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Title character count: 77
Running head character count: 48
Number of words in abstract: 95
Number of words in main text: 1617
Number of figures: 1
Number of tables: 1
1 supplementary Table

ABSTRACT

Brain mosaic mutations are a major cause of refractory focal epilepsies with cortical malformations such as focal cortical dysplasia, hemimegalencephaly, malformation of cortical development with oligodendroglial hyperplasia in epilepsy, or ganglioglioma. Here, we collected cerebrospinal fluid (CSF) during epilepsy surgery to search for somatic variants in cell-free DNA (cfDNA) using targeted droplet digital PCR. In 3/12 epileptic patients with known somatic mutations previously identified in brain tissue, we here provide evidence that brain mosaicism can be detected in the CSF-derived cfDNA. These findings suggest future opportunities for detecting the mutant allele driving epilepsy in CSF.

Keywords

Brain mosaicism, refractory epilepsy, cortical malformations, CSF, somatic variants, cell-free DNA

INTRODUCTION

Brain somatic variants have been shown to be an important etiology of structural intractable epilepsy.[1-4] We recently reported that brain mosaic variants give rise to early-onset epilepsies associated with various focal cortical malformations, including focal cortical dysplasia type 2 (FCD2), hemimegalencephaly (HME), malformation of cortical development with oligodendroglial hyperplasia in epilepsy (MOGHE), and ganglioglioma (GG), a low-grade neuronal-glial tumor.[2-11] Epilepsy associated with these aforementioned brain lesions is commonly pharmacoresistant to antiepileptics, and surgical resection of the epileptogenic zone is often the only treatment option effective at controlling seizures. While the genetic etiology of these disorders is now mostly known, molecular diagnosis requires genomic DNA derived from resected brain tissues, thus limiting a complete genetic assessment in cases not eligible for surgery.

Liquid biopsy techniques based on the sequencing of cell-free DNA (cfDNA) are emerging as non-invasive methods for tumor diagnosis and progression.[12] Interestingly, cerebrospinal fluid (CSF) has been shown to be a source of circulating tumor DNA released upon tumor cell death and a potentially powerful biomarker for the diagnosis and characterization of tumors of the central nervous system (CNS), such as gliomas.[13-15] Moreover, aberrant neuronal death has also been observed in epilepsy patients and experimental animal models.[16-18] To date, however, the clinical and diagnostic utility of liquid biopsies from patients with intractable epilepsy and brain malformations has not yet been addressed. Here, we provide evidence in epileptic patients with previously identified mutations in the brain resected tissues, that somatic mutations can be detected in the CSF-derived cfDNA collected during epilepsy surgery.

METHODS

Patient cohort

We collected brain tissues, blood and cerebrospinal fluid (CSF) samples from a cohort of 12 patients (mean age 6.3 years at surgery) with lesional refractory epilepsy from France at the Fondation Rothschild (n=5) and South Korea at the Severance children's hospital (n=7). Neuropathological diagnoses were established by expert pathologists according to the ILAE classification for cortical malformations and the WHO classification for tumors. The cohort comprised FCD2 (n=1), HME (n=5), MOGHE (n=3), and GG (n=3) cases. Informed consent for the use of biological samples was obtained from all patients. The study protocol received approval by the ethical committees of CPP Ile de France II (N° ID-RCB/EUDRACT-2015-A00671-48) and Severance Hospital and the KAIST Institutional Review Board and Committee on Human Research.

Cerebrospinal fluid sampling and DNA extraction

All CSF samples were collected after dura opening before surgery (resection or hemispherotomy). For all KR samples and FR-4 sample (8/12), CSF was collected from the subarachnoid space adjacent to the epileptogenic area; for FR-1, FR-2 and FR-3 cases, CSF was collected from the former resection cavity; for FR-5, it was collected from the inter-hemispheric fissure. The volume of collected CSF ranged from 1 to 6mL depending on the surgical site (See supplementary table for details). To remove any genomic DNA contaminant from cells, all CSF samples (except case FR-4) were centrifuged at 400g for 5 min at 4°C (FR cases) or 2000g for 5 min at 4°C followed by 10000g for 5 min at 4°C (KR cases). The supernatant was divided into 1mL aliquots and stored at -80°C. Cell-free DNA (cfDNA) samples were extracted from 1mL of CSF (except 0.5mL of CSF for KR-3 sample) with the Maxwell RSC cfDNA kit (Promega; French cohort) or the QIAamp circulating nucleic acid kit (Qiagen; South Korean cohort) and quantified using the high sensitivity dsDNA Qubit assay (Thermo Fisher Scientific). Using the same procedure described above, we also included cfDNA from 1mL of CSF, collected from the subarachnoid space after dura opening, of three other refractory epileptic

patients with FCD1, FCD2a, and TSC, to be used as mutation-negative controls in the droplet digital PCR assays.

Droplet digital PCR assays and statistical analysis

The droplet digital PCR (ddPCR) QX200 system (Bio-Rad) was used for variant specific detection. All cfDNA samples were first subjected to a mutation-site targeted 13 cycles preamplification (for each variant to test) with the SsoAdvanced™ PreAmp Supermix (Bio-Rad), optimized for unbiased target-specific preamplification of a limited amount of DNA, following the standard protocol for 20X TaqMan Assays. To avoid possible amplification errors, at least 2 independent preamplifications were done per sample. Diluted (1ng/µL) bulk brain DNAs were also preamplified to control for ddPCR accuracy. All ddPCR reactions were done in replicates (at least 2 replicates for controls, ≥3 for patients) using the ddPCR Supermix for probes according to the manufacturer's protocol. ddPCR mutation detection assays FAM+HEX (Bio-Rad) were purchased to detect the following variants: *MTOR* p.Ser2215Phe, *MTOR* p.Ala1459Asp, *AKT3* p.Glu17Lys, *PIK3CA* p.Glu545Lys, *SLC35A2* p.Ser212Leufs*9, *SLC35A2* p.Glu254*, *SLC35A2* p.Gln168*, and *BRAF* p.Val600Glu. In each assay, 3 to 5 mutation-negative cfDNAs controls (either the mutated patients used as crossed negative controls or the 3 mutation-negative controls) were preamplified and assessed by ddPCR. In all reactions, 5U of HindIII digestion enzyme was added. Amplification products were run on a QX200 droplet reader and data analyzed with the Quantasoft Analysis Pro software (version 1.0.596). To ensure sufficient sensitivity, only assays with > 100 copies/µL of WT (HEX+) DNA were kept for analysis. The VAF was calculated for each sample by the Quantasoft Analysis Pro software as the fractional abundance of mutant (FAM+) to total (wild type (HEX+) + mutant (FAM+)) DNA copies. Two-tailed t test (unequal variance) was used to assess if the average VAFs were statistically different between the mutated patient and the mutation-negative controls.

RESULTS

We assembled a collection of CSF samples from 12 neurosurgery cases of FCD2, HME, MOGHE, and GG with known mosaic pathogenic variant identified in the genomic DNA from the resected frozen brain specimen (absent from blood samples indicating their brain-specificity): brain variants in genes of the mTOR pathway (*PIK3CA*, *AKT3*, *MTOR*) in FCD2 and HME subjects (n=6);[9,10] brain somatic variants in the galactose transporter encoding gene *SLC35A2* in MOGHE cases (n=3);[8,9,11] and the recurrent brain somatic *BRAF* p.Val600Glu variant in GG patients (n=3) (**Table 1, Fig. 1)**.[3,10] We asked whether brain somatic variants can be detected in cfDNA from the patients' CSF collected from the subarachnoid or ventricle space during epilepsy surgery. Since cfDNA concentrations from CSF were generally low (mean 0.38ng/µL, range 0.05-2.7ng/µL, total amount of collected cfDNA ranged from 0.27ng to 28ng) (Supplementary Table 1), we performed a targeted preamplification for all cfDNA samples. We set up a droplet digital PCR (ddPCR) mutation detection assay to detect the presence of a given variant in the matched cfDNA sample. To exclude potential amplification bias or errors, we compared the mean VAF of patients versus mutation-negative controls. We obtained significant p-values in 3 out of 12 patients: one HME (FR-4) with a CSF cfDNA VAF of 1.358% for *PIK3CA* p.Glu545Lys; one GG (KR-7) with a CSF cfDNA VAF of 0.214% for *BRAF* p.Val600Glu, and one MOGHE (KR-4) with a CSF cfDNA VAF of 0.145% for *SLC35A2* p.Gln168*. Variant allele frequencies in the brain samples ranged from 1 to 24% in the whole cohort. There was no obvious correlation between VAFs in the brain tissue and cfDNA samples.

DISCUSSION

Currently, identification of brain somatic mutations relies on direct access to brain tissue from epilepsy surgery for both cortical malformations and GG lesions. Achieving a genetic diagnosis before surgery (or when surgery is not possible) may help in understanding mutation-related disease prognoses and adopting targeted therapies. We hypothesized that mutated DNA could be detected in CSF-derived cfDNA from patients with non-tumoral cortical malformations, as described for other CNS malignant tumors. By using a targeted ddPCR-based approach, we were able to detect somatic brain mutations in CSF-derived cfDNA in 3/12 (25%) surgical cases with a mean VAF of 0.57%. A recent study ~~with a sensitivity threshold to detect down to 0.25% mutant rates~~, reported the detection of somatic mutations from the cfDNA of 3 cases with focal epilepsies by ddPCR, ~~with a mean mutational burden of 6.8%~~: in one patient with subcortical band heterotopia (widespread mosaic variant in *LIS1* p.Lys64*, VAF at 9.4% in CSF from lumbar puncture and 13% in the blood); one patient with FCD2b (*TSC1* p.Phe581His*6, VAF at 7.8% in CSF from dural puncture and 2.8% in the resected tissue); and one with GG (*BRAF* p.Val600Glu, VAF at 3.2% in CSF from dural puncture and 20.4% in the tumor).[21]

Overall, these results raise several questions. First, we were not able to detect known somatic mutations in CSF cfDNA from a substantial proportion of patients, indicating that the detection of low VAF variants (<0.1%) still needs technical improvement before a clinical use of CSF for genetic testing in epileptic patients. Moreover, in current clinical practice the described approach can only be applied when the mutation is known or suspected (as for the recurrent *BRAF* p.Val600Glu mutation), using variant-specific approaches. Second, all CSF samples in this study were collected during brain surgery before resection (mostly from the subarachnoid space). Genetic diagnosis based on CSF-derived cfDNA would require the proof-of-concept that brain-specific variants can be detected in CSF obtained from lumbar puncture (thus in a pre-surgical assessment) and may require multiplexing ddPCR assays targeting recurrent variants. Third, it remains unknown whether mutated cells found

in FCD2/HME (e.g. dysmorphic neurons and balloon cells) and MOGHE samples undergo cell death and therefore release their DNA.

Recent advances have shown that tumor-derived cfDNA in CSF samples can be used as a biomarker for monitoring tumor progression and response to therapy.[15] Brain surgery is not always effective in treating seizures since only 30-50% of patients are seizure-free after the first surgery and multiple resections are needed in some cases. [20] Identification of mutated brain DNA in CSF may be of use in predicting possible seizure relapse and personalized therapy. Thus, this work opens new avenues for improving the diagnostic workflow of patients with various brain malformations and research on the pathological mechanisms and progression of refractory focal epilepsies.

ACKNOWLEDGEMENTS

We thank the families that took part in this study, and the neuropediatricians who referred the patients. We also thank the sequencing ICM core facility (iGenSeq). This work was funded by the European Research Council (N°682345 to SB), the "Investissements d'avenir" ANR-10-IAIHU-06 and ANR-18-RHUS-0005 program, the Suh Kyungbae Foundation (to J.H.L), the National Research Foundation of Korea (NRF) grant funded by the Korean government, Ministry of Science and ICT (No. 2019R1A3B2066619 to J.H.L), and Korean Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (H15C3143).

Author Contributions

JHL and SB contributed to the conception and design of the study; SYK, SBal, NSS, MC, GD, DSK, WSC, and VT contributed to the acquisition and analysis of data; JHL, SB, SYK, SBal and NSS contributed to drafting the text and preparing the figures.

Potential Conflicts of Interest

Nothing to report.

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Table 1. Genetic findings in cfDNA and brain tissue samples

Patient ID	Pathological Diagnosis	Age at CSF collection	Gene HGVSp	Brain VAF (%)	Mean cfDNA VAF (%)	Mean cfDNA VAF from negative controls (%)	p-value
FR-4[¶]	HME	12.4y	*PIK3CA* p.Glu545Lys	23	1.358	0.066	< 0.0001[‡]
KR-7	GG	17.3y	*BRAF* p.Val600Glu	4	0.214	0.073	0.0015[‡]
KR-4	MOGHE	4.2y	*SLC35A2* p.Gln168*	18	0.145	0.053	0.0014[‡]
FR-5	MOGHE	7.8y	*SLC35A2* p.Ser212Leufs*9	23	0.000	0.000	NA
KR-2	HME	0.7y	*AKT3* p.Glu17Lys	4	0.019	0.018	0.9205
KR-5	GG	7.1y	*BRAF* p.Val600Glu	24	0.064	0.073	0.7443
FR-1	FCD2	11.3y	*MTOR* p.Ser2215Phe	1	0.031	0.039	0.4398
KR-3	MOGHE	5.2y	*SLC35A2* p.Glu254*	16	0.000	0.003	0.3388
KR-1	HME	0.1y	*MTOR* p.Ser2215Phe	23	0.059	0.039	0.3312
FR-3	HME	3.6y	*AKT3* p.Glu17Lys	12	0.033	0.018	0.1398
FR-2	HME	1.4y	*MTOR* p.Ala1459Asp	9	0.019	0.008	0.0560
KR-6	GG	7.1y	*BRAF* p.Val600Glu	13	0.021	0.073	0.0014[$]

FCD2: Focal cortical dysplasia type 2, HME: Hemimegalencephaly, MOGHE: Malformation of cortical development with oligodendroglial hyperplasia in epilepsy, GG: Ganglioglioma, VAF: variant allele frequency, [‡]: p-values referring to significant positive result, NA: Not applicable, [¶]: FR-4 CSF sample was not centrifuged prior freezing, therefore a cellular genomic DNA contamination cannot be excluded, [$]: the significant p-value obtained in KR-6 highlights a significant lower VAF in the patient compared to the controls, thus confirming the absence of mutation detection. None of the variants was detected in the matched blood samples. Patients (except FR-4) were previously reported: KR-3 and KR-4 [8,11]; FR-1, FR-2 and FR-3 [9]; FR-5 [9,11]; KR-1, KR-2, KR-5 and KR-7 [10]; KR-6 [7,10].

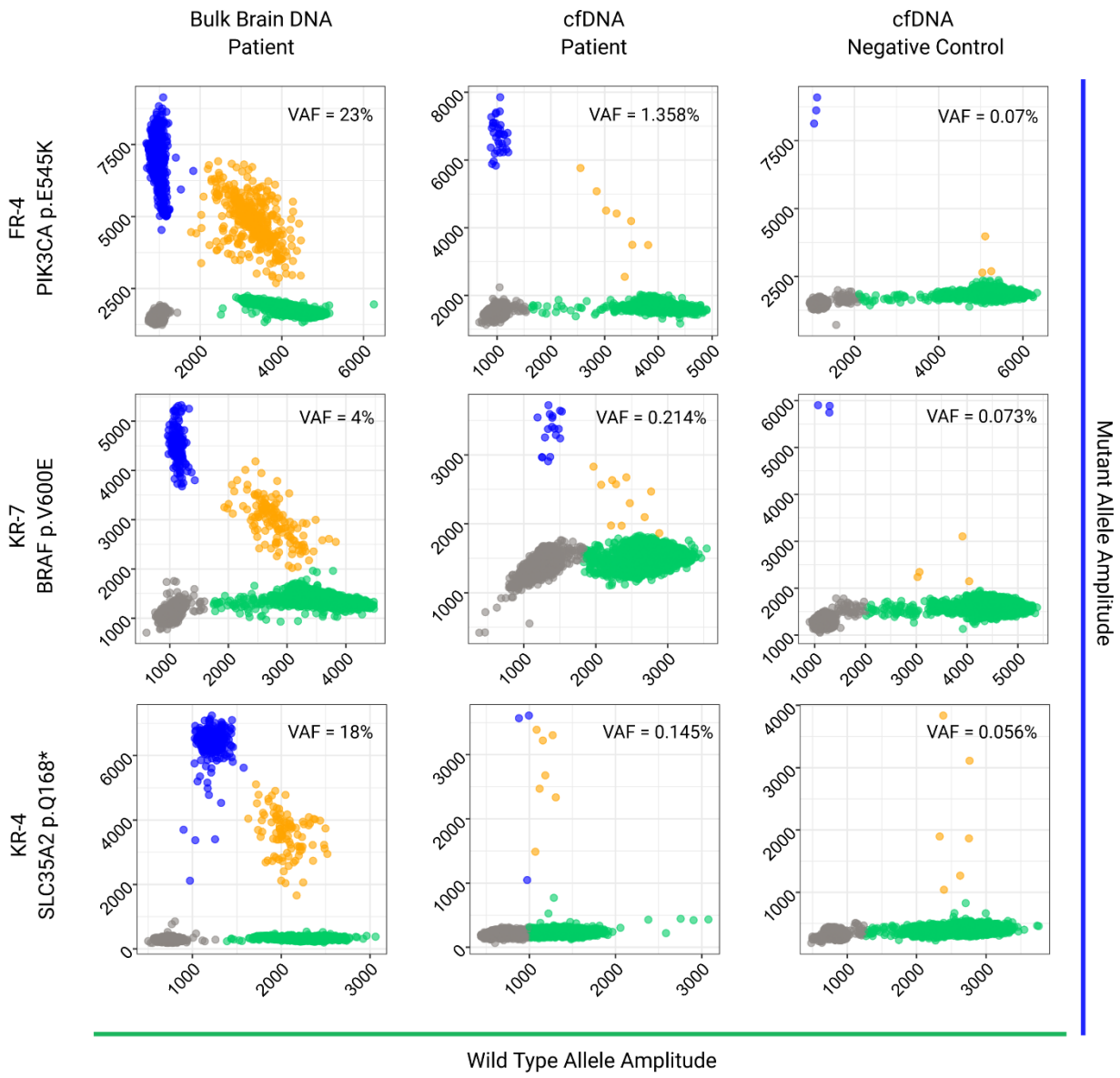

Figure 1. Representative ddPCR 2D scatter plot for the targeted detection of mutant alleles.

2D fluorescence amplitude plot shows wild-type-only droplets (HEX+ green circles), mutant-only droplets (FAM+ blue circles), double-negative droplets containing no targeted DNA templates (gray circles) and double-positive droplets containing both WT and mutant DNA templates (orange circles) from bulk brain DNA, and cfDNA for the three positive cases and mutation-negative controls. Each plot is a representation of the mean VAF. VAF: Variant Allele Frequency. Plots were generated in R using the ggplot2 package.