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Running Title: BRAF K601E in Cortical Epilepsy

Clinicopathologic characterization of a non-expansile, epilepsy-associated neocortical lesion with *BRAF* K601E mutation

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Conflicts of Interest

Running Title: BRAF K601E in Cortical Epilepsy

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Main Text

The v-Raf murine sarcoma viral oncogene homolog B1 *(BRAF)* proto-oncogene encodes the BRAF protein, an effector of the Ras/Raf/mitogen-activated protein kinase (MAPK) pathway that mediates signals from cell surface receptors to the nucleus to promote cell proliferation and survival[1]. Activating mutations in *BRAF*, particularly the V600E mutation (*BRAF*^{V600E}), are commonly seen in a wide range of tumors inside and outside the central nervous system, and are particularly common in low-grade epilepsy-associated tumors (LEATs) including pediatric-type diffuse low-grade glioma (pLGG), pilocytic astrocytoma (PA), ganglioglioma (GG), dysembryoplastic neuroepithelial tumor (DNET), pleomorphic xanthoastrocytoma (PXA) and polymorphous low-grade neuroepithelial tumor of the young (PLNTY)[2–4]. Somatic activating mutations in the MAPK pathway, including *BRAF*^{V600E}, have also been reported in epilepsy-associated, non-expansile lesions including mesial temporal lobe epilepsy (MTLE) associated with hippocampal sclerosis (HS) and in neocortically-based focal epilepsy[5,6]. In particular, a recent study identified *BRAF*^{V600E} in 5/9 cases of neocortically-based focal epilepsy. All five were reported to have CD34+ extravascular stellate cells, and none of the *BRAF*-wildtype lesions had CD34+ extravascular stellate cells[6]. These studies implicate a role for activating *BRAF* mutations in epileptogenesis independent of tumorigenesis and further suggest CD34 as a marker of MAPK-activated neocortical and hippocampal epilepsy-associated lesions. Here, we describe the clinical and histopathologic features of a case of neocortical epilepsy associated with *BRAF* K601E mutation (*BRAF*^{K601E}) with distinct histopathologic features.

The patient was referred to the UCSF Epilepsy Center in her twenties for evaluation and treatment of drug-resistant epilepsy. She was born left-handed but became ambidextrous. She reported a family history of epilepsy in two maternal uncles and seizure onset at approximately age 13 accompanied by a prominent visual aura. Scalp video EEG captured several focal and focal-to-bilateral tonic clonic seizures arising from the right posterior temporal-occipital junction, as well as frequent sleep-augmented interictal epileptiform discharges arising from the same region. No associated abnormality was identified on MRI, either prospectively or retrospectively, although preoperative MRI examinations were mildly motion degraded. On MEG, interictal epileptiform activity modeled to multiple cortical regions in the medial right temporal lobe, most notably in the right parahippocampal and fusiform gyri. A PET performed during a typical aura captured right temporal

hypermetabolism, suggesting this was an ictal study. Wada testing demonstrated left hemisphere language dominance and intact memory bilaterally. Neuropsychological testing was notable for a suspected undiagnosed learning disability, subtle chronic cognitive decline, and impaired visuospatial memory compared with verbal memory. The patient underwent a right posterior craniotomy with intracranial electrode implantation (subdural grids) that further localized the epileptogenic zone to the right basal temporal-occipital cortex, inferior temporal gyrus, hippocampus and amygdala. These areas were resected and the patient had an uneventful post-operative recovery. At last follow up (7 years post-surgery), the patient was seizure-free and off all seizure medications.

Routine histologic analysis was performed on the submitted specimens to evaluate for epilepsy-associated neuropathologic features[7,8]. Hematoxylin and Eosin (H&E) and NeuN immunostaining of the right posterior inferior temporal cortex revealed relative preservation of the laminar architecture and minimal blurring of the grey-white boundary (Fig. 1a). GFAP immunostaining highlighted subpial and subcortical astrogliosis (Fig. 1b). However, the cortical neurons were disorganized, forming clusters of abnormally shaped neurons with abundant cytoplasm (Fig. 1f-g) including scattered neurofilament-positive neurons (Fig. 1h). Ki-67 immunostaining highlighted scattered actively dividing cells scattered throughout the cortex (Fig. 1c). An immunohistochemical stain for CD45 highlighted predominantly resting microglia and rare perivascular macrophages (Fig. 1d). Immunostaining for p53 highlighted only rare positive nuclei, suggestive of wildtype TP53 status, and an immunostain for IDH1 p.R132H was negative, arguing against an IDH-mutant diffuse glioma (not shown). Unlike prior reports of epilepsy-associated lesions with *BRAF*^{V600E} [6] our case did not show any CD34+ extravascular stellate cells (Fig. 1e). Taken together, the findings were found to be most consistent with focal cortical dysplasia ILAE type IIa (FCD IIa). Histological sections of the hippocampus and amygdala revealed reactive astrogliosis. There was no significant neuronal loss to support a diagnosis of hippocampal sclerosis.

To further characterize the proliferating cell population, we performed multiplex immunofluorescence staining for Ki-67 together with different cell type markers (Fig. 2). Staining for Ki-67 together with neuronal marker NeuN and oligodendroglial marker Olig2 revealed that approximately 0.551% of cells were positive for Ki-67 (n=70/12704 in one slice). Of those cells, 52 stained for Olig2 (74.3%), two stained for both Olig2 and

NeuN (2.9%), and the remainder were negative for both NeuN and Olig2 (22.8%, 16/70). Separate staining for Ki-67 with microglial marker Iba1 and Olig2 confirmed that the majority of Ki67+ cells were Olig2+ (83.5%, n=66/79), and the remainder were Iba1+ (16.5%, n=13/79). These findings suggest that the actively dividing cells are oligodendroglia and microglia.

In addition to histopathology, cortical biopsies from the seizure focus and non-focus were flash frozen. DNA from the seizure focus and blood underwent deep whole-exome sequencing (WES) with a coverage depth of 229X and 374X, respectively. WES identified the *BRAF*^{K601E} variant in ~2% of alleles (3/143 reads) in the seizure focus and 0.4% of alleles (1/254) in the blood-derived DNA. Compared to whole-genome sequencing, WES is less sensitive for detecting gene fusions and copy number alterations[9] and we did not attempt to identify these genetic alterations in our samples. Using a digital PCR (QuantStudio 3D Digital PCR System, ThermoFisher) and a custom designed Taqman assay (LifeTechnologies) on three separate extractions of biopsies from the seizure focus and three extractions of biopsies from the brain tissue immediately adjacent to the seizure focus, we confirmed the variant to be present in the focus between 0.7%-1.4% of alleles and in the regions outside the seizure focus at 0.08%-0.36%. Blood derived DNA was confirmed to have a very low level of variant at <0.1%. After sequencing and validation, the remainder of the flash frozen tissue was submitted for histopathologic examination to further exclude the possibility of a low grade neoplasm. The frozen tissue showed similar histologic features as the main specimens previously examined. No tumor mass was identified, no CD34+ extravascular stellate cells were present, and there were scattered Ki-67+ cells throughout the cortex. In addition, the same DNA sample used for WES was submitted for methylation profiling to further assess whether it showed a methylation profile suggestive of tumor. The results mapped to the “Control Tissue, Hemispheric Cortex” subclass (Score: 0.99934) using the Heidelberg methylation classifier (v12.8.1)[10], consistent with non-neoplastic brain tissue. Of note, the classifier does not include FCD samples and methylation profiling can inaccurately classify low-cellularity neoplastic samples as “Control Tissue”.

In summary, we describe a non-expansile, epilepsy-associated lesion harboring a *BRAF*^{K601E} somatic mutation with distinct histopathologic features including mildly dysmorphic neurons and scattered Ki-67+ glial cells, but a notable absence of CD34+ extravascular stellate cells which have been reported in *BRAF*^{V600E}-

associated epileptogenic lesions[6]. This case raises important considerations regarding the overlap between LEATs and epilepsy-associated malformations of cortical development (MCDs) in the context of MAPK pathway activation. The presence of scattered Ki-67+ cells, many of which are Olig2+, raises the possibility that this lesion could represent an indolent diffuse glioma. However, elevated Ki-67 index is also a known feature of MCDs including hemimegalencephaly and focal cortical dysplasia type II (FCD II or Taylor type cortical dysplasia)[11,12]. While functional studies on *BRAF*^{K601E} are lacking, extensive work in mouse models suggests that *BRAF*^{V600E} mutation in progenitor cells during brain development leads to the acquisition of intrinsic epileptogenic properties in neuronal lineage cells, whereas tumorigenic properties are attributed to high proliferation of glial lineage cells[13,14]. However, introduction of the same mutation in mature neurons does not trigger epilepsy or abnormal cytomorphology, suggesting that changes in gene expression during development are crucial in the pathogenesis of *BRAF*^{V600E}- related epilepsy[13]. Furthermore, the neuronal phenotype induced by *BRAF*^{V600E} mutation during development is distinct from other MAPK pathway alterations, suggesting that different mutations in the MAPK pathway may lead to neuronal hyperexcitability via distinct mechanisms. Further studies will be needed to dissect the mechanisms and histopathologic phenotypes of non-*BRAF*^{V600E} MAPK pathway alterations associated with focal cortically based epilepsy.

Figures and Figure Legends

Figure 1. Histopathologic features in a case of cortically based, focal epilepsy with *BRAF* K601 mutation. Low-power (4×) images of immunohistochemical stains performed on the seizure focus reveals relative preservation of cortical layers and a well-demarcated grey-white boundary (**A**, NeuN), mild subpial and subcortical astrogliosis (**B**, GFAP), scattered Ki-67 positive cells (**C**, Ki-67), predominantly resting microglia and rare perivascular macrophages (**D**, CD45), and an absence of CD34-positive extravascular stellate cells (**E**, CD34). Higher power (40×) images reveal clusters of neurons in the superficial (**F**, H&E) and deep (**G**, H&E) cortical layers, some with abnormal morphology and ample cytoplasm. **H)** An immunohistochemical stain for neurofilament highlights scattered neurons. Scale bars: 200 μm, A–E; 20 μm, F–H. **Alt Text:** Representative images of the microscopic features in this case, including scattered Ki-67-positive nuclei, mildly dysmorphic neurons, and an absence of CD34-positive extravascular stellate cells.

Figure 2: Multiplexed immunofluorescence staining clarifies cell type identity of actively dividing cells. Multiplexed immunofluorescence staining for Ki-67 together with cell type markers reveals actively dividing microglia (A-E, arrow) and oligodendroglia (F-J, arrow). Scale bars, 10 μm. **Alt Text:** Representative images of multiplexed immunofluorescence stains for Ki-67 together with cell type markers Olig2, IbaI, and/or NeuN.

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