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

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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 patient 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 = 89–100%), 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.

A pproximately 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 DNA 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 ethnicities are summarized in Table 1. Approximately 25% (8 of 35) of the patients were diagnosed with CNS tumors outside the United States. There were 8 head and neck squamous cell carcinomas, 7 glioblastomas, 5 lymphomas, 3 medulloblastomas, 2 ependymomas, 2 gliosarcomas, 1 meningioma, 1 recurrent glioma, 1 diffuse intrinsic pontine glioma, 1 recurrent glioblastoma, 1 recurrent brain metastasis, and 1 pelvic osteosarcoma.

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 specifically 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.

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The authors declare no conflict of interest.

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preoperative symptoms are listed in Table S1. In total, 6 patients had medulloblastomas, and 29 patients had gliomas; 7, 9, 2, and 17 of the tumors were classified as World Health Organization (WHO) grades I–IV, respectively. Twenty-nine (83%) of 35 patients provided CSF during the initial surgery, whereas the remaining 6 (17%) did so during a repeat resection. The tumors were distributed throughout the brain and spinal cord, with 14 arising in the posterior fossa (including six medulloblastomas), 8 arising in the supratentorial compartment of the brain, and 13 arising in the spinal cord (Table 1).

**Identification of Somatic Mutations.** At least one mutation was identified in each of 35 tumors analyzed using a tiered approach (targeted sequencing followed by whole-exome sequencing (WES)) described in *Materials and Methods*.

With the targeted sequencing approach, we identified mutations in 13 tumors. The mutations in these samples occurred in *TP53* (tumor protein p53; n = 5), *IDH1* (isocitrate dehydrogenase 1; n = 2), or the *TERT* promoter (telomerase reverse transcriptase; n = 6) (Table S2). In the remaining 22 tumors, WES was used to identify at least one mutation per sample (Dataset S1). Genes mutated in these samples included well-known drivers, such as *NF2*, *PIK3RI*, *PTCH1*, and *PTEN* (18). The fractions of mutant alleles in tumors were generally high, averaging 46% (with an SD of 18%). This finding is consistent with the expected early development of driver gene mutations during tumor evolution and the presence of nonneoplastic cells in all tumors, even macrodissected ones, such as the samples used here. All mutations identified were confirmed to be absent in DNA from matched noncancerous (normal) cells from each patient.

The presence of one of the mutations detected in each patient’s tumor was then assessed in the CSF of the same patient using a sensitive sequencing-based method. This method reliably detects mutations with allele fractions as low as 0.01% (8, 19). An average of 4.8 mL CSF (SD of 2.6) was collected from 35 patients (Table S2). DNA could be purified from all CSF samples, although the amounts varied considerably (average of 417 ng; SD of 553 ng) (Table S2). Primers were designed to amplify each of 35 mutations as previously described (8, 19). Using this technology, we found that 74% of 35 CSF samples contained detectable levels of tumor DNA. The detectability of tumor DNA present in the CSF was not correlated with demographic characteristics, symptom duration, presence of hydrocephalus, contrast enhancement on imaging, or mutation type (Table S3). The fraction of mutant alleles in the CSF was, as expected, usually lower than the fraction in the primary tumors, and it was also much more variable than in the primary tumors. The average detectable mutant allele fraction in CSF was 12.2% (range = 0.1–77%).

**Relationship Between Mutations and Clinical Features.** The great variation in mutant allele fraction among the CSF samples suggested that there might be some anatomical or biological factor underlying the differences. The tumors were distributed among the brain and spinal cord (Table 1), and malignancies arising in both organs were detected at similar frequencies (P = 0.16; t test). High-grade (WHO grades III and IV) tumors were more likely to have detectable CSF-tDNA than low-grade lesions (P = 0.004) (Table S3), which was evidenced by the fact that all but one high-grade tumor (18 of 19) was detected. The levels of CSF-tDNA were also higher in high-grade lesions than in low-grade lesions (mutant allele fractions of 16.3 ± 21.2% vs. 2.8 ± 6.8%). Eighteen of 19 (~95%) high-grade (WHO grade III or IV) tumors had detectable levels of CSF-tDNA. However, tumor size was not a statistically significant factor in predicting CSF-tDNA detectability or level (P = 0.41) (Table S3).

Another important factor associated with CSF-tDNA levels was anatomic location. MRI scans were examined for the presence of contrast enhancement adjacent to a large CSF space (Table 1). Representative examples are provided in Fig. S1. Patients with lesions adjacent to a CSF reservoir had detectable levels of CSF-tDNA. However, tumor size was not a statistically significant factor in predicting CSF-tDNA detectability or level (P = 0.41) (Table S3).

**Genome-Wide Sequencing of DNA from the CSF.** The results described above were found after identifying at least one mutation in the primary tumor of each patient. In four patients with either brainstem or intramedullary spinal cord tumors, we also tested whether CSF-tDNA could be detected directly in their CSF by WES without prior knowledge of the tumor genotype. These four samples were selected based on the critical and highly sensitive location of the malignancies, making surgery treacherous. We found that two of four samples analyzed had levels of CSF-tDNA that were comparable with the levels identified through single-amplicon sequencing (Safe-SeqS) when the same mutation was assessed (Table 2). Both detectable cases had greater than 10% mutant allele fractions in the CSF as measured by single-amplicon sequencing. In contrast, the two cases in which WES was unable to identify CSF-tDNA had mutant allele fractions <1% as assessed by single-amplicon sequencing. As controls, we also performed WES on matched normal tissues and tumor tissues.
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,

| 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, myxopillary ependymoma | C3-6 spinal cord | Spinal subarachnoid space | Yes | Positive |
| CGLI 26 | Intramedullary spinal cord tumor | WHO II, infiltrating astrocytoma with oligodendrogial features | 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 | No | Not detected |
| CGLI 63 | Glioblastoma | WHO IV, medulloblastoma | Cerebellum | Basal cistern | Yes | 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.
Given the need for sensitive and specific markers to monitor tumor dynamics, we asked whether tumor-derived DNA could be found in the spinal fluid of individuals whose primary tumors were 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 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 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.

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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 diagnostic 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 × 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 DNA was measured using Qbiogene (Orange, CA) and NanoDrop (ThermoFisher). 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 χ2 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 set of universal 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. Exonic capture (SureSelect; Agilent) and massively parallel sequencing were carried out as described above.

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