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Cancer Diagnostics: Review

Plasma Nucleic Acids in the Diagnosis and Management of Malignant Disease

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Background: **There is a need for development of molecular markers of cancer that can be used clinically for the detection, prognostication, and monitoring of cancer. Recently, there has been much interest in the potential use of nucleic acid markers in plasma and serum for this purpose.**

Approach: **We reviewed published literature up to 2002 on the topic, with a particular emphasis on reports published between 1996 and 2002.**

Content: **The nucleic acid markers described in plasma and serum include oncogene mutations/amplifications, microsatellite alterations, and gene rearrangements. Such markers have been described in many cancer types, including lung, colon, and breast. Epigenetic alterations, such as aberrant promoter methylation, have been identified in plasma and serum. Viral nucleic acid markers, such as Epstein–Barr virus DNA in plasma and serum, are reviewed in detail with regard to their application to virus-associated cancers such as nasopharyngeal carcinoma and various lymphomas. More recently, mitochondrial DNA and tumor-related mRNAs have been identified in plasma and serum from patients with several types of tumors.**

Conclusions: **Circulating nucleic acids are an emerging class of molecular tumor markers. Their wide applicability and clinical relationship with the malignant state will likely grant them increasing clinical importance in the near future.**

The development of "conventional" tumor markers such as carcinoembryonic antigen and α-fetoprotein was driven largely by the introduction of new methods for quantifying small amounts of circulating proteins that were relatively specific for certain types of cancer. The application of immunodiffusion techniques and subsequently RIAs and ELISAs led to the discovery of several circulating tumor markers, many of which have now entered routine clinical practice. However, the shortcomings, particularly the limited sensitivity and specificity, of such an approach, are now well recognized *(1, 2)*. In seeking to increase the pool of possible serologic molecular markers, several new constituents of plasma/serum are now being examined *(3)*. PCR-based technologies that can amplify DNA copy numbers and thereby detect and quantify extremely small amounts of nucleic acids are now offering novel molecular targets for the development of molecular markers of cancer.

Many of the aberrations that are being detected in tumor-derived nucleic acids (DNA and RNA) as part of the continuing search for the genetic basis of cancer development can also be detected in the DNA and RNA present in plasma. In this area of research, the functional significance of the findings is of secondary importance; it is the extent to which our results lead to early and accurate diagnosis and prognostication as well as the ability of the novel makers to reflect tumor cell mass (and thereby provide effective monitoring of therapy) that is of primary concern.

Plasma DNA as a Molecular Marker of Cancer

The presence of DNA and RNA in plasma of cancer patients has been recognized since the 1970s *(4, 5)*, but it was not until 1989 that the neoplastic characteristics of plasma DNA in cancer patients were recognized *(6)*. Five years later, Sorenson et al. *(7)* reported the detection of tumor-derived oncogene mutations (*K-ras)* in pancreatic cancer, and Vasioukhin et al. *(8)* reported *N-ras* mutations in myelodysplastic syndrome. On the basis of these classic studies, a new field of tumor marker research has emerged.

Oncogene Mutations and Amplifications

The *K-ras* family of protooncogenes are part of the signaling pathway of several different molecules. Gain-of-func-

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tion missense mutations are often somatically acquired in colonic, pancreatic, and lung cancers, almost exclusively at three hot spots: codons 12, 13, and 61 *(7, 9)*. Because such mutations occur early in the development of cancer, are highly specific for cancer, and have a well-characterized site, they offer interesting insights into the possibilities and difficulties inherent in this approach to serologic diagnosis. Approximately one-fourth of patients with colorectal cancer and one-half of those with pancreatic cancer have mutant *K-ras* sequences detectable in plasma. If the analysis is confined to those in whom the relevant mutation is confirmed in the tissue, in both instances approximately one-half of the cases are positive in plasma *(10–12)*. These reports provided strong evidence that at least some of the DNA detected in plasma was tumor derived. The broad spectrum of tumors that exhibit *K-ras* mutations limits the specificity of the test for particular cancers, but this is counteracted by the observation that any mutation detected in plasma is very specific for cancer of some type as distinct from nonmalignant conditions. Thus, although gene mutations have been detected in healthy and other nonmalignant tissues, these do not appear in the plasma.

In many cases the mutations that have been detected in plasma DNA came from patients with advanced metastatic disease; this appears to limit the potential role of this approach in early diagnosis, although occasional cases have been described in which the mutation was detected several months before clinical diagnosis *(13)*. More recently, however, in a prospective study of patients undergoing colonoscopy, *K-ras* mutations were detected in the plasma of 83% of those who had similar mutations in tumor biopsy tissues. Among those who had no biopsy available, approximately one-fourth had *K-ras* mutations, and most of these had risk factors for colorectal cancer. Overall, 39% of patients with *K-ras* mutations in plasma had colorectal cancer compared with only 3% of those without mutations. The authors considered that this test might ultimately come to play a role as a screening test for colorectal cancer *(14)*. In another study, Ryan et al. *(15)* reported a strong concordance between the presence of *K-ras* mutations in colorectal cancer at presentation and a parallel presence in plasma/serum. An alternative or complementary approach may be to use stool samples or to combine plasma *K-ras* analysis with conventional markers such as CA 19-9 *(16–18)*. Similar approaches are being investigated for other genes frequently mutated in other cancers. For example, mutated *adenomatous polyposis coli* (*APC*) gene sequences can occasionally be detected in the plasma of patients with sporadic colorectal cancer and *erB-2* in those with prostate cancer *(19–21)*.

Microsatellite Analysis

Microsatellites are repetitive DNA sequences, ranging in size from 2 to 6 bp, that form variable-length stretches of DNA. With appropriate primers it is possible to amplify DNA fragments that can be used as microsatellite markers, and with a "panel" of such markers, tumors can be profiled. Characteristic genetic changes in the tumor tissue, in this case microsatellite alterations, are detectable in plasma DNA. The first reports of this approach involved patients with lung cancer and head and neck cancer *(22, 23)*. In the former case, microsatellite alterations were present in three-fourths of the tissues and in a similar fraction of sera; similar results have recently been described in other histologic subtypes of lung cancer *(24)*. Similar results have also been reported by Gonzalez et al. *(25)* for small cell lung cancer. In those with head and neck cancers, one-third exhibited one or more microsatellite markers in serum that matched those in the primary tumor. Again, positive results have tended to occur in patients with more advanced disease. However, using similar approaches, Taback and coworkers *(26, 27)* found a loss of heterozygosity in the serum of 21% of patients with early-stage breast cancer, based on eight markers. Although the ultimate significance of this finding remains to be demonstrated by longer follow-up of the positive patients, the strong association with other markers of poor prognosis and the demonstration of a relationship between plasma DNA and death *(26)* raise hope for clinical application in terms of early diagnosis. Microsatellite alterations have also been detected in the urine of patients with renal cell carcinoma and bladder cancer, leading workers to successfully seek the same aberrations in plasma *(28–31)*.

Chromosomal Translocations

Frickhofen et al. *(32)* demonstrated the presence of rearranged immunoglobulin gene sequences in the plasma and serum of patients with B-cell malignancies. For follicular lymphomas, characterized by a chromosome 14;18 translocation that juxtaposes the *BCL2* oncogene with the immunoglobulin heavy chain-joining region (IgH), a similar approach had also been attempted. The resulting translocation can be detected in plasma by a PCR-based technique that applies a combination of appropriate primers. Preliminary evidence suggests that detection of the translocation in plasma may be a marker for minimal residual disease after therapy *(33)*.

Epigenetic Alterations

Heritable changes in gene expression that do not depend on a DNA sequence change are termed "epigenetic". Whereas failure of expression of tumor suppressor genes is classically attributable to deletions (causing loss of heterozygosity) or point mutations, epigenetic silencing of expression results from aberrant methylation of cytosine residues of CpG islands in the promoter sequences of tumor suppressor genes *(34, 35)*. This occurs, for example, in the majority of colorectal cancers that show microsatellite instability *(36)* and has aroused considerable excitement because, at least in theory, methylation should be amenable to therapeutic intervention.

There are now several examples where this phenome-

Table 1. Epigenetic markers in lung and liver cancer *(39, 40)*.

Tumor type	Gene aberrantly methylated	Detection in tumor tissue, n (%)	Detection in serum DNA, n (%)
NSCLC[a]	*p16*	9/22 (41)	3/9 (33)
	DAP kinase	5/22 (23)	4/5 (80)
	GSTP1	2/22 (9)	1/2 (50)
	MGMT	6/22 (27)	2/6 (33)
HCC	*p15*	16/22 (73)	13/16 (81)
	p16	16/25 (64)	4/16 (25)
	p15 and *p16*	12/25 (48)	11/12 (92)

[a] NSCLC, non-small cell lung cancer; HCC, hepatocellular carcinoma.

non can be detected in plasma by the technique of methylation-specific PCR, in which bisulfite is used to convert unmethylated cytosine residues into uracil *(37, 38)*. The methylated residues remain as cytosine and can then be distinguished from the unmethylated type by application of specific primers. Aberrant methylation of the *p16* gene is common in both lung and liver cancer tissues, and two recent reports describe its detection in the plasma/serum of several of the same patients *(39, 40)* (Table 1). Similar results have also been reported by Goessl et al. *(41)* in prostate cancer. Hypermethylation of the *APC* gene can be detected in the tumor tissue of 90% of patients with esophageal adenocarcinoma and 50% of those with squamous cell carcinoma *(42)*. Among such "positive" patients, similar changes of hypermethylation could be detected in the plasma DNA in 25% and 6%, respectively. The remarkable specificity of the changes for cancer, as seen in the previously mentioned studies, was confirmed. Thus, whereas hypermethylation was sometimes found in Barrett esophagus tissue samples and even in nondiseased stomach tissues, aberrant plasma *APC* methylation was detected only in those with frank malignant disease. Less encouraging, from the point of view of early diagnosis, was the observation that detection was related to disease stage, with no stage I cases and only one stage II case being positive.

Viral DNA as a Marker of Virus-related Cancers

An additional source of DNA in plasma is that originating from viruses, and when these viruses have a close association with a particular cancer, they have the potential to be used as molecular markers. Examples include Epstein–Barr virus (EBV)[4] with Hodgkin disease, Burkitt lymphoma, and nasopharyngeal carcinoma (NPC) *(43, 44)* and the human papillomavirus with head and neck and cervical cancers *(45)*. NPC appears to be a particularly good model because of its almost universal association with EBV in southern China. With real-time PCR, EBV DNA is detectable in 95% of cases, whereas in healthy controls the frequency is only ~5% *(46)* (Fig. 1).

Although at first sight it may appear that the primary use of this test would be to screen high-incidence populations for NPC, several problems arise. The first problem is that the trials that would be needed to demonstrate a clinical benefit in terms of reducing disease-specific mortality would be formidable, and it is increasingly recognized that patients with other cancers, and occasionally apparently healthy individuals, are also plasma EBV DNA-positive, although the frequency is much lower, as are the concentrations of EBV DNA. However, once the disease is diagnosed, it appears that the test is very useful in prognostication and in monitoring disease response to treatment. Because it is this area of plasma DNA diagnostics that appears closest to clinical application, it is described in more detail below.

CLINICAL APPLICATIONS OF PLASMA EBV DNA MEASUREMENT

NPC is a common cancer in southern China. The disease is often cured by a course of radical radiotherapy, but in some cases, particularly those presenting with more advanced disease, either the primary disease is not eradicated (local persistence) or there is subsequent relapse either locally or at distant sites.

After radiotherapy, a course of which lasts 6–8 weeks, there is usually a steady decrease in plasma EBV DNA with an overall half-life of ~4 days *(47)* (Fig. 2). A similar pattern is obtained if chemotherapy is used (unpublished observations). Interestingly, there appears to be one, or sometimes two, "surge(s)" of EBV DNA after the initial course of radiotherapy *(47)*. Such might be taken to imply the rapid release of EBV DNA into the plasma from dead

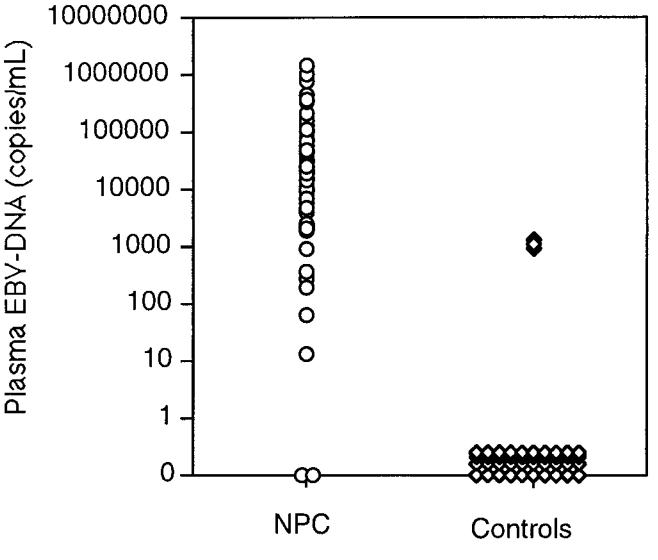

Fig. 1. Concentrations of cell-free EBV DNA, as measured by *Bam*HI W fragment PCR and presented on the *y axis* on a log scale, in 57 patients with NPC and 43 individuals without evidence of NPC.
Data reproduced with permission from Lo et al. *(46)*.

[4] Nonstandard abbreviations: EBV, Epstein–Barr virus; NPC, nasopharyngeal carcinoma; NK, natural killer; and HBV, hepatitis B virus.

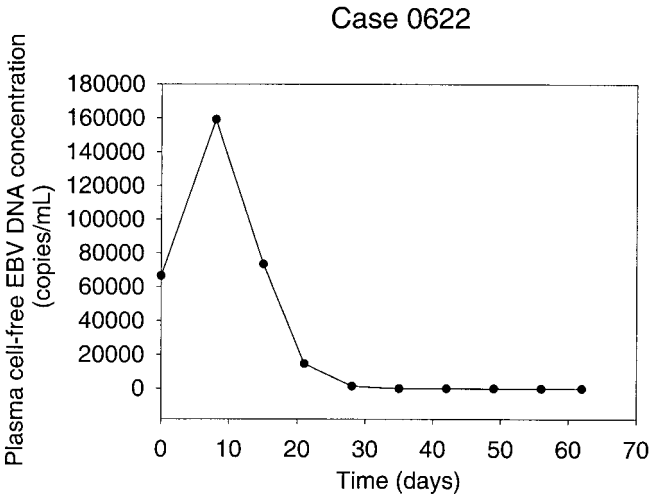

Fig. 2. Change in EBV DNA concentrations during radiotherapy.

Note the initial surge in this patient. Samples were taken weekly; when samples were taken daily during radiotherapy, such a surge was identified in the majority of cases. Data reproduced with permission from Lo et al. *(48)*.

or dying tumor cells, followed by plasma clearance. The clinical value relates to the observation that those in whom the concentrations do not reach zero (or at least decrease to a low value) subsequently relapse *(46, 48)* (Fig. 3). Furthermore, the concentrations of EBV DNA in plasma at presentation is of major prognostic value *(49)* (Fig. 4A) as are the concentrations immediately after the end of the treatment, in that an increased value implies residual disease. It seems likely that in the next few years, estimation of EBV DNA will become a routine part of the staging procedure for NPC and will directly influence therapeutic decisions.

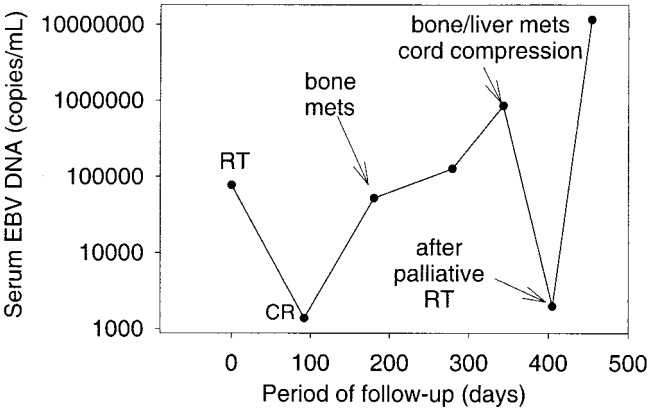

Fig. 3. Changes in plasma EBV DNA in a patient with NPC during and after radiotherapy.

Note that despite complete clinical remission (*CR*), the concentrations had not fallen to zero, and this presaged early relapse. Note also that local treatment to the spine caused an initial (although temporary) response, implying that tumor tissue is the likely source of the EBV DNA that is being detected in the serum. *RT*, radiotherapy; *mets*, metastases. Data reproduced with permission from Lo et al. *(48)*.

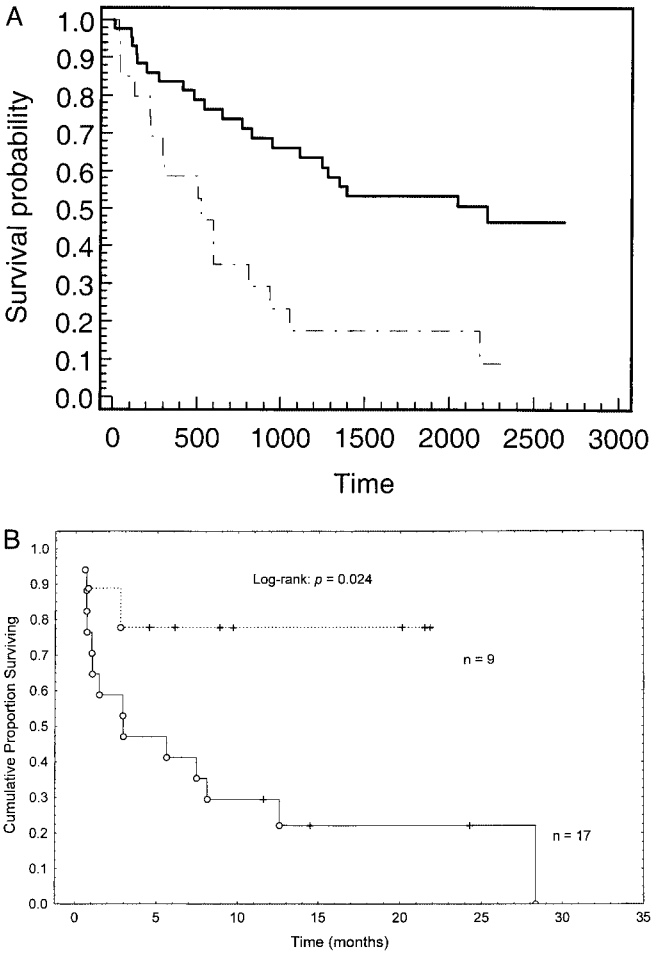

Fig. 4. Survival in relation to EBV DNA concentration in patients with advanced (stage IV) disease (*A*) and patients with nasal/NK cell lymphoma (*B*).

(*A*), all patients had advanced disease, but by separating patients on the basis of EBV DNA concentrations (*solid line*, <40 000 copies/mL; *dashed line*, ≥40 000 copies/mL), two groups with widely different survival expectation emerged (log-rank test, $P = 0.0006$). Data reproduced with permission from Lo et al. *(49)*. (*B*), similar findings were observed in patients with nasal/NK cell lymphoma, a rare tumor invariably associated with EBV infection. *Solid line*, plasma EBV DNA ≥600 copies/mL; *dashed line*, plasma EBV DNA <600 copies/mL. Data reproduced with permission from Lei et al. *(57)*.

EBV IN LYMPHOID AND OTHER MALIGNANCIES

As noted above, several other malignancies are associated with EBV infection; lymphomas, particularly Hodgkin disease, are among the most interesting of these. Both EBV DNA and EBV gene products (such as the EBV-encoded small RNAs) are readily detectable in the pathognomonic cell of Hodgkin disease, the Reed–Sternberg cell in a significant portion of the cases in the West (especially in lymphomas of the mixed cellularity subtype), and in almost all patients in countries where EBV infection in childhood is endemic *(50–53)*. EBV DNA has been detected in both adult and pediatric Hodgkin disease patients *(54, 55)*. It was detected in 90% of adult patients, and real-time quantitative PCR has been applied to measure EBV copy numbers in serum. The authors of

the latter report suggested that concentrations, particularly those obtained after treatment, were likely to be of prognostic significance *(54)*.

We and others have recently undertaken similar studies in patients with lymphoma and found similar results *(56, 57)*. The great majority of lymphomas in Asian patients are EBV DNA-positive in plasma, and the concentrations clearly change in response to treatment. It is most striking that clinically complete response precedes achievement of lowest or normal EBV DNA and that failure to achieve a negative EBV DNA, even in the presence of clinically complete response, predicts subsequent recurrence. Furthermore, in a subset of lymphomas, the natural killer (NK) cell nasal lymphomas, essentially all patients are EBV DNA-positive, and prognosis is clearly linked to the initial plasma concentration *(56, 57)* (Fig. 4B).

Mitochondrial DNA

Each human cell contains several hundred copies of mitochondrial DNA that encodes respiratory chain subunits, tRNAs, and rRNAs. Several mutations have been described in patients with colorectal carcinoma, bladder cancer, lung cancer, and head and neck cancer *(58)*. Such mutations have been detected in paired body fluids, including urine, salivary fluid, and bronchoalveolar lavage *(59)*. Recently, similar findings, although at a lower frequency, have been reported in patients with early prostate cancer, and similar mutations could sometimes be detected in plasma *(60)*. Detailed studies of the correlation of tumor-derived mitochondrial DNA mutations in plasma and clinical parameters remain to be performed.

Plasma mRNA Detection

Surprisingly, cell-free mRNA can be detected in plasma by use of a similar methodology and should, at least in theory, permit plasma-based expression profiling. It has been suggested that plasma RNA may circulate within apoptotic bodies that protect it from degradation *(61)*. In patients with cancer, RNA may arise from tumor-associated viruses [for example, DNA viruses, such as hepatitis B virus (HBV), that have a RNA genome as an obligatory part of their replicative strategy] or directly from the tumor.

EBV-encoded small RNA transcripts have been detected in the plasma of patients with NPC. The test was very sensitive for NPC (88%), but the presence of EBV-infected B lymphocytes in many healthy individuals has led to false-positive results and a lower specificity of only 70% *(62)*. HBV replicates by reverse transcription of a RNA intermediate, and this full-length transcript (fRNA) and a truncated variant (trRNA) can both be detected in plasma, where they have been proposed as markers of occult HBV infection *(63)*.

Kopreski et al. *(64)* described detection of tumor-derived tyrosinase mRNA in 4 of 6 patients with metastatic melanoma but in none of 20 healthy controls. These results have been confirmed by others *(65)*. Aberrant telomerase activity has been found in almost all human cancers *(66)*, and two research groups have recently further evaluated the usefulness of telomerase mRNA as a molecular tumor marker in the serum/plasma of patients in a wider variety of cancer types. Chen et al. *(67)* detected *hTR* (telomerase RNA template) and *hTERT* (telomerase reverse transcriptase) transcripts in 17 of 18 (94%) primary breast tumors and in 28% and 25%, respectively, of serum samples from the same patients. Using real-time quantitative reverse transcription-PCR analysis, Dasi et al. *(68)* showed that the concentrations of *hTERT* transcripts in the plasma of patients with colorectal cancer and follicular lymphoma were significantly higher than concentrations in the plasma of healthy controls. Thus, assigning the maximum concentration observed in the control group as the cutoff point for a positive result, the authors detected *hTERT* transcripts in the plasma of eight of nine colorectal cancer patients and nine of nine follicular lymphoma patients. These encouraging findings suggest that real-time reverse transcription-PCR can enhance the sensitivity of the detection of tumor-derived RNA in patients' plasma. Plasma telomerase RNA, being consistently involved in the tumorigenesis of human cancers, is clearly worthy of more detailed investigation in a wider range of malignancies and at different stages. mRNAs from other tumor types are currently under investigation as potential molecular markers *(69–71)*. For example, in lung cancer, several potentially useful mRNAs have been investigated, and either of two, *hnRNP-B1* and *Her2* RNA, was detectable in all cases studied *(72)*. Future challenges of the circulating RNA field lie in the quantitative, temporal, and clinical correlation of plasma RNA data.

Prospects and Challenges

It seems likely that over the next decade plasma DNA will join a growing band of clinically useful molecular markers of cancer. Of particular interest is the growing belief that the study of bodily fluids such as plasma/serum may provide a more global picture of the abnormalities present in the tumor. By contrast, sampling error is always a concern in conventional histologic diagnosis. Furthermore, by focusing on the direct clinical application of plasma nucleic acid detection, we should not forget that the possibility of expression profiling using plasma may be a realistic possibility and that, most exciting of all, circulating DNA may have some degree of functionality and be involved in the process of metastasis, so-called "genometastasis" *(73)*.

Exciting as the above results may be, they beg several questions. How do the tumor-derived nucleic acids enter plasma and, for that matter, other bodily fluids? Is it an active or a passive process, and why should there be an apparent predilection of the tumor-related DNA to enter the circulation? In what forms do the nucleic acids circulate; for example, in tumors associated with viral infections, is the circulating viral DNA small random frag-

ments of DNA or entire virions? What protects plasma RNA from circulating RNases? How crucial are the procedures used to prepare samples for analysis? How will the plasma-based techniques described here perform when transferred to other bodily fluids?

At a technical level, a variety of analytical and preanalytical issues remain to be addressed. For example, different groups of investigators have used different nucleic acid extraction protocols for plasma and serum. There have been relatively few quantitative studies formally comparing the performances of these different extraction protocols. For future large-scale application of plasma/serum DNA technology, automation of nucleic acid preparation would be necessary. Nucleic acid preparation systems are beginning to be evaluated by several investigators in the circulating nucleic acid field *(74)*. Preanalytical issues, such as a formal comparison of plasma and serum *(75)* and the effects of different centrifugation protocols *(76)*, are also being explored. It is hoped that the resolution of these issues will allow reliable cross-center comparison of results obtained using plasma or serum nucleic acids.

At the clinical level, the entire question of how to estimate the sensitivity and specificity of a molecular tumor marker that is potentially more sensitive than the currently available "gold standard" is fraught with difficulties, and the wide variety of methodologies used makes comparisons between different series difficult. Do the plasma concentrations of markers such as EBV DNA really reflect tumor mass? What is the significance of modestly increased serum concentrations of EBV DNA in patients with tumors in which an association with EBV DNA is not usually envisioned *(77)*? How are concentrations related to the degree of immunosuppression consequent on the underlying cancer? These and many more questions will keep the field of circulating nucleic acids in cancer an exciting area of research for many years to come.

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