pregnant with a male fetus (18). Quantification of the Y chromosome gene SRY revealed similar absolute concentrations in serum and plasma, but serum contained a larger quantity of background maternal DNA compared to plasma. Taken together, the available information suggests that a significant proportion of circulating DNA is of haematopoietic origin in healthy as well as in diseased individuals, and that excess DNA recovered in serum likely originates *in vitro* during the clotting process. Further studies will be necessary to establish

the identity of haematopoietic cells that shed DNA into circulation. Regarding the mechanism of DNA release, current evidence suggests that necrotic and apoptotic cell death are involved, but additional mechanisms might play a role.

Methodology: the need for standardization

During the last 10 years, the interest in potential applications of circulating DNA has grown rapidly. This is evidenced by the increasing number of publications in the main areas of cancer, virology, and prenatal diagnosis. However, direct comparison of the numerous available data is often prevented by differences in the parameters analyzed, and by lack of standardized methodology and analysis procedures. Differences are evident already at the stage of collection of plasma or serum. Several anticoagulants (EDTA, heparin, and lithium-heparin) can be used for plasma, while for serum, clotting has been allowed for variable periods of time. Heating of blood has been used for both plasma and serum. The recent report that single-step centrifugation is not sufficient to obtain cell-free plasma (19) has led to the introduction of additional cell removal steps in newer publications. Further variation is introduced at the stage of DNA purification. The use of the commercial QIAamp Blood Kit (Qiagen) seems currently widespread, although its performance was unfavorably rated when compared to three alternative methods (20). Additional procedures have been used by some groups (15,16,21,22). Quantitative differences in DNA yield depending on the purification method have been reported by at least two studies (16,19). Finally, a third level of variation arises from inherent differences such as the genetic target analyzed and the bias in patient choice, both of which require consistency at the level of study design. Although the general need for standardization is widely acknowledged, little effort has been invested to overcome current sources of variability. It is evident that any future application of plasma or serum DNA analysis for diagnostic purposes will depend on the reproducibility and reliability of results, both of which require the optimization and equivalence of procedures.

CIRCULATING DNA AND CANCER

The majority of reports searching for diagnostic or prognostic applications of cell-free DNA have been devoted to cancer. As explained earlier, a main hindrance to the molecular diagnosis of solid tumours is the frequent need for invasive procedures to obtain adequate testing material. Thus, the easy accessibility of plasma and serum DNA are particularly appealing in this field. To allow a better comparison of results, available data will be discussed according to the type of analysis performed. Due to space constraints and to the large number of publications, representative reports will be chosen.

Quantitative analysis of plasma and serum DNA

Several groups have measured the levels of circulating DNA in cancer patients in search for a potential prognostic application. The quantitative approaches used are a good example for the variability introduced by the use of multiple techniques. These include radioimmunoassays (3,5), a procedure based on nick translation (12), competitive PCR (13), quantitative real-time PCR (23), fluorimetric quantitation (23), spectrophotometric determination (24), and visual comparison with known standards (25). So far, all studies agree in that average DNA levels are significantly elevated in cancer patients compared to healthy controls, irrespective of the use of serum or plasma. However, the absolute amounts measured vary between studies, a difference that might be related to the cancer types analyzed and to the various methodologies used. Absolute DNA levels were lower in studies using plasma than in those analyzing serum, which is in agreement with other published data (15–17).

With regard to clinical significance, at least two reports describe a correlation between DNA levels and known prognostic factors (Table 1). In small cell (SCLC) and non-small cell lung cancer (NSCLC) patients, a close correlation was noted between plasma DNA levels, serum lactate dehydrogenase (LDH) activity and neuron-specific enolase (NSE) concentrations, with similar relationships between each marker and patient survival (12). Similarly, DNA levels correlated with clinical stage, lymph node metastasis and tumour size in breast cancer patients (24). Furthermore, two studies found partial associations between DNA levels and patient outcome: analyzing several malignancies, a partial correlation was noted between the persistence of elevated DNA levels after therapy and a lack of response (3). A similar report described decreased DNA levels in a subset of patients with relapse-free survival during follow-up (25). In patients with gastrointestinal malignancies, simultaneous measurement of DNA and carcinoembryonic antigen (CEA) increased the sensitivity and specificity of both assays (5). Finally, a recent work performed a

Table 1 Summary of studies on circulating DNA in cancer patients

Target analyzed	Cancer type	Source	Patients analyzed	Clinical correlations	Reference
DNA concentration	Various	S	173	Highest levels in metastatic disease; potential to predict therapy outcome	(3)
	Gastrointestinal	S	386	Associated with malignant disease	(5)
	Colorectal	P/S	26		(23)
	Lung	P	68	Correlation with LDH, NSE, and survival	(12)
			84	Unrelated to sex, age, histology and stage; potential to predict therapy outcome	(25)
	Various	P	30	No correlation with clinical parameters	(13)
	Breast	P	126	Correlation with clinical stage, lymph node metastasis and tumour size	(24)
ras Mutations	MDS, AML	P	10		(8)
	Pancreas	P/S	4		(9)
		P	44	Correlation with tumour stage; independent prognostic factor for survival	(27)
			21	No relation to clinicopathological features; related to tumour size and relapse risk	(28)
			21	Potential for early disease detection	(29)
	Colorectal	P	14		(30)
			240	Association with colorectal cancer risk; potential for early disease detection	(22)
		P/S	31	No correlation with sex, tumour and metastasis site, prior surgery or chemotherapy	(31)
	Gastrointestinal	P	35		(32)
p53 Mutations	Breast	P	126	Correlated with tumour size, stage, lymph node metastasis, estrogen receptor status	(24)
	Liver	P	103	Potential for early diagnosis	(33)
			20		(34)
	Colorectal	P	17	Potential to detect small tumours	(35)
Gene hypermethylation	Lung	P/S	89	Independent factor predictive of shorter survival (*APC*)	(44)
		S	22	Present at all stages; unrelated to risk of recurrence (*p16*, *DAP-k*, *GSTP1*, and *MGMT*)	(45)
	Breast	P	35	(*p16*)	(46)
	Liver	P/S	45	Potential for early detection; possibly related to aggressive phenotypes (*p16*)	(47)
			22	(*p16*)	(48)
	Esophagus	S	39	Not associated with tumour size, stage, lymph node metastasis, prognosis (*p16*)	(49)
		P	127	Significant association with disease stage and reduced survival (*APC*)	(50)
	Colorectal	S	19	Potential for early detection of relapse; discrimination of high-risk patients (*hMLH1*)	(51)
	Nasopharyngeal	P	12	Unrelated to sex, age, stage, recurrence, survival (*DAP-k*)	(52)
Microsatellite alterations	Lung	P	43		(56)
			87	Unrelated to sex, tumour type, stage, relapse; potential for early disease detection	(57)
			21	Unrelated to disease stage	(58)
			40	Potential for cancer diagnosis	(59)
	Breast	P	71	Unrelated to nodal involvement, metastasis and tumour site	(60)
		P/S	61	Unrelated to clinical and pathological features	(15)
	Melanoma	P	57	Correlation with stage, survival	(61)
			76	Correlation with stage, disease progression; unrelated to standard prognostic factors	(62)
	Bladder	P/S	40	Unrelated to stage, disease progression	(63)

	S	39	Unrelated to disease stage, tumour grade	(64)
Kidney	S	30	Associated with disease recurrence	(65)
		53	Weak association with advanced tumour stage	(67)
	P	40	Unrelated to tumour stage	(68)
Head and neck	P	91	Unrelated to tumour site, stage	(69)
	S	21	Associated with risk of metastasis	(70)
Multiple				
Lung	P	35	Correlation with survival; potential as predictor of recurrence	(21)
Breast	P	41	Unrelated to pathological parameters; persistence after mastectomy might relate to aggressive disease	(36)
		62	Association with histological features of malignancy	(37)
Colorectal	P/S	69	Potential for disease screening	(38)
	S	44	Potential for clinical prognosis	(39)
Bladder	P/S	55		(40)
	P	27	Associated with multicentric foci, tumour size, relapse ($p < 4$)	(53)
Head and neck	P	117		(41)

Abbreviations used: S, serum; *P*, plasma.

quantitative comparison of matched serum and plasma DNA in patients with colorectal liver metastases (23). Serum and plasma DNA levels were not correlated. Furthermore, while serum DNA was significantly associated with the presence of metastases, only plasma DNA was predictive of recurrence. It was thus concluded that serum DNA might represent an indirect but tumour-related process, and that plasma DNA better reflects the *in vivo* levels of circulating DNA.

Taken together, the existing data supports the existence of significant differences between DNA concentrations in the blood of healthy individuals and that of cancer patients. However, an accurate evaluation is necessary to establish whether DNA levels constitute an independent prognostic factor in light of its correlation with other established markers (e.g., NSE, LDH). Furthermore, it is important to consider that the fraction of circulating DNA contributed by tumours greatly varies among patients (13), which raises the question of what is indeed being measured, in particular in serum (see previous section). At least one study noted a correlation between elevated leukocyte counts and increased serum DNA in leukemia patients (4), and additional evidence supports the haematological origin of at least part of the DNA in circulation (16,17). Since lymphocyte and neutrophil numbers have been reported as independent prognostic factors for survival in NSCLC (26), the association of circulating DNA with blood cell counts should also be addressed. Finally, it has been noted that elevated serum DNA levels can be detected only in a subset of cancer patients (3), and increased levels are not necessarily stage-related (25). This further stresses the need for a precise evaluation to establish the possible utility of DNA concentrations as a prognostic factor in cancer patients. The requirement for standardization of techniques has been discussed in a previous section, and strongly applies for DNA quantification.

Detection of genetic and epigenetic alterations

Most studies on circulating DNA in cancer patients have searched for genetic alterations matching those detected in tumours. Besides detection, a main goal has been the assessment of sensitivity and specificity for the different analyses used. Owing to the large number of publications, data will be discussed according to the genetic targets analyzed.

Mutation of ras oncogenes

The first two reports that described tumour-specific mutations in circulating DNA involved members of the *ras* oncogene family (8,9). In both cases the

number of patients analyzed was small, which precluded the correlation of data with clinicopathological parameters. However, the finding of mutant N-*ras* sequences in the plasma of MDS and AML patients in remission suggested a high sensitivity and potential diagnostic use of the assay, since mutation of *ras* is considered a bad prognostic factor in myeloid disorders. Later studies on mutant *ras* in plasma have been devoted to gastrointestinal malignancies, where *ras* alterations are prevalent. With one exception, the number of patients studied has been rather small (Table 1). A general finding has been the high specificity of the assay owing to the consistent absence of K-*ras* mutations in plasma/serum of healthy control individuals. However, it was not unusual to find K-*ras* mutations in plasma DNA of patients with no corresponding alteration in matched tumour samples (22,31), or in a subset of patients with chronic pancreatitis and no evidence of tumour (27). Rather than a lack of specificity, this has been interpreted as a potential of the technique to detect tumour subclones that gained additional mutations during the process of metastasis, or early mutations in patients at risk for cancer, respectively. Data are less consistent regarding assay sensitivity, with values ranging from 32 to 100% in pancreatic cancer (9,27,28,32), and from 83 to 100% in colorectal cancer patients (22,30–32). Sensitivities of 100% were only observed when the number of patients was very reduced ($\leqslant$10). In some studies, tumour samples were not available for comparison, which precluded the calculation of exact sensitivities. The most consistent values (83–86%) were observed among colorectal cancer studies that included matched tumour and plasma samples and over 10 patients (22,30,32). It is interesting to note that some variability existed in the reported incidence of K-*ras* mutations in colorectal tumours (26–50%), which probably affects sensitivity values and the absolute detection rates of K-*ras* alterations in plasma. In contrast, mutation of K-*ras* was more frequent in pancreatic tumours (71–72%), but detection rates in plasma were notoriously lower. This might be the result of technical differences, or of intrinsic characteristics of each tumour type perhaps affecting the quantities of DNA released into circulation.

With regard to clinical significance, two studies reported a lack of correlation of circulating K-*ras* mutations with a series of clinicopathological parameters (28,31). However, a relation to tumour size, stage, and relapse risk was noted in pancreatic cancer patients (27,28), in which plasma K-*ras* mutations were shown to be independent prognostic factors for survival. Although no such correlations were found for colorectal cancer, plasma K-*ras* mutations were suggested as a potential marker for early disease detection owing to their association with cancer risk (22). Similarly, plasma K-*ras* mutations were identified 5–14 months before clinical diagnosis in a prospective study with pancreatic cancer patients (29). Despite these encouraging results, two studies agreed on the need for additional molecular assays to achieve diagnostic significance (30,32). This seems justified considering that the absolute detection rates of *ras* mutations in plasma ranged from 22 to 43% for colorectal, and from 23 to 83% in pancreatic cancer. Therefore, a significant number of patients will not profit from such measurement and require alternative prognostic evaluation.

Mutations of the p53 tumour suppressor gene

Compared to the analysis of *ras*, the detection of p53 alterations is especially laborious owing to the potential presence of mutations along several exons. Despite this fact, numerous groups have searched for p53 mutations in plasma/serum DNA, probably due to the prevalence of p53 alterations in most malignancies. Detection has been mostly achieved using an initial SSCP approach followed by sequencing, and in general, mutation of p53 in plasma has been analyzed in conjunction with other alterations. Among the exceptions, two reports assessed the status of a single p53 codon in patients with hepatocellular carcinoma related to aflatoxin exposure (33,34). Tumours were available only in one of these studies (34), which reported a 55% sensitivity and 100% specificity for the detection of codon 249 mutations in plasma, although in four cases mutations were present in plasma but not in matched tumours. Furthermore, plasma mutation of codon 249 was detected in 15% of cirrhosis and in 6% of control subjects in the second study (33). This was interpreted as a potential to detect individuals at risk for hepatic cancer, since no mutations were found among 60 control and hepatic carcinoma patients from areas with no risk for aflatoxin exposure. In hepatic cancer patients, the overall presence of aflatoxin-related codon 249 mutations in plasma was close to 30% in both studies.

Plasma p53 mutations as a single marker were further assessed in breast (24) and colorectal cancer patients (35). Although the lack of control subjects precluded the calculation of specificity in the breast cancer study, the data were strengthened by the larger number of patients and by the availability of matched tumour and plasma samples. The overall detection rate of p53 mutations in plasma was 24%, and the sensitivity was 65%. As shown in Table 1, correlations with clinical parameters were found that proved statistically significant. It was concluded that tumour and plasma p53 mutations were significant prognostic factors for relapse-free and

overall survival. Patients with mutations in tumour and plasma had the worst survival, independent of nodal involvement. In the colorectal study (35), specificity was high and sensitivity was 75%, with overall detection rates in plasma of 17%. As in other reports, two plasma samples showed p53 mutations that were not detected in matched tumour samples. The presence of plasma p53 mutations in a patient with a stage B tumour suggests a potential for early disease detection, although the authors classify the method as technically complex, time consuming, and expensive.

Most remaining studies have analyzed p53 mutations in plasma or serum together with other alterations, such as microsatellite changes and gene hypermethylation (Table 1) (21,36–41). As a consequence, the data have usually been evaluated with respect to the whole panel of makers, and conclusions on the value of p53 alone were uncommom. One exception is a study which analyzed p53 mutations as well as loss of heterozygosity (LOH) at the p53 gene locus in superficial bladder carcinoma (40). The data suggests that tumours harboring p53 mutations are at higher risk for recurrence and for progression into muscle-invasive phenotypes. However, the incidence of p53 mutation in tumours was only 13%, and the overall detection of mutations in plasma or serum only 3%. In agreement with others, the authors suggest that future efforts should be directed at multiplex analysis systems. Regarding LOH at the p53 locus, this alteration was more frequent in tumours with higher malignancy but the difference did not attain statistical significance. In the remaining multiplex studies, overall detection rates for p53 mutations in serum/plasma were 37% for lung (SCLC) (21), 5% for breast (36,37), 21% for colorectal (39) and 18% for head and neck cancer (41). In studies with tumour material available, detection in plasma/serum was achieved with sensitivities of 25–85%. However, it is clear from the sensitivity and overall detection rates that despite the predominance of p53 alterations in human malignancies, the analysis of p53 mutations in plasma or serum is technically cumbersome and probably not sufficient per se for prognostic purposes.

Gene hypermethylation

Epigenetic changes such as hypo- and hypermethylation of DNA play an important role in cancer development. Changes in the patterns of methylation have been associated with the altered expression of a number of genes involved in cell cycle control and apoptosis, including p14ARF, $p16^{INK4a}$, APC, H- and E-cadherins, and RASSF1A, among many others (42). The detection of methylated DNA sequences has been greatly facilitated by the introduction of methylation-specific PCR (MSP) (43), a method that appears attractive owing to its relative simplicity, high sensitivity and specificity, and the potential for quantitative measurement of methylation changes. MSP has been the main technique applied for detection of gene methylation in serum/plasma, although some authors have preferred a method based on differential restriction of methylated sequences. Although the methodology seems uniform among the different publications, it is important to note that the amounts of DNA used for bisulfite treatment prior to MSP have varied greatly (100 ng to 2 μg) or have not been specified. Furthermore, it is seldom mentioned how DNA was quantitated. Since most recent studies agree on average DNA yields in the 100 ng/ml range for plasma, great volumes of blood might often be required to obtain 1 μg or more DNA. This seems disadvantageous for assays that aim to be integrated into a diagnostic routine, especially if additional targets are to be analyzed in plasma/serum. In addition, the amount of tumour-derived DNA contained in 1 μg serum DNA will most probably be less than that in 1 μg plasma DNA. A further drawback is the quality of DNA recovered from plasma and serum. The small fragments that seem to compose the main bulk of circulating DNA (10,13) are further degraded by treatment with bisulfite (43). Therefore, any PCR approach that should amplify a representative fraction of available sequences should aim at small amplification products, preferably less than the 200 bp suggested for MSP of initially intact DNA (43).

Despite the difficulties mentioned above, numerous studies have analyzed the methylation status of cancer-related genes in plasma or serum (Table 1), and some important clinical correlations have been reported. In lung cancer, methylation of the adenomatous polyposis coli (APC) gene was shown to be a valuable disease marker due to its 96% prevalence in tumours (44). Using a quantitative MSP approach, high levels of plasma or serum APC gene methylation proved to be an independent factor that predicted for shorter patient survival, although no correlation was found with common clinico-pathological parameters. The method worked with high specificity, while sensitivity was close to 50%. A further study in NSCLC patients evaluated the methylation of the $p16^{INK4a}$, death-associated protein (DAP)-kinase, glutathione S-transferase (GSTP1), and O6-methylguanine–DNA-methyltransferase (MGMT) genes in serum (45). Despite the simultaneous analysis of four genes, only 50% of patients showed abnormal methylation of at least one gene in serum; the sensitivity for detection in serum was 33–80% for the different genes. Hypermethylation was found at all disease stages and was

not related to an early postoperative recurrence. These data suggest that even with multiple analyses, the choice of appropriate target genes is essential to attain prognostic significance.

One of the targets most frequently analyzed has been the p16 tumour suppressor gene. In breast cancer, its methylation status has been assessed as a single marker (46) or in conjunction with additional alterations (36,37). However, none of these studies identified clinical associations with p16 gene methylation per se. This might be the consequence of the low prevalence of p16 methylation in breast tumour tissue (22–23%), which resulted in overall detection rates in plasma of only 10–14%. Better results were obtained in liver cancer, where two studies found methylation of p16 in 67 and 73% of tumours, respectively (47,48). No clinical associations were described in the older work (48), but some correlations were found after incorporation of further patients into the same study (47). The data suggest that aberrant plasma/serum p16 methylation may be strongly associated with more aggressive phenotypes of hepatic carcinoma and with the development of tumour recurrence or metastasis. Furthermore, the finding of p16 methylation in low stage and well differentiated tumours and their matched plasma/serum samples suggests a potential application for early disease detection. Finally, no correlation of plasma/serum p16 methylation with clinical parameters was found in oesophageal squamous cell carcinoma (SCC) (49) or in bladder cancer (53). This is somewhat surprising regarding oesophageal SCC, where the authors report the methylation of p16 in 82% of tumours. However, detection in serum was poorly efficient with a sensitivity of only 23%. It was not discussed whether the low detection rates might arise from technical reasons or rather derive from biological hallmarks of these tumours. In contrast, p16 methylation occurred in only 18% of bladder tumours (53), and the small patient number precluded the analysis of possible clinical associations. Results were different for methylation of p14ARF in the same patient group: methylation occured in 56% of bladder tumours and the high sensitivity resulted in an overall detection of 49% in plasma (53). Furthermore, a significant association of plasma p14 methylation with multicentric foci, larger tumours, and relapse was found. Several additional clincal parameters reached close to statistical significance.

Additional targets analyzed include methylation of the APC gene in oesophageal (50), the hMLH1 gene in colorectal (51), and the DAP-kinase gene in nasopharyngeal cancer (52). Quantitative assessment of plasma APC methylation revealed a significant association of high methylation levels with disease stage and reduced survival for oesophageal adenocarcinoma but not squamous cell carcinoma (50). The high prevalence of APC methylation in esophageal adenocarcinoma tumours (90%) is similar to that reported for lung tumours (96%) using the same detection technique (44). The authors suggest that plasma methylation of the APC gene might be used to identify patients with aggressive disease in esophageal adenocarcinoma. A potential to discriminate high-risk patients has also been suggested for plasma methylation of the hMLH1 gene in colorectal cancer (51). Methylation in tumours was specifically associated with a phenotype of microsatellite instability (90%) and was not detected in nine tumours with a microsatellite stable phenotype. Although hMLH1 methylation in serum was detected with only 33% sensitivity, the authors suggest that this marker might help discriminate patients at high risk for distant metastases, and be employed for the detection of post-surgical relapse. Tumour methylation of the hMLH1 gene was not related to tumour site, stage, and patient age. Finally, methylation of the DAP-kinase gene occurred in 75% of nasopharyngeal tumours (52). Although no prognostic value was found for this marker in plasma, a possible application for clinical monitoring of residual or recurrent disease was suggested owing to the high specificity of the assay and the high prevalence of DAP-kinase methylation in nasopharyngeal tumours.

Taken together, the available data on gene methylation in serum/plasma show a relative consistency regarding the experimental techniques involved. Although the sensitivities have considerably varied among different studies, high specificity was generally reported and for some markers there is a potential for prognostic application. However, most published data remain hard to compare owing to the analysis of different targets in various malignancies. Further studies with greater patient numbers and comparable designs and technical procedures are mandatory for the validation of gene methylation as a useful prognostic/diagnostic marker in serum or plasma. This subject has been reviewed in detail elsewhere (54).

Microsatellite alterations

Of all the targets analyzed in serum/plasma DNA, the alteration of microsatellites has been the source of most controversies. The reason is less related to the choice of techniques, which have been rather homogeneous, particularly among the most recent reports. In fact, the possibility to assess microsatellite alterations by semi-automated, quantitative means such as fluorimetric measurements, should provide

an advantage over other types of more laborious and less accurate analyses. However, a main concern has been the purity of tumour-derived DNA in plasma and serum. As mentioned earlier, a significant proportion of serum DNA is thought to arise from *in vitro* lysis of normal haematological cells (16,17), and even in plasma the fraction of tumour-derived DNA is highly variable (13). For microsatellite alterations involving band shifts, it has been estimated that detection of one tumour cell in a background of up to 1000 normal cells can be achieved (55). However, most microsatellite alterations involve allele losses (LOH) rather than band shifts, and here the sensitivity of detection is significantly lower. Among the studies evaluating microsatellite alterations in plasma/serum DNA, the LOH cut-off values determining the minimal acceptable reduction in band intensity have varied between 30 and 75% (21,36,37,39–41,53,57–70). This would theoretically mean that at least one third of the DNA under analysis should be of tumour origin to permit an accurate determination of LOH. Higher proportions of normal DNA would thus be expected to reduce the detection rate of LOH, often resulting in false-negative results. Despite this limitation, microsatellite alterations including LOH have been found not only in plasma but also in serum (Table 1). Although sensitivities varied among the different studies, no obvious differences were apparent in this regard compared with other targets analyzed (see previous sections).

In addition to the above concern, a major finding has been the high frequency of unexplained positive results. Non-matching microsatellite alterations in plasma/serum have been described in virtually all studies that had tumour material available for comparison. The frequency of unexplained positives was often worrisome and hardly to be explained by the detection in plasma/serum of DNA derived from occult tumour clones. In renal cell carcinoma patients, discordant serum DNA allelic imbalances were found in 0–23% of cases depending on the microsatellite marker analyzed, and some false positive alterations were detected in the healthy control group (67). Similarly, a study in bladder cancer patients found that close to 50% of all plasma/serum alterations did not match the status of matched tumour samples (63). Artifacts were further reported in the control groups of a breast cancer (15) and a head and neck cancer study (69), the latter of which ascribes them to the low amounts of DNA recovered from plasma and serum of healthy individuals. This notion is further supported by the finding of non-specific allelic imbalances when amplifications were performed with less than 2 ng of DNA (41), although this work relied on a marker yielding a PCR product longer than 350 bp. This has been noted by other authors as a possible source of additional error (71). One study tried to overcome this limitation by interpreting plasma/serum alterations as specific only if they matched those found in tumour samples (64). However, this approach undermines the potential application of an analysis that may have the major advantage of substituting the need for tumour material for assessment of prognosis. Taken together, the data on microsatellites in plasma/serum reveals difficulties stemming from the less than optimal specificity. The low amounts of DNA are a feasible yet probably not exclusive explanation for this shortcoming.

Despite the limitations mentioned above, several studies found significant associations of microsatellite alterations in plasma/serum with clinical or pathological parameters (Table 1). In lung cancer, the assay seems particularly promising for early disease detection. Plasma microsatellite alterations were found in 43% of stage I NSCLC patients and in 45% of patients harboring tumours less than 2 cm in diameter (57). Furthermore, the analysis of symptomatic individuals at risk for lung cancer revealed a positive diagnosis in 92% of the patients harboring plasma LOH (59). Conversely, 43% of patients diagnosed with lung cancer presented with LOH in plasma, although the study was performed with only four microsatellite markers. Inclusion of additional markers could further improve the detection rates. Surprisingly, one report describes a positive impact on survival of SCLC patients when microsatellite alterations and p53 mutations were simultaneously detected in plasma (21). The authors propose that cells harboring both alterations might be more easily eliminated by chemotherapy. In contrast, no clear associations were found in two breast cancer studies (15,60). However, one of the reports suggests that the analysis of plasma microsatellites might complement cytokeratin 19 measurements to assess minimal residual disease after primary surgery (60). The use of two microsatellite markers by itself yielded too low sensitivities for a clinical application, but this could be improved by the inclusion of additional markers as already mentioned earlier. Two further reports by one group suggest that plasma microsatellite alterations together with mutation of p53 and methylation of p16 are associated with histological features of malignancy in breast cancer patients (36,37). The persistence of such alterations after mastectomy might be useful to identify patients at risk to develop a more aggressive disease. In melanoma patients, one group found an association of plasma microsatellite alterations with disease stage, progression and survival rates (61,62). The analysis was performed with 8–10 markers mapping to six chromosomal regions showing frequent LOH in tumours. Specificity was close to 100% and overall detection rates in plasma close to 60%.

Among further studies that describe correlations with clinico-pathological parameters, some tested a remarkable number of microsatellites. In renal cancer, the postoperative clinical course was evaluated for 30 patients that had been previously subjected to serum microsatellite analysis using 28 different markers (65,66). This permitted the identification of seven loci that were primarily responsible for the predictive value of postoperative recurrence. However, although 100% of tumours showed at least one alteration with the 28-marker panel, detection in serum was achieved in only 50% of the samples. This type of approach is useful in establishing which markers might be best suited for analysis in plasma/serum, a consideration relevant to all other cancer types which are each characterized by particular regions of frequent LOH or microsatellite instability. Among the remaining studies, it is worth noting that completely negative results have also been reported. In colorectal cancer, 80% of primary tumours harbored at least one microsatellite alteration when assayed with a panel of eight markers (39). However, no alterations (0%) were found in any of the matched serum samples. As possible explanations, the authors suggest either a lack of sensitivity of the method or the potential clearance of tumour DNA during its passage through the liver. Further data on microsatellites are summarized in Table 1.

In conclusion, analysis of microsatellite alterations in plasma/serum has achieved comparable sensitivities and overall results as other targets. However, technical difficulties are particularly frequent and less than optimal specificity remains a problem. In this case, the use of plasma rather than serum might be especially advisable. The need for standardization and optimization of the methodology will probably require a greater effort than for other targets, as the number of microsatellite markers potentially useful for each tumour type is vast, and screenings have to be performed to define the most useful ones. Besides considering the frequency of alteration of each microsatellite, markers should be chosen that yield short amplification products owing to the degraded nature of circulating DNA. It has been reported that the length of the amplified marker might cause artifacts by favoring the amplification of the shorter over the longer allele (55). Further technical details have been discussed elsewhere (72).

Additional approaches

The previous section was representative but certainly not exhaustive. Additional targets have been assessed in plasma/serum DNA such as mutation of other oncogenes. However, all the above targets have in common that evaluation is performed on circulating genomic DNA of endogenous origin. Recently, additional compounds recovered from blood have been tested for a potential prognostic application in cancer. These include nucleosomes, RNA, mitochondrial DNA, and viral DNA. These alternative approaches will be briefly discussed.

Circulating nucleosome levels

As mentioned earlier, nucleosomes originate from endonuclease cleavage of chromatin during apoptotic cell death, and are composed of a histone octamer core wrapped with 180–200 bp of DNA (11). It is now widely accepted that nucleosomes, and in particular mononucleosomes, are the form in which cell-free DNA circulates in the bloodstream (10). Consequently, some groups have reasoned that the measurement of circulating nucleosomes should yield comparable results to the determination of plasma or serum DNA concentrations. If nucleosomes generate by apoptosis of tumour cells in cancer patients, their levels could potentially relate to the patient's tumour burden. Technically, the determination of nucleosome levels is simplified by the existence of a commercial ELISA kit that relies on antibodies directed against DNA and histones, and this kit has been successfully used in the studies mentioned below. As with other targets analyzed, both plasma and serum have been used as source for nucleosomes.

Among the existing reports, only one focused on a particular cancer type (73). The work found a significant increase of nucleosome levels in breast cancer patients compared with healthy controls (Table 2). No correlation was found with tumour size, menopausal and estrogen receptor status, histological type, and lymphatic or venous spread, although there was a trend toward longer relapse-free survival for patients with higher nucleosome levels. These data were partly corroborated by the same authors in a subsequent study performed on breast and other cancer types (74). In breast cancer patients, a decrease in nucleosome levels was found after mastectomy. In patients receiving docetaxel, a transient increase in nucleosome levels was noted 24 h after administration which correlated with the subsequent degree of leukopenia. A marked decrease in nucleosome levels was further seen when a response was obtained, whereas no tendency to decrease was observed in either stable or progressive disease. The latter data are similar to those reported in some reports evaluating DNA concentrations (3,12,25).

The remaining work by a second group focused on nucleosomes in serum rather than plasma

Table 2 Additional circulating targets analyzed in cancer patients

Target analyzed	Cancer type	Source	Patients analyzed	Clinical correlations	Reference
Nucleosome levels	Breast	P	96	Unrelated to clinicopathological factors	(73)
	Various	P	163	Unrelated to clinicopathological factors; potential to predict therapy outcome	(74)
		S	185		(75)
		S	220	Related to disease stage	(76)
		S	418	Potential to predict therapy outcome	(77)
RNA transcripts	Melanoma	S	6		(82)
		P/S	10		(83)
	Lung	S	18		(84)
	Colorectal	P	53	Associated with disease stage and circulating tumour cells ((CEA, CK19)	(85)
	Breast	S	18		(86)
	Breast, lung	S	19		(87)
Mitochondrial DNA mutations	Prostate	P	16	Potential for early disease detection	(88)
	Liver	P	14	Potential as marker of tumour clonality	(89)
Viral DNA	Nasopharyngeal	S/P	37	Unrelated to clinicopathological parameters	(91)
		P/S	25	Potential to predict recurrence	(92)
		P	57	Correlated with disease stage, therapeutic response	(93)
	Lymphoid	P	18	Correlated with therapeutic response, survival; unrelated to age, stage, performance status, LDH	(94)
		P	13	Correlated with therapeutic response	(95)
		S/P	80	Related to prognostic index, haemoglobin levels, disease stage at presentation	(96)
	Lung	S	11	Correlated with clinical disease, response to therapy, clinical course	(97)
	Gastric	S	51	Associated with EBV-positivity of tumours	(98)
	Cervix	P	232	Correlated with clinical stage at diagnosis	(99)
			63	Associated with metastatsis	(100)
	Head and neck	S	70	Potential marker for metastatic disease	(101)

Abbreviations used: *S*, serum; *P*, plasma.

(75–77). As a first approach, the accuracy of the ELISA-based determination was examined (75). Although the variability was within an acceptable range, the presence of acute inflammations was shown to interfere with the assay. The authors caution that the assay cannot distinguish between nucleosomes derived from necrotic or apoptotic cell death. This argument has been taken in support of the use of plasma rather than serum by Kuroi *et al.* (74), who cited that plasma nucleosomes usually do not show an excess of long strands nor random DNA lengths (78). This was interpreted as an indicator that apoptosis is the main form of cell death contributing to plasma nucleosomes. Despite this observation, two further reports used serum nucleosomes owing to their apparent better stability (76,77). In fact, the authors attempted to optimize the assay to obtain a standardized procedure for clinical application. Besides finding elevated levels in cancer patients versus healthy controls, nucleosome levels were significantly higher in advanced disease stages (76). Furthermore, the timing and extent to which nucleosome levels varied during radiation or chemotherapy correlated with the clinical outcome of patients (77). The authors propose that this assay might find an application in the early estimation of therapeutic efficacy.

Circulating RNA

The fact that cell-free RNA has been considered for prognostic applications might appear surprising in light of its known propensity to degradation. Ribonuclease is present in both plasma and serum, and its levels appear to be particularly elevated in the serum of some cancer patients (79). Furthermore, RNA could not be amplified after spiking experiments in blood, suggesting a rapid degradation (80). However, there is extensive *in vitro* evidence for the release of extracellular RNA from cells, and it has been suggested that cell-free RNA might circulate in blood bound to protein or phospholipid (reviewed in (81). Thus, a few groups have attempted the identification of tumour-specific transcripts in plasma or serum of cancer patients using reverse transcription PCR (RT-PCR) (Table 2), a method that has the potential to be performed semi-quantitatively.

Using a ubiquitous control transcript, one study found amplifiable RNA in the serum of all patients and controls tested, which confirms the presence of mRNA in serum (82). In contrast, tyrosinase mRNA was specifically detected in 4/6 (67%) melanoma patients but not in control subjects. The authors could rule out the contamination of serum with circulating tumour cells, which suggests the cell-free origin of the tyrosinase transcript. A similar detection rate for tyrosinase mRNA was reported by a second work (83). However, two additional melanoma markers, gp100 and MART-1, could not be amplified despite the proven presence and integrity of RNA. It was concluded that the measurements on extracellular RNA are less sensitive than cellular-based assays. In lung cancer, disease could be traced in 18/18 (100%) patients using Her2/neu and hnRNP-B1 serum mRNA as markers (84). In contrast, other markers either lacked sensitivity (MAGE-2, PGP, and TTF-1) or specificity (CK19). Unspecific detection of CK19 and CEA mRNA was also reported in 20 and 4% of plasma samples of healthy controls, respectively (85). Despite this, a significant association was found between an advanced disease stage and the presence of CEA, CK19, or both mRNAs in plasma of colorectal cancer patients. The markers further showed a close to significant correlation with lymph node metastasis and vascular invasion. Based on this, the authors deduce that the presence of these markers might be linked to micrometastases in blood. Finally, two further reports assessed mRNA markers in the serum of breast cancer patients. The first measured the RNA telomerase subunit hTR and the mRNA encoding its protein subunit, hTERT (86). Expression of both RNAs was frequent in tumours (94% each), but detection in serum was not overly sensitive (28 and 24%, respectively). In contrast, specificity was high (100%). It is worth noting that telomerase RNA could be detected in the serum of patients with small tumours and no nodal involvement or distant spread, suggesting a potential for early disease detection and follow-up. The second work analyzed 5T4 mRNA in the serum of breast and lung cancer patients (87). Since expression of 5T4 in epithelial tumours is associated with metastatic potential and worse prognosis, the detection of this marker in serum might be particularly valuable. Reproducible amplification was obtained in serum of 42% of the patients but also in 12% of the controls, although the difference between both groups was statistically significant.

In summary, the available data points to the possibility of using extracellular RNA for cancer disease management. However, both sensitivity and specificity have been an issue and require careful evaluation, and greater patient numbers have to be analyzed. As with the techniques mentioned in previous sections, further data and standardization of methods are needed to assess the real utility of this approach.

Mitochondrial DNA mutations

Only two reports have dealt with the detection of mitochondrial DNA (mtDNA) mutations in plasma

of cancer patients (88,89). In fact, this entire research area is rather new. The authors had previously identified alterations in mtDNA from 43% of lung, 64% of bladder, and 46% of head and neck tumours (90). Despite the presence of 10^3–10^4 mitochondrial genome copies per human cell, the mutations in tumours were present in most if not all mitochondria. This homoplasmic nature of mtDNA alterations suggests the involvement of some mechanism resulting in selection or preferential expansion of the mutant mitchondria, which is certainly advantageous for the detection of mutations. Furthermore, enrichment of mutant mtDNA allowed the detection of mutations in the urine of patients with bladder cancer, and in the saliva of head and neck cancer patients. Based on the identification of mutation hotspot regions, the authors then searched for mtDNA alterations in early stage prostate cancer (88). Only 19% of patients harbored mtDNA mutations in their tumours, which is markedly lower than the frequencies described in the previous report. Although the mutations were also found in matched urine and plasma samples, mtDNA was strongly diluted and required a sensitive mismatch ligation assay to allow detection.

It was argued that prostate cancer might shed small amounts of DNA into circulation, at least in early stages. However, a significant dilution of mtDNA in plasma was further reported for hepatocellular carcinoma (89), which suggests that mtDNA might not be readily introduced into or survive long in the circulation. The frequency of mtDNA mutations in hepatocellular tumours was higher (68% of patients) and could be detected in most paired plasma samples (80%). However, more than one assay had to be employed to this end. The analysis of mtDNA mutations was suggested as a novel approach to assess tumour clonality. Taken together, the available data indicate that mtDNA might be highly diluted in the plasma of cancer patients, making the detection of previously unknown mutations, e.g., when no tumour is available, probably difficult. However, owing to the novelty of this research field, additional studies might prove the feasibility of such analyses.

Circulating viral DNA

The recognition that cell-free tumour-derived DNA could be recovered from blood encouraged investigators to test the same approach for the detection of cancer-related viruses. Viruses such as human papilloma virus (HPV) and Epstein–Barr virus (EBV) are aetiological factors in various malignancies. Since tumour cell death can lead to shedding of DNA into circulation, it was reasoned that viral DNA contained in tumour cells might be subjected to the same process. Meanwhile, several reports provide evidence for the release of viral DNA into the bloodstream (Table 2). Regarding the techniques utilized, it is worth noting that most of them have incorporated quantitative or semi-quantitative real-time PCR approaches. As will be discussed, this has often allowed the correlation of circulating viral DNA levels with tumour burden or viral load. The choice of viral sequences used for detection proved often crucial for proper functioning of the assays.

EBV is associated with the development of different types of cancer, and some of these have been subjected to analysis of circulating DNA. Nasopharyngeal carcinoma is a frequent type of cancer in Southern China and Southeast Asia, and nearly all cases harbor EBV in tumour tissues. One report analyzed EBV DNA in the serum of nasopharyngeal patients using nested PCR (91). The method detected EBV DNA in the serum or plasma of 59% of patients, and worked with as little as 10 fg DNA. Positivity in 13% of normal control subjects was explained as apparent former exposure to EBV. No false positives were obtained among patients with known negative EBV status, which was taken in favor of proper specificity.

Two further reports assessed EBV levels in nasopharyngeal cancer patients by quantitative real-time PCR (92,93). These studies provided interesting results as they performed longitudinal evaluation of patients under radiotherapy. EBV DNA was not only found in the plasma of 96% of patients, but also in 7% of normal controls. No obvious explanation was available for the latter. Importantly, plasma EBV DNA levels were significantly higher in advanced stage patients compared to early stages (93). Furthermore, plasma EBV DNA became negative in patients responding with complete remission to radiotherapy, while persistence of viral DNA was demonstrated in patients with incomplete regressions or the development of metastasis. The follow-up of patients indicated that gradual increases in serum EBV DNA preceded recurrence by up to 6 months (92).

Using quantitative PCR approaches, similar findings have been reported for EBV-associated lymphoid malignancies (94–96). Natural killer/T-cell lymphoma occurs mostly in Asian and South American countries, and is strongly associated with EBV. One group detected plasma EBV DNA in 94% of patients but never in healthy control subjects (94). Although DNA levels did not correlate with disease stage, significant reductions were observed in patients that responded to chemotherapy. Furthermore, patients with high EBV DNA levels at presentation showed significantly poorer survivals at 20 months. Changes in plasma EBV DNA

correlated with the therapeutic response in an additional group of patients with various lymphoid malignancies (95).

In EBV-associated Hodgkin's disease, the presence of plasma or serum EBV DNA correlated with a high prognostic index, lower haemoglobin, and advanced stage at presentation (96). Interestingly, positivity for serum EBV DNA was observed in a subgroup of EBV-negative patients. In these cases, scattered EBV-positive bystander cells were detected by *in situ* hybridization. Positivity was also found among some cases of infectious mononucleosis with proven IgM antibodies against EBV. Finally, EBV serum DNA levels correlated with the clinical response to treatment and the subsequent clinical course in lymphoepithelioma-like carcinoma of the lung (97), and with EBV-positivity of tumours in gastric carcinoma (98).

HPV infection is an important aetiological factor in cervical carcinoma. Importantly, progression of cervical cancer is associated with the integration of HPV DNA into the human genome. The process requires linearization of viral DNA, which leads to disruption of some viral encoded genes. This is relevant to the selection of viral targets for DNA amplification, as regions should be preferred that are not immediate to the breaking point. The issue is further complicated by the existence of several high-risk viruses which present some degree of sequence variability. The detection of all viral species will thus often require degenerate PCR primers. Using conventional PCR, one report assessed HPV plasma DNA in 232 patients with cervical cancer (99). Only 4.8% of cases had detectable plasma HPV DNA, and 1 case (1.8%) of controls also displayed positivity. Among the positive cases, quantitative analysis revealed higher levels in invasive cervical cancers as compared to one case of carcinoma *in situ*, and the viral load was correlated with clinical stage at diagnosis. Importantly, a discrepancy was observed in five cases between the HPV types recovered from plasma and those from the tumour, for which no obvious explanation was found. Sensitivity for plasma HPV detection was also low (12%) in a second study, while specificity remained high (100). No discrepancies in virus types were reported by the work, suggesting that viral DNA recovered from plasma originated from lysis of tumour cells. An association was further found between plasma HPV DNA and the presence of metastasis, and patients with plasma positivity showed a tendency to develop recurrent disease. Despite the low sensitivity of detection of plasma HPV DNA, both studies suggest a potential application to identify patients at risk of more aggressive disease. The same was suggested for head and neck cancer (101).

In summary, analysis of circulating viral DNA has yielded some promising results, in particular for EBV DNA which was detected frequently and at high levels in serum or plasma. Techniques have been rather homogeneous, although in the case of HPV the selection of the region to amplify showed great influence on the results (101). With the same approach, detection of circulating viral RNA is also a feasible alternative. This type of assays might find application in the management of virus-related cancers, in particular in endemic areas.

CONCLUSIONS

The work summarized in this review should provide the reader with an impression of the current status of research in the area of circulating nucleic acids. Certainly, important results have been attained by numerous studies, the conclusion being that assays based on cell-free DNA/RNA measurements might find potential applications in the management of cancer as well as other conditions not discussed here. However, the routine application seems still distant. Techniques are in urgent need of standardization, the utility of different targets needs careful evaluation, and data should be analyzed according to common parameters to allow comparison of different studies. Further topics to be resolved include factors limiting assay specificity and sensitivity, and the suitability of plasma versus serum. Addressing these issues will require a common effort and collaboration of groups involved in this research area. This will hopefully allow the widespread introduction of a minimally invasive procedure for diagnostic and prognostic applications in cancer and other diseases.

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