

Detection of tumor-derived DNA in cerebrospinal fluid of patients with primary tumors of the brain and spinal cord

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Contributed by Bert Vogelstein, June 18, 2015 (sent for review April 24, 2015; reviewed by Charles David James, James T. Rutka, and John H. Sampson)

Cell-free DNA shed by cancer cells has been shown to be a rich source of putative tumor-specific biomarkers. Because cell-free DNA from brain and spinal cord tumors cannot usually be detected in the blood, we studied whether the cerebrospinal fluid (CSF) that bathes the CNS is enriched for tumor DNA, here termed CSF-tDNA. We analyzed 35 primary CNS malignancies and found at least one mutation in each tumor using targeted or genome-wide sequencing. Using these patient-specific mutations as biomarkers, we identified detectable levels of CSF-tDNA in 74% [95% confidence interval (95% CI) = 57–88%] of cases. All medulloblastomas, ependymomas, and high-grade gliomas that abutted a CSF space were detectable (100% of 21 cases; 95% CI = 88–100%), whereas no CSF-tDNA was detected in patients whose tumors were not directly adjacent to a CSF reservoir ($P < 0.0001$, Fisher's exact test). These results suggest that CSF-tDNA could be useful for the management of patients with primary tumors of the brain or spinal cord.

CSF-tDNA | CNS tumors | biomarker

Approximately 25,000 individuals each year are diagnosed with a malignant brain or spinal cord tumor in the United States, and more than one-half of these patients will die from their disease (1). Although there are a number of different subtypes of primary CNS cancers, nearly all are treated with maximal safe surgical resection followed by radiation and in some cases, chemotherapy. Given the lack of clinically available biomarkers for CNS malignancies, the conventional method for disease monitoring in these patients is radiographic using either computed tomography or MRI (2). Unfortunately, anatomic changes detected by these imaging modalities are often nonspecific and slow to change, even in the face of progressing or regressing disease. Moreover, it can be difficult to discriminate between treatment effect and cancer growth with imaging alone (3). Patients must, therefore, have additional surgeries for definitive tissue diagnosis or inappropriately wait for radiographic findings to change as their disease progresses. As a result, there is a great need for more sensitive and specific tumor biomarkers in neurooncology.

The recent success of detecting circulating tumor cells in the peripheral blood of glioblastoma patients represents an important step toward this goal, with reported sensitivities between 21% and 39% (4–6). Circulating tumor DNA (ctDNA) is found in the plasma of patients with most forms of malignancies (7–11). However, brain tumors, including high-grade gliomas and medulloblastomas, are an exception, with only a minority giving rise to detectable levels of ctDNA, perhaps because of the blood–brain barrier (8).

Other studies have shown that tumor-derived DNA can be found in anatomically relevant fluids, such as urine in bladder cancer patients, sputum in lung cancer patients, stool in patients with colorectal carcinomas, and endocervical fluid in patients with gynecological malignancies (12–17). Based on this concept, we wondered whether primary brain and spinal cord tumors might shed appreciable levels of tDNA into the cerebrospinal fluid (CSF) that bathes the CNS (Fig. 1). We coined the term “CSF-tDNA” to describe tumor DNA shed into the CSF. The experiments below were designed to test this hypothesis in an exploratory study of tumors of diverse histology and locations within the CNS.

Results

Patient and Tumor Characteristics. Thirty-five patients with CNS cancers were enrolled in this study. Their ages, sexes, races, and

Significance

Outcomes for individuals with central nervous system (CNS) malignancies remain abysmal. A major challenge in managing these patients is the lack of reliable biomarkers to monitor tumor dynamics. Consequently, many patients undergo invasive surgical procedures to determine disease status or experience treatment delays when radiographic testing fails to show disease progression. We show here that primary CNS malignancies shed detectable levels of tumor DNA into the surrounding cerebrospinal fluid (CSF), which could serve as a sensitive and exquisitely specific marker for quantifying tumor burden without invasive biopsies. Therefore, assessment of such tumor-derived DNA in the CSF has the potential to improve the management of patients with primary CNS tumors.

Author contributions: Y.W., S.S., M.Z., L.A.D., N.P., K.W.K., B.V., and C.B. designed research; Y.W., S.S., M.Z., K.W.M., I.K., L.D., J.P., L.A.D., N.P., K.W.K., B.V., and C.B. performed research; H.B., K.C., G.L.G., Z.L.G., M.L.G., G.I.J., M.L., A.O., A.Q.-H., D.R., G.J.R., D.M.S., J.D.W., J.-P.W., S.M.O.-S., S.K.N.M., M.H., N.A., B.V., and C.B. contributed new reagents/analytic tools; Y.W., S.S., M.Z., K.W.M., I.K., X.Y., N.P., K.W.K., B.V., and C.B. analyzed data; and Y.W., S.S., M.Z., L.A.D., N.P., K.W.K., B.V., and C.B. wrote the paper.

Reviewers: C.D.J., Feinberg School of Medicine, Northwestern University; J.T.R., The Arthur and Sonia Labatt Brain Tumor Research Centre, University of Toronto; and J.H.S., Duke University Medical Center.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1511694112/-DCSupplemental.

Table 1. Tumor characteristics and the detection of CSF-tDNA

Patient	Diagnosis	Tumor grade	Tumor location	Location of CSF sampling	Tumor abutting CSF space	CSF-tDNA
CGLI 02	Glioma	WHO II, diffuse astrocytoma	T11 spinal cord	Spinal subarachnoid space	Yes	Not detected
CGLI 03	Anaplastic astrocytoma	WHO III, anaplastic astrocytoma	Pons	Basal cistern	Yes	Positive
CGLI 06	Pilocytic astrocytoma	WHO I, pilocytic astrocytoma	Cerebellar vermis	Basal cistern	Yes	Positive
CGLI 11	Spinal ependymoma	WHO II, ependymoma	C7-T3 spinal cord	Spinal subarachnoid space	Yes	Positive
CGLI 12	Intracranial ependymoma	WHO II, ependymoma	Fourth ventricle	Basal cistern	Yes	Positive
CGLI 13	Myxopapillary ependymoma	WHO I, myxopapillary ependymoma	L2-3 spinal cord	Spinal subarachnoid space	NA	Positive
CGLI 14	Intramedullary spinal cord lesion	Low-grade neoplasm	T7-9 spinal cord	Spinal subarachnoid space	Yes	Positive
CGLI 15	Spinal ependymoma	WHO II, ependymoma	C3-4 spinal cord	Spinal subarachnoid space	No	Not detected
CGLI 20	Medulloblastoma	WHO IV, medulloblastoma	Fourth ventricle	Basal cistern	Yes	Positive
CGLI 22	Pilocytic astrocytoma	WHO I, pilocytic astrocytoma	Cerebellar hemisphere	Basal cistern	No	Not detected
CGLI 25	Myxopapillary ependymoma	WHO I, myxopapillary ependymoma	L2-3 spinal cord	Spinal subarachnoid space	Yes	Positive
CGLI 26	Intramedullary spinal cord tumor	WHO II, infiltrating astrocytoma with oligodendroglial features	C3-6 spinal cord	Spinal subarachnoid space	Yes	Positive
CGLI 28	Anaplastic astrocytoma	WHO III, anaplastic astrocytoma	Right frontal/butterfly	Ventricle	Yes	Positive
CGLI 29	Glioblastoma	WHO IV, glioblastoma	C4-6 spinal cord	Spinal subarachnoid space	Yes	Positive
CGLI 31	Glioblastoma	WHO IV, glioblastoma	Right frontal/butterfly	Ventricle	Yes	Positive
CGLI 35	Glioblastoma	WHO IV, glioblastoma	Right temporal	Ventricle	Yes	Positive
CGLI 36	Spinal cord glioblastoma	WHO IV, glioblastoma	T10-L1 spinal cord	Spinal subarachnoid space	Yes	Positive
CGLI 39	Intramedullary spinal cord tumor	WHO II, low-grade glioma likely ependymoma	C2-3 spinal cord	Spinal subarachnoid space	No	Not detected
CGLI 40	Medulloblastoma	WHO IV, medulloblastoma	Fourth ventricle	Basal cistern	Yes	Positive
CGLI 41	Glioblastoma	WHO IV, glioblastoma	Cerebellar hemisphere	Basal cistern	Yes	Positive
CGLI 42	Spinal ependymoma	WHO II, ependymoma	T1-7 spinal cord	Spinal subarachnoid space	NA	Positive
CGLI 43	Low-grade glioma	WHO II, low-grade glioma	T10 spinal cord	Spinal subarachnoid space	Yes	Not detected
CGLI 44	Pilocytic astrocytoma	WHO I, pilocytic astrocytoma	Cerebellar vermis	Basal cistern	No	Not detected
CGLI 47	Glioblastoma	WHO IV, glioblastoma	Right temporal	Ventricle	Yes	Positive
CGLI 48	Glioblastoma	WHO IV, glioblastoma	Left temporal	Ventricle	Yes	Positive
CGLI 50	Glioblastoma	WHO IV, glioblastoma	Right temporal	Ventricle	Yes	Positive
CGLI 51	Glioblastoma	WHO IV, glioblastoma	Right frontal	Ventricle	Yes	Positive
CGLI 55	Brainstem glioblastoma	WHO IV, glioblastoma	Midbrain	Basal cistern	Yes	Positive
CGLI 56	Medulloblastoma	WHO IV, medulloblastoma	Fourth ventricle	Basal cistern	Yes	Positive
CGLI 58	Diffuse astrocytoma	WHO II, diffuse astrocytoma	T2-4 spinal cord	Spinal subarachnoid space	Yes	Not detected
CGLI 60	Medulloblastoma	WHO IV, medulloblastoma	Fourth ventricle	Basal cistern	Yes	Positive
CGLI 61	Pilocytic astrocytoma	WHO I, pilocytic astrocytoma	Cerebellar vermis	Basal cistern	Yes	Not detected
CGLI 63	Medulloblastoma	WHO IV, medulloblastoma	Cerebellum	Basal cistern	No	Not detected
CGLI 101	Glioblastoma	WHO IV, glioblastoma	Cerebellar vermis	Basal cistern	Yes	Positive
CGLI 254	Medulloblastoma	WHO IV, medulloblastoma	Fourth ventricle	Basal cistern	Yes	Positive

NA, not available.

The mutations were found in the tumors at a high frequency, but they were absent in normal tissues.

Discussion

Minimally invasive techniques to monitor disease burden have been a challenge for many diseases of the CNS, including cancer. This challenge is highlighted by the high risks associated with

neurosurgical procedures and the widely recognized limitations of current imaging modalities. In cancer patients, there is no reliable way of parsing out treatment effects from tumor recurrence, causing many patients to undergo unnecessary repeat surgeries. For example, in ~30% of patients with glioblastoma who undergo a repeat resection for presumptive recurrence, pathologic examination of the resected specimen reveals necrosis, scarring,

Table 2. Detection of CSF-tDNA using WES

Patient and sample type	Mutation	Genomic coordinate	Distinct coverage (SafeSeqS)	Mutant (%; SafeSeqS)	Distinct coverage (WES)	Mutant (%; WES)
CGLI 03						
CSF	<i>PIK3R1</i> p.N564D, c.A1690G	Chr5:67591097	57,921	0.1	76	0.0
Normal	<i>PIK3R1</i> p.N564D, c.A1690G	Chr5:67591097	284	0.0	61	0.0
Primary tumor	<i>PIK3R1</i> p.N564D, c.A1690G	Chr5:67591097	377	38.7	147	12.9
CGLI 29						
CSF	<i>TP53</i> p.R248W, c.C742T	Chr17:7577539	13,964	0.2	64	0.0
Normal	<i>TP53</i> p.R248W, c.C742T	Chr17:7577539	NA	0.0	55	0.0
Primary tumor	<i>TP53</i> p.R248W, c.C742T	Chr17:7577539	6,418	69.0	58	70.7
CGLI 36						
CSF	<i>TP53</i> p.R248W, c.C742T	Chr17:7577539	376,434	14.3	74	9.5
Normal	<i>TP53</i> p.R248W, c.C742T	Chr17:7577539	57,818	0.0	75	0.0
Primary tumor	<i>TP53</i> p.R248W, c.C742T	Chr17:7577539	44,981	65.0	25	72.0
CGLI 55						
CSF	<i>PTEN</i> p.R130*, c.C388T	Chr10:89692904	251,609	33.2	63	42.9
Primary tumor	<i>PTEN</i> p.R130*, c.C388T	Chr10:89692904	91,515	65.9	56	66.1

Genomic coordinates refer to the human reference genome hg19 release (Genome Reference Consortium GRCh37, February of 2009). NA, not available.

or other treatment-related effects rather than recurrent disease (20). Conversely, while patients are waiting for or recovering from surgery for suspicious lesions, chemotherapy or radiation therapy cannot be administered, providing time for unabated tumor growth. Finally, patients are often kept on ineffective medication regimens until definitive signs of tumor progression appear on imaging. This delay in detection precludes potential opportunities to undergo new targeted therapies that might be effective for their disease (21). The health costs of these missed opportunities will increase with the expected advances in therapeutic modalities.

Given the need for sensitive and specific markers to monitor tumor dynamics, we asked whether tumor-derived DNA could be found in the CSF of patients with primary CNS tumors. This study was stimulated by our inability to consistently detect ctDNA in the plasma of these patients (8) and inspired by previous demonstrations that tumor-derived DNA could be found in fluids located in the proximity of neoplastic lesions. For example, a recent pilot study by Pan et al. (22) suggests that tumor-derived DNA can be detected in the spinal fluid of individuals whose primary tumors have metastasized to the brain. Although lumbar puncture to obtain CSF is not a noninvasive procedure, it qualifies as minimally invasive and is currently routinely performed to follow some brain tumor patients, particularly those with medulloblastomas (23, 24). Unfortunately, the examination of these CSF samples by cytology is usually of limited use, with relatively low sensitivities achieved even using large volumes of CSF (25, 26). Only 1 of 35 patients evaluated in our study had concomitant cytologic studies of CSF, precluding direct comparison. The results of this study suggest that the rates of tumor-derived DNA found in the CSF (74%) closely approximate the levels found in body fluids adjacent to other tumor types. For example, urine in bladder cancer was found to have tumor-derived DNA in 70% of cases, whereas

sputum in lung cancer was positive in 79% of cases (27, 28). Although the rate of detection observed in this study was not 100%, its sensitivity was comparable with or superior to other noninvasive tests for malignancies in general. Moreover and as noted below, it was particularly sensitive for tumors that abutted a CSF reservoir or cortical surface. Finally, from a technological standpoint, the average fraction of mutant DNA (12.2%) far exceeded the limit of detection of the sequencing assay used (0.01%). This assay could be performed with any commercially available next generation sequencing instrument at relatively small cost.

Our study revealed a significant association between the location and type of the tumor and the presence of CSF-tDNA. In particular, we were able to detect all 13 WHO grade III or IV gliomas (also known as anaplastic astrocytoma and glioblastoma, respectively), all 5 medulloblastomas, and all 3 ependymomas that abutted a CSF reservoir or cortical surface. It is in these aggressive tumors where the need for a robust biomarker is most desperate. There are also emerging data that some brain tumors, particularly those with genotypes susceptible to targeted therapies, may be able to be treated primarily with medical therapies, thereby obviating the need for surgery if appropriate noninvasive diagnostic tools were available (29–32). It is also worth noting that surgical resection nearly always creates an opening extending from the surface to the deep-seated tumor. This passageway typically persists and may enable tDNA from any residual or recurrent tumor to enter the CSF. Even without such surgically induced openings, the vast majority of medulloblastomas and ependymomas arise within or communicate with a ventricular reservoir, making them well-suited for CSF monitoring (24–26, 33, 34). Future studies will be required to directly compare CSF-tDNA with CSF cytology. Rather than replacing cytology, we envision that CSF-tDNA will be used in combination with it and other biomarkers under

development as well as with radiographic and clinical parameters (35–38). This could substantially increase the accuracy of the estimates of tumor burden at various points during the management of patients.

Given the invasive and risky nature of surgical interventions on the brain and spinal cord, it would be useful to be able to identify a neoplastic process without performing surgery. Our results provide a glimpse of the potential for this form of diagnosis in the future. We evaluated four patients: one patient with a tumor in the midbrain, one patient with a tumor in the pons, and two patients with a tumor in the spinal cord. Using WES, we were able to detect CSF-tDNA in two of four cases by comparing the data with those obtained by targeted sequencing with SafeSeqS. The results were consistent with expectation, in that the mutant fractions revealed by genome-wide sequencing were in accord with those identified by targeted sequencing (Table 2). Additional cases will need to be tested to elucidate the potential of this approach in patients in whom biopsies are challenging, but our results show that genome-wide analysis of the DNA from CSF is feasible in at least some cases.

Although the results described above are promising, we caution that this is an exploratory study designed primarily to determine whether it was possible to detect CSF-tDNA in patients with primary CNS tumors. A secondary goal was to document the anatomical and pathologic characteristics of the tumors that shed DNA into the CSF. The most important technical limitation of our study is that CSF samples were obtained at the time of surgery, and they were often from the ventricles rather than from a lumbar puncture. CSF has been shown to quickly circulate throughout the ventricles and spinal reservoirs (39, 40). It is, therefore, very likely that the DNA in the spinal fluid obtained through lumbar puncture will be similar to that of the ventricles, although the fluid obtained from lumbar puncture is farther away from the site of malignancy. An additional consideration is that, in individuals with a bulky mass that obstructs spinal fluid flow or elevates intracranial pressure, a lumbar puncture might be unsafe. However, these patients will almost always require surgical decompression to reduce the mass effect generated by the tumor, and CSF could be safely obtained after opening the dura. The exact method and location of CSF sampling in patients with CNS neoplasms will need to be individualized, and they will be based on a number of factors, including tumor location, ease of CSF sampling, and clinical characteristics. For example, patients may initially undergo CSF sampling from an intracranial space at the time of surgery to determine baseline levels of CSF-tDNA, but lumbar punctures could be used to longitudinally monitor CSF-tDNA levels.

Now that it has been documented that most primary brain tumors release tDNA into the CSF, the stage is set for a longitudinal study of the clinical use of this biomarker. Our results suggest specific guidelines for such a follow-up study. The optimal patients to follow would be those with medulloblastomas, ependymomas, or high-grade gliomas that abut a CSF space, because the CSF-tDNA assay is particularly sensitive in such cases and these tumor types are relatively common. CSF-tDNA should be evaluated intraoperatively to establish a baseline, and a concomitant lumbar puncture should be performed when possible to ensure concordance between the two fluid samples. Subsequent evaluations of CSF obtained through lumbar puncture or an implanted reservoir should be compared with other clinical and laboratory features, with the goal of determining the use of CSF-tDNA to detect minimal residual disease. For example, patients whose mass persists on MRI but CSF-tDNA is undetectable might be spared a second biopsy. Alternatively, patients in whom residual disease is evident on CSF-tDNA analysis but equivocal on imaging analysis might be well-served by additional therapy. In the future, it is likely that most brain tumors will be routinely assessed for mutations in various genes

of interest for both prognostic and therapeutic purposes (41–43). The availability of such sequencing data should make the approach described here more cost-effective and easier to implement.

Materials and Methods

Patient Samples. All samples were collected after approval was obtained from the Johns Hopkins Institutional Review Board and informed consent was provided. Whole blood and CSF were collected at the time of surgery before surgical manipulation of the tumor. A WBC pellet was prepared from the blood sample after hypotonic lysis of RBCs by centrifugation at $200 \times g$. CSF was frozen in its entirety at -80°C until DNA purification, and the entire volume of CSF (cells plus fluid) was used for DNA purification. The amount of CSF used averaged 4.8 mL (range = 0.75–10 mL). When fresh tumor tissue from surgical specimens was available, it was immediately frozen at -80°C . When frozen tissue was not available, formalin-fixed, paraffin-embedded tissues were used for DNA purification. In either case (fresh frozen or formalin-fixed, paraffin-embedded), tumors were macrodissected to ensure neoplastic cellularity exceeding 50%. DNA was purified from the white cell pellet, CSF, and tumor using an ALLPrep Kit (Qiagen).

Statistical Analysis. Clinical characteristics were compared between the CSF samples with and without detectable CSF-tDNA with Fisher's exact test or t test. Correlation coefficients among outcomes were estimated using Pearson correlation statistics. A logistic regression model was used to estimate the odds of detecting CSF-tDNA under different conditions. All P values are two-sided, and all analyses were conducted using SAS software (version 9.2; SAS Institute).

Tumor Mutational Profiling. A tiered approach was used to determine a somatic mutation within each tumor. Initially, a PCR-based approach testing for mutations in codons 130–139 of *IDH1*; codons 126–155, 144–178, and 250–262 of *IDH2*; all coding exons of *TP53*; and the *TERT* promoter was used (44–48). If no mutations were present within these genes, paired-end libraries of DNA from the tumors and WBC pellets were prepared and captured (SureSelect; Agilent) as previously described (47). Massively parallel sequencing was carried out on an Illumina HiSeq Instrument at either the Goldman Sequencing Facility at Johns Hopkins Medical Institutions or Personal Genome Diagnostics. Mutations were identified as previously described (47, 49–52).

Mutation Detection in CSF. DNA from tumor, WBCs, and CSF was used to validate the somatic mutations identified by targeted sequencing and determine whether these mutations could be found in the CSF; 3–5 ng tumor and WBC DNA was used for each assay, whereas all DNA from the CSF (for cases with <20 ng CSF DNA available) or 20 ng CSF DNA was used for each assay (Table S2). For this purpose, primers were designed to amplify an ~ 100 -bp region surrounding each mutation. The two primers had universal sequences at their 5' ends, allowing a second round of PCR to be performed using a second set of primers containing these sequences (19, 47). The sequences of the primers used to assess each mutation are listed in Table S5. Oligonucleotides used in this study were synthesized by TriLink Biotechnologies. The final PCR products (after two rounds of PCR) were purified with AMPure (Beckman) and sequenced using an Illumina MiSeq Instrument. The data were analyzed with the SafeSeqS Pipeline, allowing mutations occurring as infrequently as 0.01% to be detected and quantified with confidence using the experimental conditions applied (19). In every case, DNA from the normal cells served as a control to ensure that the mutations were not the result of errors generated during the DNA purification, amplification, or sequencing processes. Four paired-end libraries for CSF samples were also generated and exome-captured (Table 2). Preparation of the genomic library was performed using the TruSeq DNA Sample Prep Kit (Illumina) according to the manufacturer's recommendations. Exomic capture (SureSelect; Agilent) and massively parallel sequencing were carried out as described above.

ACKNOWLEDGMENTS. We thank our patients for their courage and generosity. We also thank N. Silliman, J. Schaefer, C. Blair, K. Judge, and M. Popoli for technical assistance. This work was supported by a Burroughs Wellcome Career Award for Medical Scientists, Alex's Lemonade Stand/Malia's Cord Foundation, the Pediatric Brain Tumor Foundation, a Johns Hopkins Clinician Scientist Award, Doris Duke Charitable Foundation Grant 2014107, The Virginia and D. K. Ludwig Fund for Cancer Research, The Banyan Gate Foundation, Swim Across America, The Sol Goldman Sequencing Facility at Johns Hopkins, and NIH Grants CA43460 and NS70024.

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