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# Serum DNA can define tumor-specific genetic and epigenetic markers in gliomas of various grades

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**We evaluated whether cell-free circulating DNA can be used as a noninvasive approach for detection of genetic/epigenetic alterations in brain tumors during the course of the disease. Paired tumor-serum samples from 70 patients with either high-grade astrocytomas ($n = 41$) or oligodendrogliomas of various grades were analyzed. The median interval between surgery and serum sampling was 1 month (range 0.5–168 months). DNA was extracted from whole blood, serum, and paraffin-embedded tumor sections. Loss of heterozygosity (LOH) in chromosomes 1p, 19q, and 10q was assessed by polymerase chain reaction (PCR)-based microsatellite analysis. The methylation status of $O^6$-*methyl guanine methyltransferase* (*MGMT*) and *phosphatase and tensin homolog* promoters was studied by methylation-specific PCR. LOH and/or methylation that could identify DNA as tumor-specific was found in 80.5% of astrocytic tumors and in all oligodendrogliomas. The rate of serum detection of these biomarkers was 51% and 55%, respectively, with specificity around 100%. The rate of serum detection did not differ between low- and high-grade oligodendrogliomas. Statistically significant tumor-serum concordance was found for *MGMT* methylation in both astrocytic tumors (83%; $P < .001$) and oligodendroglial tumors (72%; $P < .003$) and for LOH of 10q (79%; $P < .002$) and 1p (62%; $P < .03$) in oligodendrogliomas. We conclude that serum DNA in glial tumors is informative for both LOH and aberrant gene promoter methylation analysis during the course of the disease. The sensitivity is moderate and specificity is high for both low- and high-grade tumors. Future studies should identify a panel of biomarkers that bear the highest potential for clinical application.**

**Keywords:** allelic loss, astrocytoma, chromosomal deletion, circulating DNA, glioblastoma multiforme, glioma, MGMT, promoter methylation, PTEN

Extracellular nucleic acids occur ubiquitously, because they are released by dying cells. There are several sources of extracellular DNA. One of them is necrotic cell death that releases high-molecular-weight DNA into the bloodstream and another is programmed cell death (apoptosis) that continues throughout life and represents the most common form of cell death.[1] Apoptotic cells release truncated DNA fragments that are usually 185–200 bp in length.[2] Cell-free circulating DNA levels are elevated in many medical conditions, including autoimmune disorders, infectious diseases, pregnancy-associated disorders, trauma, and cancer.[3,4] Almost all extracellular DNA in the blood of healthy patients is associated with the surface of blood cells, which contains nucleic acid-binding proteins.[5] In contrast, cancer patients have an increased amount of the circulating extracellular DNA in their plasma, with only a negligible portion of this DNA bound to blood cell membranes.[4,6] It has been demonstrated that at least part of this free circulating DNA is tumor derived, based on evaluation of strand stability, tumor-specific gene aberrations, and tumor-related epigenetic alterations such as hypermethylation of tumor suppressor genes.[1,3–5,7–11] The ability to identify tumor-specific DNA alterations in the plasma leads to the possibility of utilizing circulating nucleic acids as a new generation of tumor markers. Recent studies have evaluated the potential application of tumor-derived circulating DNA as a diagnostic tool for either early detection of systemic cancer, prediction of tumor progression, or as a means to monitor the response to therapy.[3–5,7–10,12–14]

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Mechanisms for clearing circulating DNA include plasma nucleases and hepatic and renal clearance.[15] Injected DNA is cleared rapidly from plasma, almost entirely by the liver. The degradation of single-stranded DNA begins immediately after injection, whereas removal of double-stranded DNA proceeds less rapidly because macrophages in the liver play an important role in the latter.[16] Although brain tumors may shed free DNA into the extracellular space at the same rate as systemic tumors, several anatomic and physiologic differences make it uncertain how much of this DNA may reach systemic circulation while it is still detectable. Primary brain tumors are confined to the cranial vault, where their extracellular space drains largely into the cerebrospinal fluid (CSF), which, following circulation, will eventually clear into the bloodstream. This sink effect of the CSF may substantially dilute the amount of detectable circulating nucleic acids in the blood samples of patients with brain tumors. In addition, the lack of hallmarks of systemic tumors such as circulating tumor cells and metastatic spread to other organs probably reduces the bulk of tumor that has the potential to release DNA into proximal vascular compartments. Two previous small studies evaluated the levels of circulating DNA in primary brain neoplasms. One study contained 28 patients with glioblastoma multiforme (GBM) and the other included 10 patients, 6 of them with GBM.[12,17] Both series obtained blood samples at the time of brain tumor surgery and showed that gene promoter methylation can be detected in the bloodstream. So far, the feasibility of analyzing chromosomal allelic losses in the circulating DNA of patients with brain tumors has not been studied.

Numerous challenges exist for the possible use of brain tumor-derived circulating DNA as a diagnostic and research tool. It is not clear whether extracellular DNA of brain tumors can be used for detection of both aberrant gene promoter methylation and microsatellite alterations. It is also unclear whether these alterations can be detected in the bloodstream only at the time of surgery or anytime during the course of the disease. The relevance of changes seen in circulating DNA to a tumor's DNA aberrations has not thoroughly been evaluated before. Furthermore, it is unknown whether free circulating DNA is equally informative in low-grade gliomas as in high-grade tumors. Finally, as there is no standard sample preparation protocol for circulating DNA analysis, it is not known which DNA fragments, high-molecular-weight or low-molecular-weight DNA, yield maximal detection.

In the current study, we evaluated paired tumor and serum DNA of 70 glial tumors of various grades and processed it for detection of chromosomal allelic losses and aberrant gene promoter methylation. All serum samples were obtained weeks away from surgery and concordance between serum and tumor DNA was evaluated. The yield of both low- and high-molecular-weight DNA extractions was evaluated in relation to tumor-derived circulating detectable markers.

## Patients and Methods

### *Serum Samples and Clinical Information*

Paired tumor and serum samples of 70 patients with glial tumors were analyzed. The study was based on samples sent routinely to the laboratory for analysis of chromosomal allelic losses and gene promoter methylation status. DNA was extracted from formalin-fixed paraffin-embedded sections of the tumor and from whole blood and serum samples that were obtained at the time that the treating physician requested the analysis. All patients gave consent for DNA analysis of the tumor samples, whole blood, and serum according to guidelines set forth by Hadassah Hebrew University Medical Center institutional review board. We analyzed 70 consecutive cases based on the availability of good-quality DNA.

All astrocytic tumors ($n = 41$) were of high grade whereas the oligodendroglial tumors ($n = 29$) included 15 (52%) low-grade neoplasms. Table 1 describes the clinical information in relation to patients' demographics, tumor type, imaging characteristics, and treatment. The information was obtained from the primary treating physicians who were requested to fill out a questionnaire, or from the patient's hospital file. As the study was based on routine clinical demands for the genetic analysis, 60% (42/70) of the patients were diagnosed and treated at other medical centers and therefore some data related to patients' characteristics are missing.

The median interval between tumor and serum sampling was 1 month for both astrocytic and oligodendroglial tumors, but the wide range reflects the presence of patients with long follow-up times. Blood samples were obtained more than 12 months after surgery in 5 (12%) patients in the astrocytic group and more than 48 months after tissue sampling in 6 (21%) patients with oligodendroglial tumors. All serum samples were obtained after surgery and, in 40% of the patients, prior to radiotherapy.

### *DNA Extraction from Tumor and Blood*

Genomic DNA was isolated from whole blood, serum, and tumor samples. DNA was extracted from 5 mL of whole blood using the standard salting-out method (Miller SA Nucleic Acids Research 1988). DNA was purified from 200 μL of serum, either by the salting-out method to yield high-molecular-weight DNA or by "High Pure Viral Nucleic Acid Kit" (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol, to get low-molecular-weight DNA. Tumor DNA was extracted from paraffin-embedded tissue using the Paraffix kit (Syntezza Bioscience, Jerusalem, Israel) according to the manufacturer's instructions.

DNA isolated from the serum of 20 healthy donors served as normal controls for the assessment of circulating DNA genetic and epigenetic aberrations.

**Table 1.** Patients characteristics at time of serum sampling

| | Astrocytic tumors | Oligodendroglial tumors |
|---|---|---|
| Number of patients | 41 | 29 |
| Tumor grade | | |
| WHO II | — | 14 (48%) |
| WHO III | 12 (29%) | 15 (52%) |
| WHO IV | 29 (71%) | — |
| Gender | | |
| Male | 26 (63%) | 14 (48%) |
| Female | 15 (37%) | 15 (52%) |
| Age (y) | | |
| Median (range) | 58 (19–76) | 42.7 (28–68) |
| Surgical procedure | | |
| Craniotomy | 28 (68%) | 15 (52%) |
| Stereotactic biopsy | 8 (20%) | 5 (17%) |
| Not specified | 5 (12%) | 9 (31%) |
| KPS | | |
| Median (range) | 75% (40%–90%) ($n = 34$) | 90% (60%–100%) ($n = 26$) |
| Preoperative MRI | | |
| Contrast enhancement | 30 (73%) | 12 (45%) |
| No enhancement | 6 (15%) | 8 (28%) |
| Not specified | 5 (12%) | 8 (28%) |
| Interval between surgery and serum sampling (mos) | | |
| Median (range) | 1 (0.5–158) | 1 (0.5–168) |
| Treatment at time of serum sampling | | |
| Postsurgery pre-RT | 24 (59%) | 4 (14%) |
| Ongoing RT | 3 (7%) | 1 (3%) |
| Ongoing chemotherapy | 5 (12%) | 1 (3%) |
| Surveillance | — | 13 (45%) |
| Not reported | 9 (22%) | 10 (34%) |

KPS, Karnofsky performance status; RT, radiotherapy.

### *Microsatellite Analysis*

For the PCR-based loss of heterozygosity (LOH) analysis, 8 primer pairs of the microsatellte loci labeled with 1 of 3 fluorochromes, FAM, HEX, or NED, were obtained from Applied Biosystem (Foster City, California) on chromosomes 1p, 19q, or 10q as follows: For 1p LOH: D1S199: 5′-GGTGACAGAGTG AGACCCTG-3′, 5′-CAAAGACCATGTGCTCCGTA-3′ (reverse primer); D1S226: 5′-GCTAGTCAGGCA TGAGCG-3′ (forward primer), 5′-GGTCACTTGACA TTCGTGG-3′ (reverse primer). D1S186: 5′-TAGCT CATCCCCCCCTTTCT-3′ (forward primer), 5′-CCCC TCCTTCCTGCCGCT-3′ (reverse primer); D1S312: 5′-CAGCCTTCCCCACAACTTTA-3′ (forward primer), 5′-TTCCAAACAGCAGGGGAG-3′ (reverse primer). For 10q LOH: *phosphatase and tensin homolog* (*PTEN):* 5′-GTTAGATAGAGTACCTGCA CT-3′ (forward primer), 5′-TTATAAGGACTGAG TGAGGGA-3′ (reverse primer); D10S1765: 5′-ACA CTTACATAGTGCTTTCTGCG-3′ (forward primer), 5′-CAGCCTCCCAAAGTTGC-3′ (reverse primer). For 19q LOH D19S112: 5′-GCCAGCCATTCAGTCAT TTGAAG-3′ (forward primer), 5′-CTGAAAGACACG TCACACTGGT-3′ (reverse primer); D19S918 5′-AAA GGCTTGATTACCCCCGA-3′ (forward primer), 5′-GA TTACAGGCGTGAGCACCG-3′ (reverse primer).

Genomic DNA isolated from the peripheral blood lymphocytes of all tumor patients served as the internal control for LOH. In addition, DNA isolated from serum of healthy donors served as the normal control for circulating LOH. PCR was performed on each patient's samples (normal lymphocyte DNA, tumor DNA, and serum DNA) in a final volume of 25 μL containing 2 primer pairs (3 pmol of each primer), 25 ng of DNA, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 0.2 mM deoxyribonucleoside triphosphates, 2.5 mM $MgCl_2$, and 0.6 unit of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, California). PCR cycling conditions are 95°C for 9 minutes once, 42 cycles at 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 60 seconds, followed by a final elongation step of 45 minutes at 60°C. Amplified PCR products were electrophoresed in denaturing 5% polyacrylamide gels on an ABI Prism 310 automated DNA sequencer (Applied Biosystems, Foster City, California). The collected data analysis was performed by GeneScan Analysis software version 3.1 (Applied Biosystems). LOH was inferred by a 70% reduction of

allele signal intensity in tumor samples relative to matched corresponding blood DNA specimens.

### *Analysis of Promoter Methylation Status of $O^6$-Methyl Guanine Methyl Transferase and PTEN*

Methylation status of *$O^6$-Methyl guanine methyl transferase (MGMT)* and *PTEN* promoters was determined on tumor DNA and serum DNA. Genomic DNA (500 ng) from each sample was chemically modified by sodium bisulfite to convert all unmethylated cytosines to uracils while leaving methylcytosines unaltered (EZ DNA methylation kit; Zymo Research, Orange, California). Two to four microliter aliquots of converted DNA were subjected to methylation-specific PCR (MSP) using specific primer pairs designed for amplifying the methylated allele of each promoter. The relative level of methylation was normalized using the ratio obtained from the values of the gene of interest over the values of the unmethylated reference gene (β-actin).

*MGMT* methylated reaction: 5′-TTTCGACGTTCG TAGGTTTTCGC-3′ (forward primer) and 5′-GCACT CTTCCGAAAACGAA ACG-3′ (reverse primer). *PTEN* methylated reaction: 5′-GTTTGGGGATTTTTTTTT CGC-3′ (forward primer) and 5′-AACCCTTCCTAC GCCGCG-3′ (reverse primer); unmethylated reference gene (β-actin): 5′-AGGGAGTATATAGGTTGGGGA AGTT-3′ (forward primer); 5′-AACACACAATAACAA ACACAAATTCAC-3′ (reverse primer).

PCR was performed using the following conditions: an initial melting step of 10 minutes at 95°C; followed by 50 cycles of 20 seconds at 95°C, 20 seconds at 59°C, and 45 seconds at 72°C; and a final elongation step of 4 minutes at 72°C in Gene Amp 9700 thermocycler (Applied Biosystems) using AmpliTaq Gold DNA polymerase (Applied Biosystems). Amplified products were separated on 3.5% methaphore gel and visualized under UV illumination. Genomic DNA isolated from the serum of healthy donors served as normal controls for the assessment of tumor-related gene methylation.

### *Statistical Analysis*

Concordance between biomarkers (*MGMT* and *PTEN* promoter methylation, LOH of 10q, 1p, 19q, and 10q) in tumor tissue and serum was assessed by Kappa measure of agreement. Association was tested using $\chi^2$ test. Sensitivity was defined as the probability of detecting positive serum findings among patients with the presence of the tumor's biomarker and specificity as the probability to detect negative serum findings among patients without tumor biomarkers. A $P$ value less than .05 was considered significant.

## Results

The amount of circulating genomic DNA that was extracted from normal controls and from all brain-tumor patients was sufficient to perform the assays and to yield results. The average DNA amount extracted from serum of healthy donors was 265 μg/mL (ranging between 149 and 365) and the average amount extracted from brain-tumor patients was 258 μg/mL (ranging between 183 and 280).

Tumor markers that were assessed in the serum samples match those routinely analyzed per request of the referring physicians. For astrocytic tumors, the markers included promoter hypermethylation of both *MGMT* and *PTEN* and LOH of 10q. For oligodendroglial tumors, *MGMT* promoter methylation and LOH analysis of 10q, 1p, and 19q were assessed. For each tumor, both total serum DNA (composed of both high- and low-molecular-weight DNA) and low-molecular-weight extracts were analyzed for the presence of tumor biomarkers. Comparison of the rate of detection of these biomarkers in the two DNA extracts revealed no significant difference in promoter hypermethylation. However, LOH detection was inferior in total DNA extract compared with low-molecular-weight serum DNA. For example, analysis of total DNA extracts for 10q and 1p yielded no detection of LOH in oligodendroglial tumors in 43% and 53% of tumors, respectively, whereas in GBM 10q was not detected in 70% of cases. Therefore, all data below refer to the results of serum low-molecular-weight DNA analysis. It is important to note that in our study, neither hypermethylation nor LOH were detected in circulating low-molecular-weight DNA extracted from serum of normal donors. The rates of detection for each marker in the study cohort, as well as the concordance between tumor and serum findings, are presented in Table 2. High and significant concordance was noted for *MGMT* promoter methylation, regardless of tumor type, and for LOH of 10q and 1p in oligodendroglial tumors. It is worth noting that there were 19 discordant findings of 10q LOH in astrocytic tumors and 6 in the oligodendroglioma group. Interestingly, deletion of 10q was detected in serum, but not in 3 (16%) astrocytic tumors or in 1 (17%) oligodendroglial tumor.

There were 33 (80.5%) astrocytic tumors that contained biomarkers (*MGMT* promoter methylation and/or 10q LOH) that are potentially detectable in circulating serum DNA (excluding *PTEN*, which had null serum detection). Interestingly, all oligodendroglial tumors carried detectable biomarkers (*MGMT* promoter methylation and/or 10q LOH and/or 1p/19q LOH). The disparity in the ratio of tumors containing detectable markers had no significant impact on the rate of serum findings in either astrocytic or oligodendroglial tumors, which was 17 of 41 (41.5%) and 16 of 29 (55%), respectively. In fact, the 51% (17 of 33) rate of positive serum findings in astrocytic tumors containing detectable tumor-specific biomarkers was remarkably similar to the rate in oligodendroglial tumors (55%). Table 2 shows that despite the fact that the sensitivity of serum testing is moderate across all analyzed markers, the specificity is remarkably high, indicating that this approach is a potentially attractive research tool to be tested in the future. We have analyzed the possibility that the use of multiple markers as opposed to a single tumor marker will improve the

**Table 2.** Detection of tumor-specific biomarkers in tumor and serum samples

| Biomarker | Biomarker detected | | Tumor–serum concordance | Sensitivity (%) | Specificity (%) | *P*-value, Kappa[a] |
|---|---|---|---|---|---|---|
| | Tumor | Serum | | | | |
| Astrocytic tumors | | | | | | |
| 10q LOH | 26/41 (63%) | 12/41 (29%) | 21/41 (51%) | 35 | 80 | NS, 0.12 |
| *MGMT* methylation | 17/41 (41.5%) | 10/41 (24%) | 34/41 (83%) | 59 | 100 | <.001, 0.63 |
| *PTEN* methylation | 8/34 (23.5%) | 0/34 (0%) | 26/34 (76.5%) | 0 | 75 | NA |
| Oligodendroglial tumors | | | | | | |
| 10q LOH | 12/29 (41%) | 8/29 (27.5%) | 23/29 (79%) | 58 | 94 | <.002, 0.55 |
| 1p LOH | 16/29 (55%) | 5/29 (17%) | 18/29 (62%) | 31 | 100 | <.03, 0.28 |
| 19q LOH | 15/28 (54%) | 1/23 (4%) | 7/23 (30%) | 7 | 50 | NS, −0.05 |
| *MGMT* methylation | 15/29 (52%) | 7/29 (24%) | 21/29 (72%) | 47 | 100 | <.003, 0.46 |

[a]Kappa measure of concordance.
LOH, loss of heterozygosity; NA, not applicable.

**Table 3.** Use of a single versus multiple tumor biomarkers and sensitivity of their serum detection in 29 oligodendroglial tumors

| Biomarkers | No. of biomarkers tested | Biomarkers in tumor samples | Serum | | *P*-value, Kappa[a] |
|---|---|---|---|---|---|
| | | | Sensitivity | Specificity | |
| 1p LOH | 1 | 55% (16/29) | 31% (5/16) | 100% | <.03, 0.28 |
| 1p LOH and/or 10q LOH | 2 | 86% (25/29) | 44% (11/25) | 75% | NS, 0.08 |
| 1p LOH and/or *MGMT*-M | 2 | 76% (22/29) | 50% (11/22) | 100% | <.018, 0.33 |
| 10q LOH and/or *MGMT*-M | 2 | 83% (24/29) | 54% (13/24) | 100% | <.03, 0.29 |
| 1p LOH and/or 10q LOH and/or *MGMT*-M | 3 | 100% (29/29) | 55% (16 /29) | NA | — |

[a]Kappa measure of concordance.
LOH, loss of heterozygosity; MGMT-M, MGMT methylation; NA, not applicable.

tumor-serum concordance and the sensitivity of the test. To that end, each marker was tested against the combined use of 2 or 3 markers. Table 3 shows the results for oligodendrogliomas. We tested 1p LOH, which is the single most abundant marker in oligodendrogliomas (detected in 55% of tumors), against the combined use with 10q and/or *MGMT*. The sensitivity of serum detection increased from 31% when 1p LOH was used alone to 55% with the use of all 3 markers. Thus, the concurrent use of 3 markers increased the sensitivity of the analysis and tripled the rate of detectable tumor-specific biomarkers in the serum from an overall 17% (5 of 29) to 55% (16 of 29).

Assuming that rapid turnover of cellular components is associated with shedding of tumor-specific DNA into the circulation, it could be possible that, in low-grade tumors, free circulating DNA will be less readily detectable. In this study, 52% of the oligodendrogliomas were of low grade and interestingly the rate of detection of tumor-specific serum markers was remarkably similar in low-grade (8 of 15; 53%) and high-grade (8/14; 57%) oligodendrogliomas. These rates resembled those found in high-grade astrocytic tumors.

The study of *PTEN* promoter methylation status found 20 of 34 (58.8%) unmethylated promoters, 8 of 34 (23.5%) fully methylated promoters, and 6 of 34 (17.6%) partially (less than 50%) methylated promoters in tumor samples. However, there was no detection of *PTEN* promoter methylation in the serum and, therefore, it is not recommended as a tumor-specific serum biomarker for future use. Analysis of oligodendroglial tumor markers revealed a significant statistical association between 10q and *MGMT* promoter methylation status ($\chi^2 = 5.86$; $P = .016$) and between 10q and 1p status ($\chi^2 = 5.4$; $P = .02$). Most tumors with a methylated *MGMT* promoter contained no 10q loss, and a 1p deletion was notable in tumors with an intact 10q chromosome. No significant association was found between the status of the 10q chromosome and *MGMT* promoter in astrocytic tumors.

We retrospectively evaluated the available data for concordance between the presence of tumor biomarkers in the serum and a documented tumor mass on MRI study that has been performed within 2 weeks of serum sampling. Table 4 shows that of the 62 tumors that contained at least 1 potentially detectable biomarker, such MRI reports were available for 38 (61%). Measurable tumors were present in 29 patients (76%) and the others showed only postoperative changes. The sensitivity of serum testing in patients harboring a measurable tumor by MRI criteria is 65.5% ($\kappa = 0.33$; $P < .02$), which is higher than the overall sensitivity presented above. There was no significant difference in the rate of detection between astrocytic and oligodendroglial tumors. The serum of 2 patients out of the 9 with no measurable tumors was positive

**Table 4.** Concordance between serum findings and MRI assessment of tumor mass

| | Astrocytic tumors | Oligodendroglial tumors | All tumors |
|---|---|---|---|
| Number of tumors containing at least 1 biomarker | 33/41 (80.5%) | 29/29 (100%) | 62/70 (88.5%) |
| MRI performed within 2 wk of serum sampling (available reports) | 22/33 (66.6%) | 16/29 (55%) | 38/62 (61%) |
| MRI shows a measurable tumor | 15/22 (68%) | 14/16 (87.5%) | 29/38 (76%) |
| Serum positive for tumor biomarkers | 10/15 (66.6%) | 9/14 (64%) | 19/29[a] (65.5%) |
| MRI shows no measurable tumor | 7/22 (32%) | 2/16 (12.5%) | 9/38 (24%) |
| Serum negative for tumor biomarkers | 6/7 (86%) | 1/2 (50%) | 7/9[a] (78%) |

[a]Kappa measure of concordance = 0.33; $P < .02$; sensitivity = 65.5%; specificity = 78%

for tumor biomarkers (both with anaplastic WHO III tumors). The meaning of these findings in terms of imminent tumor recurrence is unclear and requires prospective evaluation in future studies.

## Discussion

The results reported here indicate that circulating DNA is a potentially promising source of tumor-specific biomarkers in patients with gliomas of various grades. Unlike systemic tumors that may secrete specific proteins that currently serve as tumor markers (eg, carcinoembryonic antigen, alpha fetoprotein, and prostate-specific antigen), brain tumors release no such biomarkers into the bloodstream. The development of molecular techniques has opened up the potential of utilizing circulating nucleic acids as prospective tumor markers for brain tumors as suggested by our study. Similar findings have been recently demonstrated for systemic malignancies.[8,10,13,14,18–20] This approach may also provide personalized identification of tumor-specific biomarkers in serum samples once genetic and epigenetic aberrations have been characterized in the tumor specimen. We have demonstrated that these circulating tumor-specific biomarkers can be detected at any time during the course of the disease and once detected indicate that a tumor is probably present. The latter assumption is based on previous studies that evaluated tumor-specific circulating DNA in colorectal tumors and estimated that the half-time of circulating DNA after surgery is 114 minutes.[19,21] With such a short postsurgery half-life, the continuous presence of tumor-specific DNA in the circulation indicates that the tumor is probably constantly shedding DNA fragments into the bloodstream. In our series, all blood samples were obtained weeks after surgery, with the earliest samples collected 2 weeks after brain tumor removal (Table 1). At that time, the DNA that was presumably released during the procedure into the bloodstream should have completely been cleared out of the circulation. We cannot exclude that in some patients the circulating DNA represents the effect of treatment on the tumor, but in the majority of glial tumors neither radiation therapy nor chemotherapy can eradicate this malignancy. In fact, we were able to show that a statistically significant concordance exists between positive serum findings and the presence of a measurable tumor on recent MRI studies (Table 4). However, this evaluation was performed retrospectively and therefore it requires further verification. We plan future prospective studies that will correlate circulating tumor-specific DNA with clinical and imaging parameters based on the findings of this study, which showed that it is feasible to detect both genetic and epigenetic aberrations in the serum of brain tumor patients at any point during the course of the disease.

The results presented in the current study constitute a framework for a possible future practice in which circulating tumor-specific DNA will be utilized for tumor monitoring. For that purpose, the sensitivity and specificity of the test should be well established. We found an overall moderate sensitivity for detection of both chromosomal allelic deletions and *MGMT* promoter methylation (Table 2) that is probably escalated once a measurable tumor mass is detectable on MRI (Table 4). However, the specificity is very high, approaching 100%, and both built-in controls in LOH studies and cell-free circulating DNA of normal donors never shows these genetic and epigenetic abnormalities. This is not surprising as the selected molecular markers are not expected to occur in the absence of malignant transformation. Our findings in brain tumors are similar to previous studies that evaluated the sensitivity of circulating tumor-specific DNA in systemic malignancies.[3,7,10,14,20,22,23] Regardless of the type of molecular biomarkers tested, the average reported sensitivity in systemic tumors is less than 40% with a range of 6%–88%.[3,7,14] The relatively low detection rate is probably related to several factors. First, it should be noted that circulating tumor-specific DNA represents only a tiny fraction of the total circulating nucleic acids, sometimes <0.01%.[8,21] Therefore, the reliable detection of such small DNA fragments is still challenging. Second, when the biomarker is a gene's promoter methylation, an additional factor, the bisulfite conversion step, becomes an issue. The bisulfite conversion, which is used in MSP, might be associated with substantial degradation of DNA that could encompass up to 84%–96% of the DNA.[24] In addition, incomplete modification of the DNA is one of the most common sources of false results.[25] Last, when a limited panel of genetic and epigenetic alterations is used as molecular biomarkers, like in this study, it carries the default that a certain proportion of tumors may not express them. These drawbacks have recently been addressed by several investigators who studied systemic tumors and have tried to increase the sensitivity and reliability of circulating tumor-specific DNA detection.[18,19,21] A recent

workshop that focused on identifying and overcoming the existing barriers in the application of methylated genes as cancer biomarkers has tried to promote validation studies of these biomarkers.[25]

As there is no standard protocol for sample collection and processing for the assessment of circulating nucleic acids, it seems that future efforts should focus on standardization, improved sensitivity, and validation studies of various biomarkers selected for clinical applications. Issues related to methodology should be specifically addressed in the future to improve sensitivity and reliability of outcome. For example, published studies that evaluated circulating nucleic acids used either plasma or serum, utilizing diverse protocols for sample collection and handling. Several studies showed that serum is a better source for circulating DNA than plasma because serum contains significantly higher amounts of DNA with a low level of contaminating extraneous DNA released from leukocytes.[13,26–28] As the time delay and storage temperature of blood before centrifugation has an impact on total DNA concentration, we selected to assess serum rather than plasma because the majority of the samples in our series were collected and shipped to our laboratory from other medical centers.

Another methodological issue is related to the method used for DNA extraction and whether to use total versus low-molecular-weight DNA for the analysis of brain tumor molecular biomarkers. Studies that evaluated systemic tumors suggested that an elevated level of long DNA fragments is present in the circulation and that this may be positively correlated to the size of the invasive cancer.[1,13] Likewise, large variations in the amount and composition of DNA were found in the circulation.[1] In our hands, low-molecular-weight DNA extract yielded a better rate of biomarker detection, but this matter may be subjected to future methodological modifications. Another aspect that is being increasingly recognized is the necessity to have a non-gel-based assay for methylation, especially if the studied markers are used for diagnostic procedures.[25] A quantitative measurement is important for clinical application as it may improve comparison of results across laboratories and provide prognostic significance and means for monitoring residual disease or early recurrence. Numerous new techniques are being applied, assessed, and validated for quantitative analysis of tumor samples and body fluids.[18–21,25,29,30] We have no doubts that future studies in brain tumors that seek to assess circulating tumor-specific DNA should incorporate such quantitative methods.

The panel of circulating biomarkers that were evaluated in our study matched the demands of neuro-oncologists for molecular analysis of tumor samples. These demands are driven by the independent prognostic information that these biomarkers carry (eg, methylation status of *MGMT* and 1p/19q LOH status) for either high-grade astrocytic tumors or oligodendrogliomas, and largely fall into the category of prognostic biomarkers.[31] However, these biomarkers are not necessarily the most common genetic and epigenetic alterations that can be identified within the tumor as *MGMT* promoter methylation characterizes only about 40% of all grade IV astrocytomas and 1p/19q LOH are detected in about 60% of oligodendrogliomas.[30,32–35] Recent studies have shown that other markers in astrocytic tumors, such as RASSF1A, RUNX3, SOCS1, TES, and CDKN2A, commonly present promoter hypermethylation,[36,37] yet their applicability as serum biomarkers has never been examined. In the current study, the limited panel of biomarkers used flagged the DNA of all oligodendrogliomas and 80.5% of astrocytic tumors as tumor-specific based on the presence of at least 1 biomarker in the analyzed tissue sample. Detection of any of these markers in the serum practically tags the circulating DNA as tumor specific as their presence in non-neoplastic tissue is not expected. We also found that the use of multiple markers increased the rate of serum detection (Table 3), but it is not yet known which combination of probes would be the most efficient for maximizing the sensitivity of the serum assay. We have also showed that the sensitivity of serum testing is highest (65.5%) in the presence of a measurable tumor on imaging, indicating that, in the future, serum assessment may serve as a confirmatory test for tumor activity. Future studies should address this issue along with others discussed above.

Finally, we found that the rate of detection of circulating tumor-specific DNA was similar in low-grade oligodendrogliomas and in high-grade tumors. This finding suggests that the serum analysis might turn out to be a noninvasive and sensitive indicator for the presence of brain tumor, not necessarily indicating its aggressiveness. Previous studies in systemic cancers noted that circulating tumor-specific DNA can be identified in early localized tumors as well as in metastatic ones and that quantitative evaluation probably carries prognostic implications as it may correlate with the bulk of the tumor and its invasiveness.[7,8,18–20,22] Whether similar correlations can be demonstrated in glial tumors is still unknown. Our study proved that DNA of gliomas of various grades can be detected in the circulation during the course of the disease and that it is feasible to study brain tumor DNA noninvasively. In the future, more sensitive and accurate detection may provide multiple applications in the follow-up and management of brain tumor patients.

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