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Cerebrospinal fluid methylome-based liquid biopsies for accurate malignant brain neoplasm classification

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Abstract

Background. Resolving the differential diagnosis between brain metastases (BM), glioblastomas (GBM), and central nervous system lymphomas (CNSL) is an important dilemma for the clinical management of the main three intra-axial brain tumor types. Currently, treatment decisions require invasive diagnostic surgical biopsies that carry risks and morbidity. This study aimed to utilize methylomes from cerebrospinal fluid (CSF), a biofluid proximal to brain tumors, for reliable non-invasive classification that addresses limitations associated with low target abundance in existing approaches.

Methods. Binomial GLMnet classifiers of tumor type were built, in fifty iterations of 80% discovery sets, using CSF methylomes obtained from 57 BM, GBM, CNSL, and non-neoplastic control patients. Publicly-available tissue methylation profiles ($N = 197$) on these entities and normal brain parenchyma were used for validation and model optimization.

Results. Models reliably distinguished between BM (area under receiver operating characteristic curve [AUROC] = 0.93, 95% confidence interval [CI]: 0.71–1.0), GBM (AUROC = 0.83, 95% CI: 0.63–1.0), and CNSL (AUROC = 0.91, 95% CI: 0.66–1.0) in independent 20% validation sets. For validation, CSF-based methylome signatures reliably distinguished between tumor types within external tissue samples and tumors from non-neoplastic controls in CSF and tissue. CSF methylome signals were observed to align closely with tissue signatures for each entity. An additional set of optimized CSF-based models, built using tumor-specific features present in tissue data, showed enhanced classification accuracy.

Conclusions. CSF methylomes are reliable for liquid biopsy-based classification of the major three malignant brain tumor types. We discuss how liquid biopsies may impact brain cancer management in the future by avoiding surgical risks, classifying unbiopsiable tumors, and guiding surgical planning when resection is indicated.

Key Points

- CSF methylome-based models can accurately classify major malignant brain tumor types.
- CSF methylomes address limitations associated with other liquid biopsy techniques.
- CSF liquid biopsies may impact future malignant brain tumor patient care pathways.

Importance of the Study

We show that the major three intra-axial malignant brain tumors can be classified using cerebrospinal fluid (CSF) derived circulating tumor DNA methylation signatures with accuracy that is higher than existing plasma methylome-based and CSF genomic alteration-based methods, approaching the accuracy of standard-of-care tissue-based diagnosis. We also present how the management of brain cancer patients may be impacted by CSF-based liquid biopsies to avoid the risks of invasive neurosurgical biopsies in patients amenable to non-surgical treatment with chemoradiotherapy. Additionally, in patients where a surgical resection is indicated, comprehensive pre-operative planning may also be done by tailoring the surgical approach to a patient's non-invasive classification. CSF-based liquid biopsies may also allow for tumor classification to be made in patients not amenable to surgical biopsies, who otherwise may not undergo optimal treatment.

A major clinical dilemma in the neurosurgical management of brain tumors is the resolution of the differential diagnosis between brain metastases (BM), glioblastomas (GBM), and central nervous system (CNS) lymphomas (CNSL), which are the three main intra-axial tumor types, to decide on the treatment regimen that is indicated for a patient. These malignant entities cannot be reliably distinguished using standard-of-care magnetic resonance imaging (MRI)[1] and, given the vastly divergent management approaches for each tumor, invasive surgical biopsies are typically necessary to establish diagnoses and determine optimal treatment.[2,3] Non-invasive approaches to distinguish between these tumor types would change patient management by allowing us to avoid the risks and morbidities associated with surgical biopsies, in particular for patients both with BM where treatment is now principally radiosurgery as well as CNSL that are treated medically or with radiotherapy.[3,4] These approaches would also lead to more personalized operative planning for patients who do have an indication for surgical resection, where the specific diagnosis could impact the neurosurgical approach utilized. Overall, identifying diagnostic biomarkers that can obviate the need for surgical tissue diagnoses will impact malignant brain tumor patient care by allowing surgical procedures to be reserved for patients where resection will have a clear oncological benefit.

It is well-established that malignant brain tumors have distinct diagnostic and discriminatory tumor-derived DNA methylome signatures that can be used to refine CNS tumor classification.[5–7] We have previously shown that plasma cell-free (cf) circulating tumor (ct) DNA methylomes can also be utilized to assist in distinguishing between many systemic and CNS cancers.[8–10] We postulated that cerebrospinal fluid (CSF) is an enriched source for brain tumor ctDNA compared to other biofluids that may allow for improved non-invasive brain tumor classification using circulating methylomes. Furthermore, existing CSF liquid biopsy literature to date has mainly focused on the identification of genomic alterations in CSF ctDNA, however, these genomic studies have had limited clinical applicability since known mutations that are able to be tested for occur only in subsets of patients, their mutation status may not be sufficient to rule out the other major differential diagnoses, and they have low abundance in circulating biofluids.[11–13] CSF methylomes have not been evaluated for these tumors to date, and we hypothesized that their use may establish value for pre-operative CSF sampling in the management of brain tumor patients.

Here, we assessed the utility and accuracy of a ctDNA methylome recovery approach, the cell-free methylated DNA immunoprecipitation plus high-throughput sequencing (cfMeDIP-seq) protocol, to classify these main malignant brain tumor types using CSF methylation signatures in patients with tumors identified on MRI. We validated these results by confirming that the CSF methylome signals are able to distinguish between these tumor entities in an independent external tumor tissue dataset. Finally, we integrated tumor tissue methylation profiles together with CSF methylomes to assess whether the use of tumor-specific methylation signals to differentiate tumors in CSF improves classification accuracy. We present how CSF methylome-based malignant brain tumor liquid biopsies can impact malignant brain tumor patient care in the future to enable personalized decision making regarding treatment.

Materials and Methods

Study Design

A total of 52 biobanked frozen BM, GBM, and CNSL CSF samples were obtained based on availability and according to research ethics board approval. These three tumor types were specifically chosen as they are considered the three major intra-axial pathologies and exist within a typical clinical differential diagnosis.[1] The sample sizes per tumor type were all within the range of samples sizes used successfully for brain tumor cfMeDIP-seq work previously.[9] CSF was sampled during placement of Ommaya reservoirs during clinical care for subsequent therapy delivery, cryopreserved, and biobanked. cfDNA was extracted from 0.5 to 2 mL of CSF (QIAamp Circulating Nucleic Acid Kit, Qiagen) as we have utilized previously for plasma samples.[8,9,14] All GBM samples were confirmed to be from patients with IDH wildtype tumors, consistent with the current World Health Organization classification.[15] All lymphoma samples were primary CNSL. Brain metastases were from patients with primary breast ($N = 10$), lung ($N = 4$), esophageal ($N = 1$),

and ovarian ($N = 1$) cancers along with one brain metastasis of unknown origin. Five additional CSF samples were acquired from patients with normal pressure hydrocephalus, as non-neoplastic controls, during clinical care and biobanked as above.

CSF Methylome Sequencing

Between 2.5 and 10 ng of cfDNA from each sample quantified by a Qubit fluorometer (Qubit 4, Thermo Fisher Scientific) was processed according to our cfMeDIP-seq protocol as has been described and used effectively by our group for 1–10 ng cfDNA per sample.[9,16] In summary, cfDNA underwent library preparation with Kapa HyperPrep Kits (Roche), immunoprecipitation of methylated cfDNA using the MagMeDIP Kit (Diagenode), cfDNA purification with version 2 of the IPure Kit (Diagenode), and library polymerase chain reaction amplification and cleanup. Resulting libraries underwent sequencing on the Illumina Novaseq6000 with 100 bp pair ended reads and a median of 68 million reads per sample, similar to that used previously, after optimal fragment size selection was confirmed using 2100-Bioanalyzer traces (Agilent).

CSF cfMeDIP-seq data were processed as described previously.[8] In brief, sequencing reads were first aligned with the human genome using *Bowtie2*. Reads were deduplicated and then indexed using *SAMtools*. Data was reduced to 300 bp genomic windows that map to regulatory features (covering CpG islands, shores, shelves, and FANTOM5 enhancers) with the *MEDIPS* package. Reads per kilobase million and count per million (CPM) values were calculated for these windows.

Tumor Tissue Cohort and Methylation Profiling

A total of 167 BM, GBM, and CNSL tumor tissue sample methylation profiles were obtained by combining existing publicly-available Illumina 450k array-based methylation datasets.[5,6] Additionally, 30 normal brain parenchyma methylation profiles were acquired as non-neoplastic controls.[5] Raw methylation data for all samples was processed using *minfi* and normalized with the single-sample Noob approach. CpG sites located on X or Y chromosomes, overlapping single-nucleotide polymorphisms, or considered cross-reactive were removed from further analysis. All samples passed final quality control assessment with sample detection P-values < .05. Processed beta values for all samples were utilized for all analyses.

Tumor Classification Using CSF Methylomes

To assess whether BM, GBM, and CNSL are distinguishable using cfMeDIP-seq window data, the 52 CSF samples were separated into 50 random discovery sets with 80% of samples as well as validation sets with 20% of samples. Within each of the discovery sets, binomial GLMnet models were developed for each tumor class versus others. These models were trained using the top 300 differentially methylated regions (DMRs) from pairwise comparisons between each tumor class versus all others using moderated t-statistic with limma-trend in discovery set samples. The ensemble of one-class versus other models for each tumor class were optimized with ten-fold cross validation across three iterations.

For each model iteration, performance was assessed using validation set data that was independent from the corresponding discovery set data used to generate that model. Area under receiver operating characteristic (ROC) curve (AUROC) values were calculated for each model in validation set data. Median AUROC values were calculated to address expected variability in accuracy between models trained with different randomly selected subsets of the dataset, similar to what we have shown previously.[9] An multidimensional scaling (MDS) plot using the combined DMRs from discovery sets was utilized to visualize CSF sample clustering.

Utilization of CSF-Based Features in Tissue Dataset

CpGs within DMRs between tumor types in CSF (fold change[FC] > 2, $P < .01$) were used as features for MDS plotting of the external tissue sample data. This approach allowed for CSF-based features to be assessed in an external dataset that is both independent of feature selection and tumor-specific without the presence of potential non-tumor cfDNA contributors to circulating biofluids. Additionally, the CpGs within the DMRs identified during CSF model building (described in the section above) were used within GLMnet models in the external tissue data, allowing for further assessment of the utility of the CSF-based features in tissue samples with AUROC calculations and associated 95% confidence intervals (CIs).

CSF-to-tissue Methylation Signal Correlation and Differentiation of Tumors from Non-neoplastic Controls

To assess the correlation between tissue methylation signatures and CSF methylation signals, scatterplots were computed using fold changes in CPM values at each window in CSF and deltas of median tissue beta values for CpGs within these windows in tissue, for each tumor class compared to others. Pearson's correlation coefficients and associated P-values were calculated for each scatterplot, one for each tumor type.

Furthermore, tumor tissue hypermethylation signatures were developed for each tumor type by identifying hypermethylated CpGs compared to the other tumor types (delta beta > 0.3, FDR < 0.01). Median CPM values in windows corresponding to these tissue-based hypermethylated CpGs were calculated for each CSF sample.

Additionally, DMRs between tumor types and non-neoplastic controls in CSF (fold change[FC] > 2, $P < .01$) were identified and used for MDS plotting of all CSF samples. CpGs within these DMRs were used as features for MDS plotting of external tumor and non-neoplastic control tissue sample data.

Classification Using Tumor-specific CSF Methylomes

An additional set of CSF-based models was built where the DMRs selected in CSF discovery sets were restricted to those windows that were also differentially methylated between tissue sample tumor types (delta beta > 0.3, FDR < 0.01). This approach ensured that the features used to develop the models were tumor-specific (present in tissue-based comparisons) and also identified in discovery set CSF samples. Representative clinical malignant brain tumor cases are displayed with standard-of-care MRI, along with boxplots showing class probability distribution from the subset of one-class versus other CSF-based models restricted to tumor-specific windows, where cases were partitioned into the validation sets and therefore not used for model training.

Statistical Analysis

Those *P*-values with two-tailed $P < .05$ were considered statistically significant, after multiple comparison correction with a false discovery rate (FDR) where relevant, unless stated otherwise. Nonparametric methods were used to compare cfDNA yield from biofluids (Wilcoxon's rank-sum test) as well cfMeDIP-seq CPM values between tumor groups (Kruskal–Wallis test). Boxplots depict medians with central bars, upper and lower distribution quartiles with box edges, and data within the 1.5× interquartile range using whiskers.

Data and Materials Availability

All data generated in this manuscript are available on request from the corresponding authors to comply with the institutional ethics regulations.

Results

Quantification of CSF cfDNA

A total of 52 CSF samples separated across BM, GBM, and CNSL diagnoses (see Supplementary Figure S1A) were identified for cell-free methylated DNA assessment. Measured extracted cfDNA yields from CSF are shown in Figure 1A with median (interquartile range) ng of cfDNA per mL of CSF being similar across tumor types: 20.0 (14.1–30.2) for BM, 21.1 (13.2–44.5) for GBM, and 20.6 (12.3–37.6) for CNSL. Supplementary Table S1 contains cfDNA yields as well as clinical characteristics for each patient. The cfDNA quantities acquired in CSF samples were comparable to those of cancer patients in our published plasma-based cfMeDIP-seq dataset.[8]

Tumor Classification Using CSF Methylomes

The cfMeDIP-seq profiles of these 52 CSF samples were then generated and our machine-learning pipelines (see Supplementary Figure S1B) were applied to this cohort using fifty iterations of randomly split 80% discovery and 20% validation sets. One-tumor class versus others binomial GLMnet classifiers were built using the top 300 DMRs within each CSF discovery set, identified in pairwise comparisons between one-tumor class and each of the other tumor types.

An MDS plot of the dataset, using the combined DMRs identified in discovery sets, showed clustering of CSF samples by tumor type (Figure 1B). Model performance was assessed within independent validation set data using AUROC metrics. Models showed high accuracy in classifying these malignant brain tumor types using CSF methylomes as shown in Figure 1C, with median (95% CI) AUROCs of 0.93 (0.71–1.0) for BM, 0.83 (0.63–1.0) for GBM, and 0.91 (0.66–1.0) for CNSL.

Utilization of CSF-based Features in Tissue Dataset

Tumor tissue methylation data for 167 tissue samples across BM, GBM, and CNSL diagnoses (see Supplementary Figure S1A) was utilized as an external tumor-specific dataset with which to evaluate CSF-based features independently and without the presence of potential non-tumor contributors to circulating biofluids.[5,6] To further validate the utility of CSF methylomes in distinguishing these entities based on ctDNA signatures, the Figure 2A MDS plot shows that differentially methylated features identified between tumor types in CSF distinctly cluster the independent external tissue sample dataset by tumor type. The same set of features identified in each of the CSF discovery sets and used to build the CSF-based models were then evaluated in tissue-based classifiers, which accurately distinguished all tumor classes in tissue (Figure 2B: median AUROCs of 1.0 for all groups).

Correlation Between CSF and Tissue Methylation Signals

Both tissue-based differentially methylated CpGs and CSF-based DMRs between each tumor type versus others were computed. Resulting CSF cfMeDIP-seq tumor type signals were observed to correlate well with tissue methylation data tumor signatures for all three entities (Figure 2C). Pearson correlation coefficients were: $r = 0.20$ for BM ($P < 2.2 \times 10^{-16}$), $r = 0.30$ for GBM ($P < 2.2 \times 10^{-16}$), and $r = 0.45$ for CNSL ($P < 2.2 \times 10^{-16}$). These correlations between tissue to CSF methylome signals are greater than what we have shown previously in a similar comparison between plasma and tissue methylomes.[8]

Additionally, BM, GBM, and CNSL tissue-based hypermethylation signatures were developed using windows with hypermethylated CpGs between tumor types in the tissue methylation dataset. Hypermethylated BM tissue signatures were enriched in BM CSF samples ($P = .012$) and hypermethylated GBM tissue signals were enriched in GBM CSF samples ($P = .00017$), when compared to tissue signatures from the other tumor types within these samples (see Supplementary Figure S2). The hypermethylated CNSL tissue signature was not enriched in CSF samples, suggesting that the classification models for this entity may also utilize hypomethylated features and/or interactions between methylation signals across multiple individual regions.

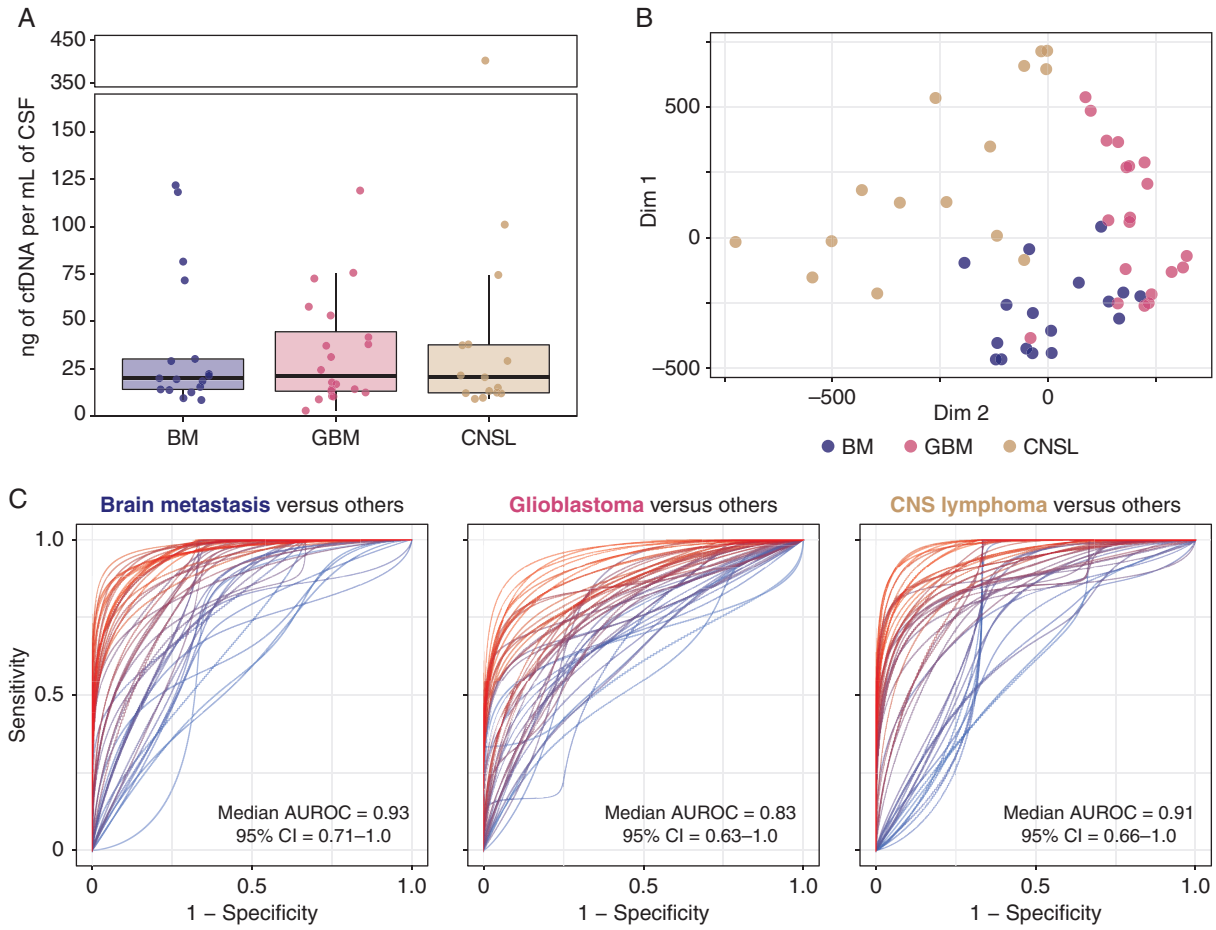

Figure 1. CSF methylome-based accurate discrimination of malignant brain tumors. (A) cfDNA yield per mL of CSF used was similar between tumor types. (B) MDS plot using DMRs identified between tumor types in CSF discovery sets shows clustering by tumor class within CSF samples. (C) Ensemble of ROC curves for 50 iterations of one-class versus others classifiers trained using CSF-based features showing a high discriminative capacity for the main three intra-axial brain tumor types within CSF validation set samples.

Enhanced Classification Using Tumor-specific CSF Methylomes

Given the correlation between tissue DNA methylation profiles and CSF cfMeDIP-seq signals, an additional set of tumor-specific CSF-based models were developed. For these models, the selection of top DMRs was restricted to the subset of CSF methylome windows that were also differentially methylated between tumor types in tissue methylation data. Accordingly, these models were built using features specific to tumor DNA, as they were differentially methylated between tumor types in tissue samples, and then also selected as top DMRs between tumor types in CSF discovery sets.

The performance of these tumor-specific models within CSF sample validation sets is shown in Figure 3A. AUROC (95% CI) values for the tumor-specific models were notably high at 0.95 (0.76–1.0) for BM, 0.88 (0.66–1.0) for GBM, and 0.95 (0.74–1.0) for CNSL in CSF validation sets. These tumor-specific CSF models were more optimal than the Figure 1C CSF models that were trained on features present in CSF only. Both sets of CSF-based classifiers were observed to be significantly more accurate than our published plasma-based results for cancers that metastasize to the brain and gliomas and with less variability across model iterations.[8,9]

Illustrative Clinical Cases of CSF-based Malignant Brain Tumor Classification

Representative clinical cases of malignant brain tumors that cannot be reliably distinguished using standard-of-care MRI are depicted in Figure 3B. These tumors were accurately identified using probability outputs from the subset of tumor-specific classifiers shown in Figure 3A, where cases were randomly separated into validation sets and therefore not used to train the models. Flow diagrams illustrating the current patient care pathway as well as a potential future biomarker-driven care pathway are shown

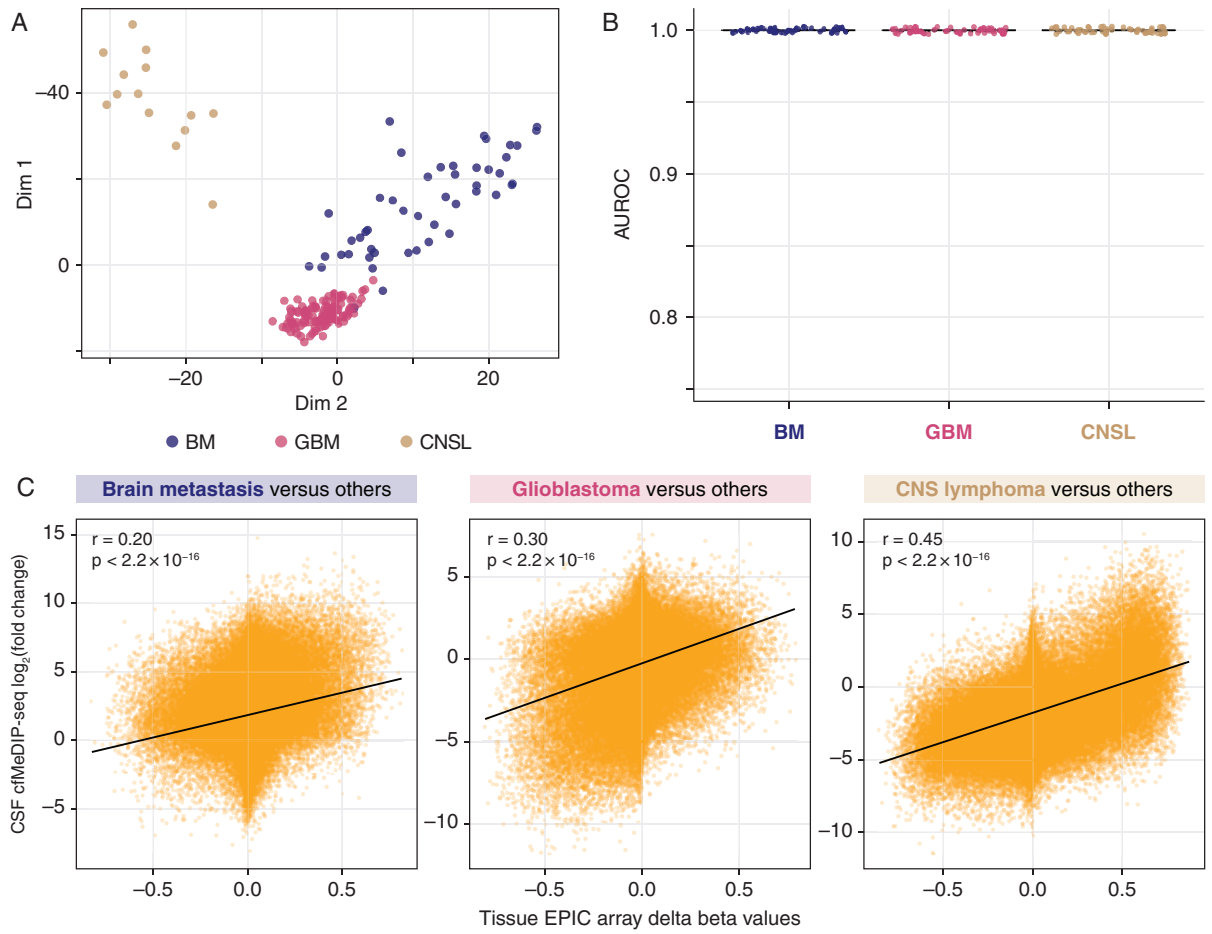

Figure 2. Evaluation of discriminatory CSF methylome-based features in external tumor tissue dataset. (A) MDS plot showing distinct clustering of external BM, GBM, and CNSL tissue samples using independent DMRs identified between tumor types within CSF methylomes. (B) Boxplots depicting AUROC value distribution of 50 iterations of one-class versus others classifiers built and evaluated in tissue samples using the sets of CSF-based features from each iteration of CSF model building shown in Figure 1C. (C) Scatterplots of the differences in CSF methylation signals between tumor classes versus the differences in tissue methylation levels between each tumor type versus others, showing high CSF-to-tissue correlations for all entities.

to demonstrate how our models may impact overall management for such cases with a more advanced personalized care approach in the future (Supplementary Figure S3).

Malignant Brain Tumor Differentiation from Non-neoplastic Controls

Given the reliable classification of tumor types in CSF and tissue using CSF-based features, it was then assessed whether tumors could be distinguished from non-neoplastic control samples. It was observed that CSF-based features between BM, GBM, CNSL, and non-neoplastic controls were able to cluster tumor types from controls in MDS plots of both CSF (Supplementary Figure S4A) and tissue (Supplementary Figure S4B) cohorts.

Discussion

In this study we demonstrate liquid biomarker based classification of malignant brain tumors using CSF methylomes, which may have utility for biomarker-driven decision making on the optimal patient treatment for the specific diagnosis. We have shown here the first use of non-invasive methylome-based models to distinguish the main three intra-axial malignant brain tumor types using CSF with a level of accuracy approaching that of standard-of-care tissue sampling but with significantly less patient risk, as the 0.5–2 mL of CSF required can be feasibly obtained via lumbar punctures.[4] The methylation signatures identified in CSF samples and used for CSF-based classification also showed utility outside of our dataset and within an external

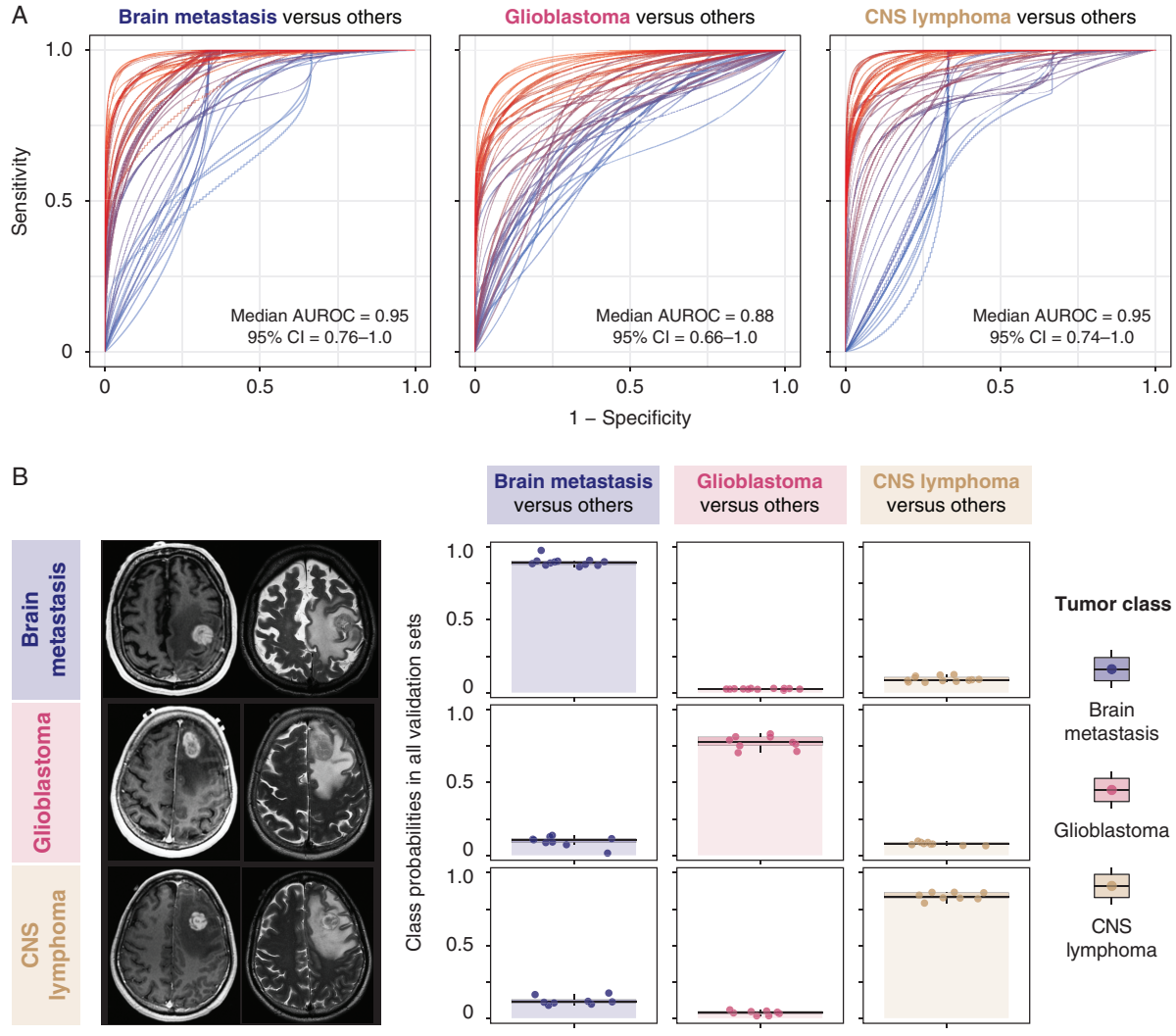

Figure 3. Enhanced classification accuracy using tumor-specific CSF methylomes. (A) Ensemble of ROC curves for 50 iterations of one-class versus others classifiers trained using tumor-specific features (present in both tissue and CSF discovery set comparisons), showing enhanced accuracy in classifying tumor types within CSF validation set samples in comparison to Figure 1C. (B) Distribution of validation set probabilities for representative clinical cases of malignant brain tumors that cannot be reliably distinguished using standard-of-care MRI, but are accurately classified using CSF methylomes.

tissue dataset, demonstrating their independent validation and tumor specificity.

Our approach leverages robust sets of genome-wide methylation-based signatures that do not depend on the upfront knowledge of the status of any one marker or its individual ctDNA abundance in order to enable sensitive biomarker detection, which are limitations of other approaches previously studied.[11–13]The identification of tumor tissue DNA methylation signals within CSF methylomes, along with the enhanced discrimination of malignant brain tumors in CSF when using circulating tumor-specific signatures only, supports both the enrichment of circulating tumor DNA in CSF compared to plasma and the value of CSF samples for liquid biopsy-based classification of these tumors. It was also observed that CSF-based brain tumor classification is also significantly more accurate than what we have shown previously for distinguishing both gliomas and systemic malignancies at risk of metastasizing to the brain using plasma methylomes.[8,9] Accordingly, CSF liquid biopsy approaches are expected to be incorporated into future diagnostic workflows for these patients after further prospective validation.

This work is expected to impact how we approach patient care for malignant brain tumor patients in the future, towards biomarker-driven approaches. Specifically, methylation-based liquid biopsies hold promise as a reliable approach to avoid the risks of invasive diagnostic neurosurgical tumor biopsies in

the subset of patients amenable to non-surgical management (many BM and most CNSL patients) and requiring the imaging-based diagnosis to be clarified. Additionally, CSF liquid biopsies may be used to classify unbiopsiable tumors, which currently may receive suboptimal treatment if the diagnosis is unclear, and to enable pre- as well as intra-operative surgical planning based on tumor entity in patients who may benefit from a surgical resection based on their CSF-based entity. However, in patients with metastatic cancer where a diagnosis of BM is highly likely, with large solitary GBM or BM requiring surgery to relieve mass effect, with glioblastomas that will ultimately require resection, or where imaging features are highly suggestive of one particular entity, liquid biopsies may not impact management decisions apart from cases where confirming the diagnosis pre-operatively may impact preoperative or intra-operative surgical planning. Additionally, tissue biopsies or CSF ctDNA sequencing may be required following CSF methylome-based classification in patients that require additional molecular profiling, for example for IDH mutation status testing in gliomas or targetable mutation status in brain metastases not known from the primary tumor.

It is important to note that the sample size utilized here was relatively low, although within the range of what we have used previously for our methylome-based analyses[9] and what has been published on CSF genetic biomarkers[17] for classification models. Our study utilizes retrospective samples and it will be important for future studies to validate the utility of these models prospectively on larger cohorts of patients with newly diagnosed malignant brain tumors plus patients without cancer, to determine the optimal CSF acquisition approach for use in clinical practice, and to characterize biomarker abundance in CSF throughout the course of disease. It may also be useful for future work to study patients with leptomeningeal disease in order to build additional models for this subset of patients and to build models that subclassify tumor types, for example brain metastases from different sources. Future work building composite classifiers that incorporate clinical variables, including imaging features, together with CSF methylomes may further enhance non-invasive classification accuracy.

Overall, this study highlights the value of liquid biopsies for accurate classification of the main three malignant brain tumors, using models built using tumor-specific ctDNA signals in CSF. These results may also lead to future work developing approaches for CSF-based minimally invasive prediction of response to therapy and identification of early recurrence for early intervention and corresponding improved patient outcomes for these devastating malignant brain tumors.

Supplementary material

Supplementary material is available online at *Neuro-Oncology* (http://neuro-oncology.oxfordjournals.org/).

Keywords

brain cancer | cerebrospinal fluid | diagnostic biomarkers | DNA methylation | liquid biopsy

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Authorship

Study design: JAZ, VP, AC, DDDC, GZ. Biospecimen acquisition: BZ, MG, AM. Laboratory methodology: JZ, MV, SM, SYS. Data processing: JAZ, VP, AC. Initial data interpretation: JAZ, VP, AC, DDDC, GZ. Initial manuscript preparation: JAZ, VP, GZ. Manuscript revision: JZ, VP, SM, MV, AC, SYS, FN, NM, MT, AS, BZ, MG, DDDC, AM, GZ.

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