PI3K/AKT pathway mutations cause a spectrum of brain malformations from megalencephaly to focal cortical dysplasia

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Malformations of cortical development containing dysplastic neuronal and glial elements, including hemimegalencephaly and focal cortical dysplasia, are common causes of intractable paediatric epilepsy. In this study we performed multiplex targeted sequencing of 10 genes in the PI3K/AKT pathway on brain tissue from 33 children who underwent surgical resection of dysplastic cortex for the treatment of intractable epilepsy. Sequencing results were correlated with clinical, imaging, pathological and immunohistological phenotypes. We identified mosaic activating mutations in PIK3CA and AKT3 in this cohort, including cancer-associated hotspot PIK3CA mutations in dysplastic megalencephaly, hemimegalencephaly, and focal cortical dysplasia type IIa. In addition, a germline PTEN mutation was identified in a male with hemimegalencephaly but no peripheral manifestations of the PTEN hamartoma tumour syndrome. A spectrum of clinical, imaging and pathological abnormalities was found in this cohort. While patients with more severe brain imaging abnormalities and systemic manifestations were more likely to have detected mutations, routine histopathological studies did not predict mutation status. In addition, elevated levels of phosphorylated S6 ribosomal protein were identified in both neurons and astrocytes of all hemimegalencephaly and focal cortical dysplasia type II specimens, regardless of the presence or absence of detected PI3K/AKT pathway mutations. In contrast, expression patterns of the T308 and S473 phosphorylated forms of AKT and in vitro AKT kinase activities discriminated between mutation-positive dysplasia cortex, mutation-negative dysplasia cortex, and non-dysplasia epilepsy cortex. Our findings identify PI3K/AKT pathway mutations as an important cause of epileptogenic brain malformations and establish megalencephaly, hemimegalencephaly, and focal cortical dysplasia as part of a single pathogenic spectrum.

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Abbreviations: CLOVES = congenital lipomatous overgrowth, vascular malformations, epidermal nevi and spinal/skeletal anomalies; DMEG = dyplastic megalencephaly; FCD = focal cortical dysplasia; HMEG = hemimegalencephaly; MEG = megalencephaly; mTOR = mammalian target of rapamycin; MIP = molecular inversion probe

Introduction

The PI3K/AKT/mTOR signalling pathway has been shown to be centrally important in both normal brain development and human neurological disease. Loss of function mutations in the tuberous sclerosis complex TSC1 or TSC2 genes, resulting in dysregulated mammalian target of rapamycin (mTOR) activity, are associated with brain lesions, epilepsy, cognitive impairment, and autism (European Chromosome 16 Tuberous Sclerosis Consortium, 1993; Slegtenhorst et al., 1997). Loss of function mutations in phosphatase and tensin homologue (PTEN), resulting in dysregulated AKT activity, cause Cowden and Bannayan-Riley-Ruvalcaba syndromes (also known as PTEN hamartoma tumour syndrome), associated with megalencephaly (MEG), cognitive impairment, autism and epilepsy (Arch et al., 1997; Liaw et al., 1997; Pilarski et al., 2013). Recently, we identified mosaic-activating mutations in AKT3, PIK3CA, which encodes the p110 catalytic subunit of phosphoinositide 3-kinase (PI3K), PIK3R2, which encodes the p85 regulatory subunit of PI3K, and CCND2, which encodes the AKT downstream target cyclin D2, in megalencephaly-capillary malformation syndrome and megalencephaly-polydactyly-hydrocephalus (MPPH) syndrome (Riviére et al., 2012; Mirzaa et al., 2014).

Mosaic AKT3, PIK3CA and MTOR mutations have been found in brain tissue resected from individuals with hemimegalencephaly (HMEG) (Lee et al., 2012; Poduri et al., 2012). The neuropathological phenotypes of tuberous sclerosis complex, PTEN mutation syndromes, and HMEG exhibit many similarities, including cortical dyslamination, cytomegaly and dysmorphic neurons (Huttenlocher and Wollmann, 1991; Richardson, 1991; Flores-Sarnat, 2002; Merks et al., 2003). These histological characteristics are shared by focal cortical dysplasia (FCD), a condition causing the majority of intractable localization-related epilepsy in childhood (Blumcke et al., 2011; Hauptman and Mathern, 2012). Activation of the PI3K/AKT pathway in FCD has been suggested by previous immunohistochemistry studies (Ljungberg et al., 2006; Schick et al., 2006, 2007).

We therefore performed next generation sequencing of brain tissue from a cohort of 33 children with either HMEG or FCD using molecular inversion probe (MIP) capture technology targeting 10 genes involved in the PI3K/AKT signalling pathway. Mutation status was correlated with clinical, imaging, pathological and immunohistological phenotype. In addition to identification of novel causative mutations, we demonstrate that mosaic mutation in a single gene, PIK3CA, can result in either MEG, HMEG, or FCD type IIa. Furthermore, increased PI3K/AKT/mTOR pathway signalling was evident in all type IIa and IIb FCD specimens in the absence of detected mutations, implicating other causative mechanisms leading to a common pathophysiology. These findings solidify MEG/HMEG/FCD as a single neurobiological spectrum disorder and suggest new potential therapeutic approaches.

Materials and methods

Paediatric cortical brain specimens

Specimens were collected from 33 children with intractable epilepsy due to HMEG or FCD who underwent resective surgery at Seattle Children’s Hospital between 2004 and 2012. Four non-dysplasia epilepsy specimens with pathological diagnoses of gliosis were also examined. A control, non-epilepsy cortical specimen from a 4-year-old child was obtained during surgical approach for tumour resection. Informed consent for the use of a portion of the resected tissues for research purposes was obtained with the approval of the hospital’s Institutional Review Board. Immediately after excision, the tissue was frozen in liquid nitrogen and stored at –80°C until use. Formalin-fixed, paraffin-embedded sections (4μM) were prepared from tissue adjacent to each frozen specimen. The results of routine stains done for diagnostic purposes were reviewed and classified by R.F.H.

Multiplex targeted sequencing

Genomic DNA was isolated from 50–200 mg of frozen resected cortex using the Qiagen Gentra Puregene® Tissue kit. Targeted sequencing was performed using 12N (degenerate tag) single molecule MIP capture technology (Hiatt et al., 2013). A pool of 350 single molecule MIP oligonucleotides targeting the coding sequences of 10 PI3K/AKT pathway genes was designed and optimized (PIK3CA, PIK3CB, PIK3CD, PIK3R1, PIK3R2, PIK3R3, AKT1, AKT2, AKT3, and PTEN). These upstream members of the PI3K/AKT pathway were chosen based on our previous findings in the megalencephaly syndromes megalencephaly-capillary malformation and MPPH (Riviére et al., 2012). The MIPs tiled the coding exons and splice-junctions, a total of 12.6 kb of genomic sequence. Targeted capture was performed as previously described using 500 ng of DNA as template (O’Roak et al., 2012). Following the post-capture PCR amplification, samples were subjected to massively parallel sequencing using the Illumina platform. Reads were mapped as described previously (O’Roak et al., 2012). Read-pairs with identical tags (originating from the same capture event) were removed, leaving the read-pair with the highest estimated quality. We then identified all sites with at least two Phred-quality 30 non-reference
bases and compared the observed read counts to a site-specific empirical error model built from unrelated samples (binomial). Sites showing strong signal for mutation were evaluated for possible pathogenicity. The mean coverage of the target bases was 567×, with 86% of target bases at 50× coverage or greater and 92% of target bases at 10× coverage or greater. Full details regarding our single molecule MIP technology can be found in Hiatt et al. (2013).

**Sanger sequencing**

Standard Sanger sequencing was used to confirm all identified variants using custom intronic primers and standard PCR protocols (primer sequences available on request). Amplicons were Sanger sequenced at the Seattle BioMed Sequencing Core Facility. All mutations were tested in at least two independent amplification and sequencing reactions in the proband and available relatives. Sequence traces were analysed using Mutation Surveyor v3.97 (SoftGenetics).

**Quantitative PCR**

Quantitative PCR was performed using the Power SYBR® Green PCR Master Mix (Applied Biosystems) following the manufacturer’s protocol. Three independent primer pairs across the AKT3 gene were used to check for copy number variation. Primer sequences are available on request. Quantitative PCR was performed on an ABI 7900 HT real-time PCR system (Applied Biosystems) in a 384-well format using SDS 2.3 software. Each sample was analysed in triplicate in a total reaction volume of 10 μl using 20 ng of DNA. PCR conditions were 95°C, denaturation for 10 min, then 40 cycles each of 95°C for 15 s and 60°C for 1 min. Data analysis was carried out with RQ manager 1.2 using the ΔΔCT method to obtain the relative quantity values after normalizing with two reference genes (ALB, RPP30). Primers were designed using Primer3 (http://frodo.wi.mit.edu/primer3/).

**Western blot analysis**

Frozen cortical tissue (100–200 mg) was separated from underlying white matter and homogenized in 5 mM Tris/HCl (pH 7.4) with 0.32 M sucrose. The homogenates were centrifuged at 3000g for 5 min at 4°C. The supernatant was then centrifuged at 40000g for 1 h at 4°C to pellet the membrane fraction. Thirty micrograms of protein from the cytosolic fraction was electrophoretically separated and transferred to an Immobilon®-FL PVDF membrane (Millipore). The membrane was blocked with Odyssey Blocking Buffer (LI-COR Biosciences), incubated in primary antibodies overnight at 4°C, and then incubated in goat anti-rabbit or goat anti-mouse infrared dye-labelled secondary antibody (IRDye®-LI-COR) or HRP-conjugated secondary antibody (Bio-Rad) for 2 h at room temperature. Infrared fluorescence (Odyssey Infrared Imaging System, LI-COR) or chemiluminescence (Kodak Gel Logic 2200 imaging system) was used for signal detection. Primary antibodies used for western blotting included rabbit monoclonal phospho-Akt (Ser473) XP, rabbit monoclonal phospho-Akt (Thr308), and mouse monoclonal Akt3 (all from Cell Signaling Technology). Binding of mouse monoclonal antibodies against β-tubulin (Novus Biologicals) was used as a loading control. Each blot was repeated twice and the results averaged.

**Immunohistochemistry**

Specimens were deparaffinized by incubation in xylene, followed by rehydration through graded ethanol/water solutions and equilibration in PBS. Antigen retrieval was performed by heating in 0.01 M sodium citrate (pH 6.0) in a 90°C water bath for 45 min. Specimens were then blocked in PBS with 10% donkey serum, 0.1% Triton® X-100 and 2% bovine serum albumin for 30 min and incubated overnight at 4°C in primary antibodies diluted in blocking solution. Primary antibodies used in these studies included mouse monoclonal MAP2 (Millipore), rabbit monoclonal phospho-Akt (Ser473) XP, rabbit monoclonal phospho-Akt (Thr308), and rabbit monoclonal phospho-S6 ribosomal protein (Ser235/246) XP (all from Cell Signaling Technology). Slides were then incubated in fluorescent secondary antibodies (Alexa Fluor®) at room temperature for 2 h. Following incubation in DAPI nuclear counterstain and Sudan Black to block endogenous fluorescence, sections were examined and photographed using a Zeiss Axio Imager Z1 or Nikon Eclipse Ni-U epifluorescence microscope. Maximal cell soma diameters of all neurons with combined MAP2 and phospho-S6 immunofluorescence were measured manually in at least five microscopic fields using Nikon NIS Elements software.

**AKT kinase activity measurements**

Frozen cortical brain tissue (50 to 300 mg) was separated from underlying white matter and homogenized in buffer containing (in mM) glycine 200, NaCl 150, EGTA 50, EDTA 50, and sucrose 300 (pH 9), plus 10 μl of protease inhibitor cocktail. Samples were centrifuged and protein concentrations determined in the supernatant using a Bradford protein assay. AKT kinase activity was measured using the Cell Signaling Technology assay kit (Cat. no. 9840). AKT was immunoprecipitated from 100 μg of extracted protein with bead-conjugated phospho-Akt (Ser473) rabbit monoclonal antibody. Precipitated AKT was then incubated with a GSK-3 fusion protein substrate and ATP in kinase buffer at 30°C for 2 h. GSK-3 fusion protein phosphorylation was detected and quantitated by western blotting using phospho-GSK-3α/β (Ser21/9) antibodies and infrared detection (Odyssey Li-Cor Imager). For each specimen the measured density of the phosphorylated GSK-3 fusion protein band was normalized to that of the anti-AKT IgG light chain (gel loading control) and expressed relative to the control surgical specimen.

**Results**

**Cortical dysplasia cohort characteristics**

Thirty-three children who underwent surgical resection for intractable epilepsy due to cortical dysplasia were studied, in addition to one child with imaging features of bilateral HMEG (termed dysplastic megalencephaly, DMEG). Males and females were equivalently represented (16 males,
polymicrogyria (4/6). Interestingly, aggregates of immature morphic neurons, but the absence of balloon cells. Other lamination, blurring of the grey–white junction, and dys-
cortical abnormalities, including localized undersulcation, cortical thickening, and/or blurring of the grey–white junction. Localized abnormalities in white matter signal were also frequent in FCD, present in 15/24. Ventricular and extra axial space enlargement was rare in the FCD patients. Also seen in the affected hemispheres were dilation of the lateral ventricle and enlargement of the extra axial space with prominent vasculature. In contrast, the majority of the patients with FCD type I and II (19/24) had mild to moderate cortical abnormalities, including localized undersulcation, cortical thickening, and/or blurring of the grey–white junction. Localized abnormalities in white matter signal were also frequent in FCD, present in 15/24. Ventricular and extra axial space enlargement was rare in the FCD patients. All FCD IIA and IIB patients had at least one abnormality evident on retrospective review of MRI images, whereas 2/5 FCD I patients were thought to have completely normal imaging studies (Supplementary Table 2).

The results of routine histopathological analysis were reviewed for all surgical specimens and classified based on the International League Against Epilepsy (ILAE) consensus classification (Blumcke et al., 2011), as shown in Supplementary Table 3. All HMEG specimens in our cohort (6/6) demonstrated histopathological features of FCD type IIA, as indicated by the presence of cortical dyslamination, blurring of the grey–white junction, and dysmorphic neurons, but the absence of balloon cells. Other common histopathological features of HMEG cortex were calcifications (3/6), microscopic heterotopia (2/6), and areas of layer 1 (molecular layer) fusion, similar to that seen in polymicrogyria (4/6). Interestingly, aggregates of immature small diameter neurons were found at relatively high frequency in FCD IIA (5/13) but not FCD IIB (0/6), whereas layer 1 fusion was also found in some cases of FCD IIA (2/13) but not FCD IIB (0/6).

Identification of PI3K/AKT pathway mutations

We performed multiplex targeted sequencing of the PIK3CA, PIK3CB, PIK3CD, PIK3R1, PIK3R2, PIK3R3, AKT1, AKT2, AKT3 and PTEN genes in genomic DNA isolated from frozen brain tissue resected from the 33 children with HMEG/FCD and intractable epilepsy. This sequencing identified pathogenic mutations in 4 of 33 patients (12%), in addition to the patient with DMEG and systemic features (buccal DNA). These patients are presented in detail below. In the remaining 29 HMEG/FCD patients, no pathogenic sequence variants were found. Full details regarding pathogenic mutations identified by sequencing are presented in Supplementary Table 4. Based on the report of Poduri et al. (2012), analysis for AKT3 gene duplication by quantitative PCR was performed on samples from the three HMEG patients without identified mutations; this analysis was negative.

Patient LR12-033: mosaic PIK3CA mutation in a patient with DMEG, capillary malformations and segmental overgrowth

This female child had severe congenital megalencephaly associated with asymmetric enlargement of the face, dramatic overgrowth of both hands and both feet, extensive capillary malformations of the skin, and several epidermal nevi, features consistent with CLOVES syndrome (congenital lipomatous overgrowth, vascular malformations, epidermal nevi and spinal/skeletal anomalies; Kurek et al., 2012). Her brain MRI, briefly described in an earlier report (Riviere et al., 2012), showed diffuse cortical malformation resembling HMEG on both sides, ventriculomegaly, and white matter dysplasia with relative enlargement of the left hemisphere as compared with the right (Fig. 1A and Supplementary Table 2). Deep sequencing of genomic DNA isolated from a buccal swab identified a mosaic p.Glu545Lys PIK3CA mutation in 26% of reads, which was absent from blood (Table 1). This mutation was also detected by Sanger sequencing of buccal DNA. Given the bilateral severe cortical malformation, she was not considered a candidate for epilepsy surgery and brain tissue was therefore not available for analysis.

Patient LR12-241: mosaic PIK3CA mutations in a patient with HMEG and linear epidermal nevus

This male child developed focal-onset and myoclonic seizures within the first few days of life that became intractable to anticonvulsant medications. Linear epidermal nevi were identified on his right upper extremity, trunk, and face. Brain MRI revealed enlargement of the right occipital, parietal and posterior temporal lobes (Fig. 1B) with irregular
cortex, undulation of the grey–white matter junction, and underlying dysplastic white matter (Supplementary Table 2). He underwent hemispherectomy at 17 months of age, which resulted in seizure freedom. Deep sequencing of genomic DNA isolated from the resected temporal lobe identified two mosaic mutations in PIK3CA: p.Glu542Lys and p.Thr544Asn (Table 1). Both were present in ~31% of reads and were confirmed by Sanger sequencing (Fig. 2A). Manual examination of the deep sequencing reads revealed these two mutations occurred on the same DNA strand, likely representing a single mutational event.

Figure 1 MRI findings in dysplastic brain malformations with and without detected mutations. T2-weighted images are shown. Filled arrows indicate regions of abnormality, including ventricular enlargement, variable degrees of cortical dysmorphism (i.e. pachygyria, polymicrogyria, or undersulcation), periventricular nodular heterotopia, abnormal white matter signal, and/or widening of the extra-axial space. (A) DMEG; (B, E–H) HMEG; (C) FCD type IIa; (D) bihemispheric asymmetric dysplasia. The open arrow indicates unilateral ocular enlargement in Patient LR11-443, who exhibits severe abnormality of the left hemisphere (HMEG) along with milder abnormalities of the posterior portion of the right hemisphere.
Figure 2  Sanger sequencing confirmation of mutations detected in HMEG brain by molecular inversion probe capture technology. Sequence changes are highlighted by the blue bars. (A) Mosaic PIK3CA p.E542K and p.T544N mutations from HMEG brain LR12-241. (B) Mosaic AKT3 p.E17K mutation from HMEG brain LR11-443 operculum and hippocampus, which was absent in overlying dura. (C) Germline PTEN p.Y68H heterozygous mutation detected in LR12-123 brain.

Table 1  Sequencing results

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Gene</th>
<th>Mutation</th>
<th>Type</th>
<th>Level and tissues tested</th>
<th>Sanger validation</th>
<th>Parental testing</th>
</tr>
</thead>
<tbody>
<tr>
<td>LR12-033</td>
<td>1</td>
<td>F</td>
<td>DMEG</td>
<td>PIK3CA</td>
<td>p.Glu545Lys</td>
<td>Mosaic</td>
<td>26% (buccal) negative (blood)</td>
<td>Yes</td>
<td>n/a</td>
</tr>
<tr>
<td>LR12-241</td>
<td>1.4</td>
<td>M</td>
<td>HMEG</td>
<td>PIK3CA</td>
<td>p.Glu542Lys</td>
<td>Mosaic</td>
<td>31% (brain)</td>
<td>Yes</td>
<td>n/a</td>
</tr>
<tr>
<td>LR12-251</td>
<td>5.3</td>
<td>F</td>
<td>FCD IIa</td>
<td>PIK3CA</td>
<td>p.Glu542Lys</td>
<td>Mosaic</td>
<td>31% (brain)</td>
<td>Yes</td>
<td>n/a</td>
</tr>
<tr>
<td>LR11-443</td>
<td>1</td>
<td>F</td>
<td>HMEG</td>
<td>AKT3</td>
<td>p.Glu1Lys</td>
<td>Mosaic</td>
<td>10-18% (brain) negative (dura, skin, fibroblast cx)</td>
<td>Yes</td>
<td>Negative</td>
</tr>
<tr>
<td>LR12-123</td>
<td>9.2</td>
<td>M</td>
<td>HMEG</td>
<td>PTEN</td>
<td>p.Tyr68His</td>
<td>Germline</td>
<td>50% (brain, blood, saliva)</td>
<td>Yes</td>
<td>Mother negative, father unavailable</td>
</tr>
</tbody>
</table>

*Validated on repeat single molecule MIP sequencing. n/a = not available.
Patient LR12-251: mosaic PIK3CA mutation in a patient with localized FCD IIa

This female patient developed medically refractory epilepsy at 3 years of age and was found on MRI to have a localized area in the right occipital lobe of undersulcation, blurring of the grey–white matter junction, and underlying increased T2 signal in the white matter (Fig. 1C and Supplementary Table 2). She was developmentally normal and had no extra-CNS lesions. She underwent right occipital resection at 5 years of age with resolution of her seizures. Histopathology was consistent with FCD IIa (Supplementary Table 3). Deep sequencing of resected cortical tissue identified a PIK3CA p.Glu17Lys mutation in 4.7% of reads (Table 1). This level of mosaicism was undetectable by Sanger sequencing, but was confirmed on repeat single molecule MIP analysis.

Patient LR11-443: mosaic AKT3 mutation in a patient with HMEG, cutaneous vascular malformations and unilateral ocular enlargement

At birth, this female patient was found to have enlargement of the left eye and vascular skin lesions on her leg and back resembling cutis marmorata telangiectatica congenita. She developed seizures at 3 days of life. MRI demonstrated enlargement and dysplasia of the entire left cerebral hemisphere, including an undersulcated gyral pattern with pebbling of the grey-white matter junction and underlying dysplastic white matter, as well as more subtle abnormalities of the right hemisphere (Fig. 1D and Supplementary Table 2). Her epilepsy progressed to intractable clonic seizures and infantile spasms. She underwent a left hemispherectomy at 1 year of age. Deep sequencing of genomic DNA isolated from resected frontal, temporal, opercular, and hippocampal tissue identified a mosaic p.Glu17Lys AKT3 mutation in 10–18% of reads (Table 1 and Supplementary Table 4). The presence of the mutation was also visible by Sanger sequencing (Fig. 2B), even though this level of mosaicism is traditionally below the level of Sanger sensitivity. The AKT3 mutation was absent from dura overlying the area of resection (single molecule MIP analysis), as well as from epidermis and fibroblasts cultured from a biopsy of unaffected skin (Sanger sequencing).

Patient LR12-123: germline PTEN mutation in a patient with isolated HMEG

This male patient developed focal onset seizures shortly after birth which proved resistant to anticonvulsant therapies. Brain MRI revealed right frontal overgrowth with pachygyria and dysplastic underlying white matter and lateral ventricle (Fig. 1E and Supplementary Table 2). He underwent hemispherectomy at 9 years of age and became seizure-free. Deep sequencing of resected cortical tissue identified a germline PTEN p.Tyr68His mutation that was confirmed by Sanger sequencing (Fig. 2C) and was also present in peripheral blood (Table 1). The PTEN mutation was not present in his mother; DNA samples from his father were not available. No peripheral stigmata of the PTEN hamartoma tumour syndrome were found at the time of his most recent evaluation.

PI3K/AKT pathway activation in HMEG/FCD specimens with and without identified mutations

The activation state of kinases in the PI3K/AKT pathway can be examined through the use of antibodies specific for the phosphorylated forms of their target proteins. AKT is phosphorylated on Thr308 (or its equivalent) by PDK1 (3-phosphoinositide dependent protein kinase 1) and on Ser473 by the mTORC2 complex (Fig. 7). Increased AKT T308 phosphorylation results from either activation of PI3K or PDK1 or loss of activity of the inhibitory protein PTEN, while increased AKT S473 phosphorylation results from activation of mTOR. Phosphorylation of AKT at both sites is required for full kinase activity (Alessi et al., 1996). In contrast, phosphorylation of ribosomal protein S6 serves as a downstream readout of activation at any level of the PI3K/AKT/mTOR pathway (Bellacosa et al., 2005; Bhaskar and Hay, 2007; Manning and Cantley, 2007). In these experiments, we assayed the phosphorylation state of AKT and ribosomal protein S6 in 20 HMEG/FCD brain specimens by western blot and fluorescence immunohistochemistry analysis. As shown in Fig. 3, nearly all HMEG and FCD specimens (19/20) exhibited an increase in both T308 and S473 pAKT expression in comparison to control cortex as measured by western blotting of cortical homogenates. While all HMEG specimens had elevated pAKT expression, the samples with the highest levels were those with identified PIK3CA, AKT3, and PTEN mutations (Fig. 3). Among the FCD IIa and FCD IIb specimens, a range of pAKT levels was found, from mildly to markedly elevated (Fig. 3B and C, and Supplementary Fig. 1). The level of pAKT in the FCD Ila specimen with the PIK3CA mutation was only mildly increased, likely reflecting the low level of mosaicism (4.7%) in this specimen. Interestingly, in non-dysplasia epilepsy cortex, S473 pAKT was elevated (suggestive of downstream pathway activation) whereas T308 pAKT was not (indicating lack of upstream pathway activation). Total AKT3 levels were similar among all samples (Fig. 3A and Supplementary Fig. 1).

The phosphorylation state of AKT and ribosomal protein S6 were next analysed in HMEG and FCD specimens by double label fluorescence immunohistochemistry to determine which cell types were affected by dysregulated PI3K/AKT pathway signalling. In each HMEG, FCD Ila and FCD IIb specimen analysed, prominent phospho-S6 expression was found in the cytoplasm of MAP2-positive neurons (Fig. 4). The proportion of affected neurons ranged from 10% to nearly 50% in samples both with and without identified PI3K/AKT pathway mutations. In the specimen with the germline PTEN mutation (which should be present as a single copy in all cells), only a small percentage...
(~10%) of neurons exhibited increased phospho-S6 expression (Fig. 4D). This suggests that a second, unidentified somatic mutation may be required for excessive PI3K/AKT pathway activation, similar to what has been found in the tumours of Cowden syndrome (Bonneau and Longy, 2000). Our single molecule MIP sequencing data makes a mosaic intragenic mutation of PTEN unlikely, but a low level deletion has not been excluded. In FCD IIb specimens, phospho-S6 was present in both neurons and balloon cells (Fig. 4I). Astrocytic phospho-S6 immunofluorescence was visible in all HMEG/FCD specimens as well as in non-dysplasia epilepsy cortex (Fig. 4 and Supplementary Fig. 2A and B).

We observed that neuronal size seemed to vary substantially between different HMEG/FCD specimens (Fig. 4). Phospho-S6 positive neuronal diameter was therefore quantified in 12 HMEG/FCD specimens and compared with neuronal diameter measured in four control paediatric samples (Supplementary Fig. 3). In all HMEG/FCD II samples, phospho-S6 immunofluorescence was present in neurons with enlarged diameters (>25 μm) as well as in smaller neurons. The most striking neuronal hypertrophy was in the specimen with the AKT3 p.Glu17Lys mutation (LR11-443) (Fig. 4C). Lesser hypertrophy of phospho-S6 positive neurons was seen in the two specimens with PIK3CA mutations (LR12-241 and LR12-251, Fig. 4A and B), whereas neurons in the specimen with the PTEN mutation were intermediate in size (Fig. 4D). This heterogeneity in phospho-S6 positive neuronal size extended to the mutation-negative HMEG and FCD specimens (Supplementary Fig. 3).

We next used fluorescence immunohistochemistry to determine which cell types exhibited evidence of upstream PI3K/AKT pathway activation in HMEG/FCD. T308 pAKT immunofluorescence was present in neuronal cytoplasm and nuclei in the HMEG/FCD specimens with identified PIK3CA, AKT3 and PTEN mutations (Fig. 5). Neuronal T308 pAKT immunofluorescence was also present in many HMEG/FCD specimens without detected mutations, consistent with western blot results (Fig. 3 and Supplementary Fig. 4). Dysplastic specimens with and without detected mutations also exhibited T308 pAKT labelling in astrocytes (Supplementary Fig. 2). In contrast to T308 pAKT, in HMEG/FCD as well as non-dysplasia epilepsy specimens S473 pAKT expression was present in the neuronal soma, but not nucleus, and was also prominent in the cortical neuropil (Supplementary Fig. 5).

In addition to analysing the phosphorylation state of AKT at T308 and S473, we directly measured AKT kinase activity in vitro in cortical extracts. Marked increases in AKT kinase activity were found in specimens with AKT/PI3K pathway mutations as compared with control and non-dysplasia epilepsy brain (Fig. 6). Importantly, a substantial increase in AKT kinase activity was found for the FCD IIa specimen with the low-level mosaic PIK3CA p.His1047Arg mutation (LR12-251), even though pAKT

Figure 3 Western blot analysis of T308 pAKT, S473 pAKT, and total AKT3 levels in dysplastic brain specimens. (A) Representative western blot showing increased expression of T308 pAKT and S473 pAKT in six HMEG brain specimens as compared to control, non-epileptic brain (all surgical specimens). The highest levels of pAKT were seen in the HMEG specimens with detected PIK3CA, AKT3 and PTEN mutations. (B and C) Summary of relative T308 pAKT (B) and S473 pAKT (C) levels in 17 dysplastic brain specimens (HMEG, FCD IIa, and FCD IIb) as compared with control brain and three non-dysplasia epilepsy brain specimens. The ratio of T308 pAKT or S473 pAKT band intensity to total AKT3 band intensity was determined for each specimen and is expressed relative to that of specimen LR12-241, which was run concurrently on each blot. 1LR11-443 AKT3 mutation; 2LR12-123 PTEN mutation; 3LR12-241 PIK3CA mutation; 4LR12-251 PIK3CA mutation.
T308 and S473 levels in whole-tissue homogenates for this sample were only mildly elevated (Fig. 3).

**Discussion**

We report a detailed phenotypic study of 34 children with DMEG, HMEG or FCD and critical genetic information that expands our understanding of this spectrum of disorders. This is the first study of the cortical dysplasias that combines thorough review of imaging studies, massively parallel sequencing using single molecule MIP technology for detection of low levels of brain mosaicism, and examination of affected tissue by a combination of histopathology, immunohistochemistry, western blotting, and in vitro kinase assays. All of these data converge to demonstrate a shared pathogenesis for these developmental disorders.

**Neuroimaging**

In our surgical cohort, brain imaging studies demonstrated wide variation in distribution of cortical malformation...
from lobar to multilobar to hemispheric, similar to published cohorts (Barkovich and Chuang, 1990; Nakahashi et al., 2009; Colombo et al., 2012). The involved areas usually demonstrate subtle irregularity of the pial surface, variably thick (5–15 mm) cortex with poor differentiation of the grey-white border, and shallow sulci. The underlying white matter appears abnormal. More severe malformations in our cohort were characterized by ventricular...
dilation, widened extra-axial spaces with enlarged dysplastic blood vessels, markedly thick (10–15 mm) cortex, visible white matter cysts, and variable enlargement of other brain structures such as the deep nuclei, brainstem and cerebellum. We also report imaging data on a female child with severe bilateral brain and extra-CNS involvement associated with a mosaic mutation of \textit{PIK3CA} found in saliva-derived DNA, as we believe that this completes the spectrum of brain involvement of this class of cortical malformations. Rather than using the problematic term ‘bilateral’ HMEG, we classify her malformation as DMEG. Although rare, bilateral malformations of cortical development with features of HMEG or FCD have been reported (Jahan et al., 1997; Fauser et al., 2009; Kometani et al., 2010).

### Histopathology

Microscopic review of brain tissue demonstrated histopathological features of FCD I \((n = 5)\), FCD IIa \((n = 19)\), FCD IIb \((n = 6)\), or FCD III \((n = 3)\) in all 33 brains, as this was one of our inclusion criteria. The histology of the six children with HMEG as well as another 13 patients with more focal lesions was consistent with FCD IIa including loss of cortical lamination, dysmorphic neurons, and blurred grey–white matter junction with increased heterotopic neurons in white matter (Blumcke et al., 2011). Six patients exhibited both dysmorphic neurons and balloon cells, consistent with FCD IIb. In our FCD IIa/IIb cohort, we also found several variable features including disparity in size of dysmorphic neurons and presence or absence of layer 1 fusion, calcifications, neuronal heterotopias and clusters of immature cells.

### Sequencing

Our sequencing studies detected mutations in upstream components of the PI3K/AKT pathway in 4 of 19 patients with FCD type IIa histopathology (including three of six with HMEG and 1 of 13 with localized FCD IIa) and none of six with FCD IIb. No mutations were identified in the eight specimens with FCD types I or III. Combining our data with prior reports, brain DNA mutations of core PI3K/AKT/mTOR pathway components have now been found in 12 of 34 (35%) patients with HMEG including five with mutations of \textit{PIK3CA}, five with mutations of \textit{AKT3}, and one each with mutations of \textit{PTEN} or \textit{MTOR} (Lee et al., 2012; Poduri et al., 2012). All were mosaic...
except for the PTEN mutation (which we discuss below), representing 8–40% of reads corresponding to 16–80% of cells, as the mutations are heterozygous. We also report the first patient with localized FCD (FCD IIa by pathology) and mosaic mutation of PIK3CA, although this result is not surprising given the similarities between FCD and HMEG on brain imaging, histopathology and immunohistochemistry. The level of mosaicism in the FCD specimen was only 4.7%, lower than any reported patients with HMEG. Although more data are needed to assess the relationship between phenotype and level of mosaicism, these data suggest that localized dysplasias (i.e., FCD) could be associated with lower levels of mosaicism than extensive dysplasias, due to the occurrence of somatic mutations later during brain development.

The sequencing panel used in our study, which focused on the upstream members of the PI3K/AKT pathway, was designed based on previous results from the megalencephaly syndromes reported in (Riviére et al., 2012). Sequencing of MTOR, other downstream PI3K/AKT pathway members, and additional genes related to this pathway may identify further causative mutations. Accordingly, in future studies we plan to screen the mutation-negative specimens from this cohort as well as additional specimens with an extended gene panel.

### PI3K/AKT pathway activation in HMEG/FCD

We used western blotting, immunohistochemistry, and in vitro kinase assays to look for activation of the PI3K/AKT pathway in our HMEG/FCD specimens. We found the highest levels of T308 (PI3K driven) AKT phosphorylation and AKT kinase activity in specimens with identified mutations, but also found elevated T308 AKT phosphorylation in the majority of patients with HMEG or FCD IIa/IIb, regardless of mutation status. Previous studies of AKT phosphorylation in HMEG/FCD have not examined the T308 phosphorylation site (Ljungberg et al., 2006; Schick et al., 2006, 2007; Aronica et al., 2007; Sarnat et al., 2012). Interestingly, we also found evidence of increased S473 phospho-AKT in epilepsy specimens without dysplasia, supporting a role for the downstream PI3K/AKT/mTOR pathway in seizures of many causes, as has been seen in rodent models (Zeng et al., 2009; Berdichevsky et al., 2013; Macias et al., 2013) and recent human epilepsy studies (Liu et al., 2014). In our immunohistochemistry studies we analysed protein S6 phosphorylation at Ser235/236, which is mediated by the Ras/ERK signalling cascade as well as by the PI3K/AKT/mTOR pathway (Roux et al., 2007), so an additional contribution of the Ras/ERK pathway to our findings cannot be ruled out.

### Genotype–phenotype analysis

Our studies strongly support a single pathobiological spectrum of disease for DMEG, HMEG and FCD, but also demonstrate more heterogeneity within each category than apparent from previous studies. Although all of our HMEG and FCD type II specimens displayed cortical dyslamination and dysmorphic neurons, we found that activating mutations of different PI3K/AKT pathway core components had different effects on neuronal morphology. Mutations of PIK3CA were associated with only mildly enlarged neuronal size, whereas mutation of AKT3 was associated with dramatically enlarged neuronal size. These data support previous in vitro experiments, in which transfection of a constitutively active form of AKT into cultured hippocampal neurons caused more prominent neuronal hypertrophy than transfection of a constitutively active form of PIK3CA (Kumar et al., 2005). The same variation was seen among mutation-negative brain samples, which suggests further aetiological heterogeneity. Studies of larger groups of specimens with identified mutations are needed to confirm these initial observations. We further predict that differences in morphology will be associated with differences in biochemical and neurophysiological phenotype, differences in the causal genes, and potentially differential sensitivity to targeted therapies.

### Type 1 mosaicism

Genetic mosaicism may be classified as either type 1 or type 2 (Happle, 1997). Type 1 mosaicism is caused by heterozygous post-zygotic mutations. Our data and recent reports (Lee et al., 2012; Poduri et al., 2012) prove that HMEG,
and now DMEG and FCD IIa, can represent type 1 mosaicism. HMEG and FCD demonstrate most of the features previously associated with mosaicism. These include (i) nil or very low recurrence risk; (ii) asymmetric and often patchy pattern of abnormalities; (iii) variable extent of involvement; and (iv) lack of diffuse involvement (Happle, 1987). Many mosaic disorders including HMEG also have increased or decreased growth (Tinkle et al., 2005). To these features we add discordant occurrence in monozygotic twins, which has been reported for FCD (Briellmann et al., 2001).

The post-zygotic mutations leading to developmental disorders such as HMEG and FCD most likely occur early in embryogenesis, and the resulting phenotypes depend on the developmental timing and cell type affected as well as the gene and specific mutation. The best examples to date involve PIK3CA. Three activating mutations of PIK3CA have been identified as ‘hot spots’ in cancer, p.Glu542Lys, p.Glu543Lys, and p.His1047Arg (Samuels et al., 2004; Zhao et al., 2005). Germline expression of these mutations would likely result in embryonic lethality. Adding our data to other recent reports, post-zygotic PIK3CA hotspot mutations have been observed in syndromes affecting multiple tissues in large areas of the body (i.e. CLOVES syndrome), in single tissues or areas with limited extension to other tissues (i.e. HMEG with epidermal nevus), and in very localized tissues or areas (i.e. seborrheic keratoses, benign lichenoid keratoses, type 1 macroactively, HMEG and FCD IIa) (Hafner et al., 2007; Groesser et al., 2012; Kurek et al., 2012; Lee et al., 2012; Lindhurst et al., 2012; Rios et al., 2013; Keppler-Noreuil et al., 2014). It follows that mosaic mutations leading to CLOVES syndrome are likely to occur very early in embryogenesis, and mutations causing the other phenotypes somewhat later. Consistent with this prediction, several patients with both CLOVES syndrome and DMEG (e.g. Patient LR12-033) or HMEG have been reported (Gucev et al., 2008; Poretti et al., 2013). Less severe activating mutations of PIK3CA, also heterozygous and mosaic but found at higher levels in more tissues, result in megalencephaly-capillary malformation syndrome with or without cortical dysplasia (Riviè re et al., 2012). This demonstrates that the specific mutation is also important in determining the final phenotype.

Although fewer affected individuals have been reported, a similar spectrum has been seen with mutations of AKT3. Germline duplication of AKT3 has been associated with megalencephaly (Wang et al., 2013). Two children with mosaic duplication of 1q (including AKT3) and two with the severe activating mutation p.Glu17Lys were identified in previous studies of HMEG (Lee et al., 2012; Poduri et al., 2012). Mosaic duplication of AKT3 was also recently discovered in resected FCD type Ib tissue (Conti et al., 2014). We found the p.Glu17Lys AKT3 mutation in brain from a female child with left HMEG, right posterior FCD, left unilateral enlarged eye, and striking vascular malformations of the skin that resemble a severe port-wine stain with subcutaneous hypotrophy or a variant of cutis marmorata telangiectatica congenital. These extensive findings suggest earlier embryonic timing of her mutation compared to previously reported patients with mosaic p.Glu17Lys AKT3 mutation and isolated HMEG.

### Type 2 mosaicism

Our data and several previous reports also support the occurrence of type 2 mosaicism in HMEG and FCD. Type 2 mosaicism occurs when a germline heterozygous mutation is followed by a post-zygotic mutation of the other allele, resulting in a more severe condition in the affected segment (Happle, 1997; Itin and Buechner, 1999). We unexpectedly found a germline mutation of PTEN (p.Tyr68His) in a 9-year-old male with HMEG but no other abnormalities at the time of his last evaluation. This same mutation has been identified in individuals with PTEN hamartoma tumour syndrome (Marsh et al., 1998; Pilarski et al., 2011), and recombinant PTEN containing this mutation demonstrates defective phosphatase activity (Han et al., 2000). HMEG and FCD have not been described in large series of patients with PTEN mutations (Eng, 2003; Pilarski et al., 2011, 2013). However, rare reports have appeared of germline mutations of PTEN associated with HMEG (Merkx et al., 2003) or FCD (Elia et al., 2012; O’Rourke et al., 2012; Child and Cascino, 2013), including one remarkable example of a mosaic mutation of PTEN in dysplastic neurons in FCD IIb, but not in nearby normal neurons or blood-derived DNA (Schick et al., 2006). We hypothesize that all of these children have type 2 mosaicism, with a second hit in the other PTEN allele or possibly another gene affecting the PI3K/AKT pathway. Type 2 mosaicism for PTEN has been reported in other segmental overgrowth syndromes (Caux et al., 2007). Mice with heterozygous loss of PTEN in brain have no apparent phenotype, whereas those with homozygous PTEN loss exhibit neuronal hypertrophy and seizures (Backman et al., 2001; Kwon et al., 2001; Ljungberg et al., 2009). These findings, in concert with our identification of cellular hypertrophy and elevated protein S6 and T308 AKT phosphorylation in only a subset of neurons in cortex from our germline heterozygous PTEN mutation subject, support the hypothesis that a second mosaic mutation is necessary to cause cortical dysplasia.

### Summary and implications

In this study, we present diverse data from brain imaging, histopathology, immunohistochemistry and deep sequencing of a large cohort of children with dysplastic cortical malformations. Our results show that MEG, HMEG and FCD comprise a complete series of overlapping phenotypes associated with upregulation of the PI3K/AKT/mTOR pathway, as shown in Fig. 7. We accordingly propose that they be classified as a single malformation of cortical
development with variable expressivity rather than as separate disorders.

In the genetics literature, mosaic phenotypes localized to an anatomically recognizable portion of the body are designated as segmental mosaicism (Happle, 1997; Youssoufian and Pyeritz, 2002; Biesecker and Spinner, 2013). Respecting this usage, we have begun using the term PI3K/AKT-associated segmental cortical dysplasia to refer to the complete spectrum, with DMEG, HMEG and FCD used as anatomical subsets when clinically relevant. A thorough classification scheme of these segmental dysplasias would incorporate the distribution of the malformation as determined by brain imaging (bihemispheric symmetric, bihemispheric asymmetric, hemispheric, multilobar, or intralobar), the histopathology (FCD type Ia–c, Ia–b, or IIIa–d) (Blumcke et al., 2011), and the identified aetiology. As an example, for our Patient LR11-443, the nomenclature would be ‘bihemispheric asymmetric cortical dysplasia, histologic type IIa, due to mosaic AKT3 p.Glu17Lys mutation’. This classification could then evolve as new causative mechanisms for dysplastic brain malformations are identified.

Multiple selective inhibitors of PI3K and AKT are currently in clinical trials for treatment of cancer (Bellacosa et al., 2005; Engelmann, 2009; Willems et al., 2012). It is tempting to hypothesize that these agents may be useful in the management of epilepsy and associated conditions in the segmental cortical dysplasia spectrum, similar to what has been suggested for the use of mTOR inhibitors in tuberous sclerosis (Krueger et al., 2013; Wiegand et al., 2013; Cardamone et al., 2014). Testing of these compounds in mouse and in vitro models of abnormal neuronal PI3K/AKT pathway activation will be an important first step in evaluating the possible benefits of these compounds for treatment of children with dysplastic brain malformations.

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Supplementary material

Supplementary material is available at Brain online.

References


