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Mammalian Target of Rapamycin Pathway Mutations Cause Hemimegalencephaly and Focal Cortical Dysplasia

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Focal malformations of cortical development, including focal cortical dysplasia (FCD) and hemimegalencephaly (HME), are important causes of intractable childhood epilepsy. Using targeted and exome sequencing on DNA from resected brain samples and nonbrain samples from 53 patients with FCD or HME, we identified pathogenic germline and mosaic mutations in multiple PI3K/AKT pathway genes in 9 patients, and a likely pathogenic variant in 1 additional patient. Our data confirm the association of DEPDC5 with sporadic FCD but also implicate this gene for the first time in HME.

Our findings suggest that modulation of the mammalian target of rapamycin pathway may hold promise for malformation-associated epilepsy.
appear to represent different manifestations of aberrant mTOR signaling, with complex combinations of germline and mosaic mutations, suggesting that therapies targeting this pathway may prove useful across a range of MCDs.

Patients and Methods

Patient Cohort

The study was approved by the institutional review boards of Boston Children’s Hospital, Beth Israel Deaconess Medical Center, Boston, and University of California, Los Angeles. Informed consent was obtained when appropriate. Fifty-three patients were included; 14 had FCD and 39 had HME based on magnetic resonance imaging and neuropathology. Surgically resected brain samples and in some cases buccal or blood samples were available for 39 patients; only buccal or blood samples were available for the remaining 14 patients.

Next Generation Sequencing and Analysis for PI3K/AKT Pathway Variants

Genomic DNA was extracted from patient samples using standard methods. Whole exome sequencing (WES) was performed for 33 samples (10 FCD, 23 HME) and analyzed using standard methods. Molecular inversion probe sequencing (MIPS) was performed for 44 samples (6 FCD, 38 HME). Twenty-four samples were analyzed using both techniques. Rare variants (minor allele frequency ≤ 1%) in genes in the PI3K/AKT pathway were filtered using dbSNP 137 (http://www.ncbi.nlm.nih.gov/SNP/), the 1000 Genomes Project (http://browser.1000genomes.org/index.html), and the Exome Variant Server (http://evs.gs.washington.edu/EVS/). Previously reported mutations were identified using the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php), ClinVar (http://www.ncbi.nlm.nih.gov/clinvar/), and the Leiden Open Variation Database (http://chromium.liacs.nl/LOVD2/home.php) for TSC1/2. We used PROVEAN (http://provean.jcvi.org/index.php), SIFT (http://sift.jcvi.org/), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), and Mutation Taster (http://www.mutationtaster.org) to assess for pathogenicity. Variants were considered mosaic if (1) next generation sequencing showed an alternate allele frequency (AAF) < 50% and we validated the AAF using droplet digital polymerase chain reaction (ddPCR) or subcloning for cases where only 1 tissue was available or (2) the variant was present in brain tissue but not in

TABLE I. Pathogenic Mutations and Likely Pathogenic Variant Detected in PI3K/AKT Pathway in Patients with FCD and HME

<table>
<thead>
<tr>
<th>Case</th>
<th>Diagnosis</th>
<th>Gene</th>
<th>Mutation</th>
<th>HGVS</th>
<th>Type (alternate allele frequency)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCD-1</td>
<td>FCD IIb</td>
<td>DEPDC5</td>
<td>Fs</td>
<td>p.N261Kfs*11</td>
<td>Germline (55%)</td>
<td>Loss of function</td>
</tr>
<tr>
<td>FCD-2</td>
<td>FCD IIb</td>
<td>DEPDC5</td>
<td>Sp</td>
<td>c.624+1G&gt;A</td>
<td>Germline (50%)</td>
<td>Loss of function</td>
</tr>
<tr>
<td>HME-1</td>
<td>HME</td>
<td>DEPDC5</td>
<td>Fs</td>
<td>p.N45Qfs*3</td>
<td>Germline (61%)</td>
<td>Loss of function</td>
</tr>
<tr>
<td>HME-2</td>
<td>HME</td>
<td>MTOR</td>
<td>Ms</td>
<td>p.C1483Y</td>
<td>Mosaic (14%)</td>
<td>Previously identified in HME2</td>
</tr>
<tr>
<td>HME-3</td>
<td>HME</td>
<td>PIK3CA</td>
<td>Ms</td>
<td>p.E542K</td>
<td>Mosaic (28%)</td>
<td>Previously identified in CLOVES,15 rs121913273</td>
</tr>
<tr>
<td>HME-4</td>
<td>HME</td>
<td>PIK3CA</td>
<td>Ms</td>
<td>p.E545K</td>
<td>Mosaic (18%)</td>
<td>Previously identified in HME2 and MCAP,5 rs104886003</td>
</tr>
<tr>
<td>HME-5</td>
<td>HME</td>
<td>PIK3CA</td>
<td>Ms</td>
<td>p.E545K</td>
<td>Mosaic (17%)</td>
<td>Previously identified in HME2 and MCAP,5 rs104886003</td>
</tr>
<tr>
<td>HME-6</td>
<td>HME</td>
<td>PIK3CA</td>
<td>Ms</td>
<td>p.H1047R</td>
<td>Mosaic (13%)</td>
<td>Previously identified in CLOVES,15 rs121913279</td>
</tr>
<tr>
<td>HME-8</td>
<td>HME</td>
<td>MTOR</td>
<td>Ms</td>
<td>p.A1669S*</td>
<td>Mosaic (44% brain, 0% blood)</td>
<td></td>
</tr>
<tr>
<td>HME-11</td>
<td>HME</td>
<td>TSC2</td>
<td>Ms</td>
<td>p.R1713H</td>
<td>Germline (50%)</td>
<td>Previously identified in TSC,17 proven pathogenic,16 rs45517395</td>
</tr>
</tbody>
</table>

*Likely pathogenic mutation.

FCD = focal cortical dysplasia; Fs = frameshift; HGVS = Human Genome Variation Society; HME = hemimegalencephaly; MCAP = megalencephaly–capillary malformation syndrome; Ms = missense; Sp = splicing.
nonbrain tissue for some cases where multiple tissues were available.

Validation of PI3K/AKT Pathway Variants

Rare and protein-altering (nonsynonymous, nonsense, splice-site, frameshift, and insertion–deletion) variants in the target genes were validated using Sanger sequencing, and parental samples were tested when available. For potential mosaic variants, the original DNA was amplified using PCR, subcloned into a TOPO TA vector (Invitrogen), Carlsbad, CA, and transformed into TOP10 chemically competent Escherichia coli cells (Invitrogen); multiple clones were then isolated and sequenced.

ddPCR Screening for PIK3CA Mutations


The following cycles: 10 minutes at 95°C, 722 Volume 77, No. 4

20 rare and protein-altering variants in the PI3K/AKT pathway genes DEPDC5, MTOR, PIK3CA, PIK3CB, PIK3C2G, PIK3C3, and TSC2 in 16 patients (Supplementary Tables 1 and 3). Variants were considered pathogenic if they were loss-of-function mutations, predicted deleterious nonsynonymous mutations proven pathogenic by functional studies, or mutations previously identified in HME or related syndromes (Table 1). With the exception of 1 mosaic nonsynonymous mutation classified as likely pathogenic due to its somatic nature, the remaining variants were classified as variants of unknown significance (VUS; Supplementary Table 2), although further work is likely to identify some of these as causative.

In 2 patients with FCD type IIb, we identified germline loss-of-function mutations in DEPDC5: a frameshift (p.N261Kfs*11) in Patient FCD-1 with a right frontal FCD (Fig 1A) and a splice-site mutation (c.624+1G>A) in Patient FCD-2 with a left parietal FCD (see Fig 1B). We also identified a germline missense mutation (c.1355C>T, p.A452V) in FCD-3 previously reported as causative in patients with familial focal epilepsy with variable foci, which we conservatively classified as a VUS given recent preliminary functional studies suggesting the variant may not be pathogenic.

In 7 patients with HME, we identified loss-of-function or damaging missense mutations in DEPDC5, MTOR, PIK3CA, and TSC2. HME-1 has a germline frameshift in DEPDC5 (p.N45Qfs*3; see Fig 1C, D), and imaging shows right HME with abnormally thick gray matter, abnormal signal in the white matter, and an enlarged right ventricle. HME-7, who has generalized tonic–clonic seizures, speech delay, and mild right hemiparesis, has a germline inherited missense variant in DEPDC5 (c.1265G>A, p.R422Q), and imaging shows left HME with blurring of the gray–white matter junction and cortical irregularity most striking in the left parietal lobe (Supplementary Fig. 1). Two patients showed mosaic missense mutations in PIK3CA, E542K (c.1624G>A) in HME-3 and H1047R (c.3140A>G) in HME-6, both previously identified in CLOVES syndrome. HME-6 also harbors a germline missense variant in PIK3CA (c.1432G>T, p.D478Y). HME-8, who has complex partial seizures, harbors a mosaic missense variant in MTOR (c.5005G>T, p.A1669S) present at an AAF of 44% in the brain but not detectable in blood. HME-11 harbors a germline missense mutation in TSC2 (c.5138G>A, p.R1713H) previously shown to be pathogenic. In an additional 3 patients, we detected the same mosaic mutations in PIK3CA (c.1633G>A, p.E545K) and MTOR (c.4448G>A, p.C1483Y) that had been previously reported in other cases.

Discussion

Our data show that FCD and HME are allelic disorders, reflecting activating mutations in the PI3K/AKT pathway. DEPDC5 mutations have only recently been shown to be associated with FCD, originally reported in familial focal epilepsies with FCD in a few family members; our data confirm this association and extend it to sporadic FCD, also implicating DEPDC5 mutations for the first time in HME.

The mTOR pathway is critical for sensing nutrients and other metabolic cues and regulating protein synthesis and cell growth. Activating mutations in positive regulators of the pathway, including MTOR, PIK3CA, and PIK3R2, lead to excessive mTOR signaling. DEPDC5 encodes a member of the GATOR1 complex, and, along with TSC1 and TSC2, acts as a negative regulator of the mTOR complex 1 (mTORC1). We observed 1 pathogenic mutation and 2 additional potentially pathogenic variants in TSC2 in HME patients that we conservatively classified as VUS (see Supplementary Table 2). Thus, the loss-of-function and damaging
nonsynonymous mutations identified here are all predicted to result in hyperactivation of the mTOR pathway.

Both TSC1 and TSC2 provide critical regulation of mTORC1 through the GTPase-activating protein (GAP) activity of the TSC protein complex toward the RHEB GTPase. Loss of either of these protein complexes through loss of any of their critical protein components leads to high-level activation of mTORC1, and downstream effects on anabolic processes, including synthesis of all components needed for organelle synthesis, protein translation, and an increase in cell size. Hence, it is not surprising that mutation in any of TSC1, TSC2, or DEPDC5 could cause a neurologic syndrome in which giant cells are a primary feature.

Several of the variants identified here were germ-line, but the focal nature of both HME and FCD suggests the possibility of a somatic “second hit,” either in the other allele of the gene with a germline mutation or in another gene in the same pathway. Given the

![FIGURE 1: Magnetic resonance imaging (MRI) of focal cortical dysplasia (FCD) and hemimegalencephaly (HME) mutation-positive patients. (A) This axial inverted T2 image from the MRI of Patient FCD-1, with the germline DEPDC5 p.N261Kfs*11 frameshift mutation, shows a right frontal FCD II (arrows), seen as blurring of the gray–white matter junction and abnormal gray matter signal extending toward the ventricle. (B) This T2-weighted axial image from the MRI of Patient FCD-2, harboring the germline DEPDC5 c.624+1G>A splice-altering mutation, shows an FCD II characterized by blurring of the gray–white matter junction and abnormal deep gyral configuration in the left parietal region (arrows). The wide arrow points to a region of dysplastic cortex. (C, D) These T2-weighted axial (C) and coronal (D) images from Patient HME-1, with the germline DEPDC5 p.N45Qfs*3 frameshift mutation, illustrate right hemimegalencephaly, with abnormally thick gray matter, abnormal signal in the white matter, and an enlarged, dysmorphic right ventricle. Images are shown using MRI convention (L = left; R = right).](Image1)
previously identified patients with familial DEPDC5 mutations, most of whom lack cortical malformations, we strongly suspect a somatic second hit giving rise to the FCDs in a few family members. For example, in 1 case with an inherited DEPDC5 variant (p.R422Q), it is possible that the variant-carrying parent, who is phenotypically unaffected, represents nonpenetration and that the patient carries a second mutation. Similar to a second hit in TSC giving rise to cortical tubers in the presence of a germline TSCI or TSC2 mutation, a somatic mutation in a neural progenitor at a different developmental time point could give rise to either FCD or HME in combination with a germline mutation or on its own. However, identification of such somatic mutations will require very high-coverage next generation sequencing, ideally of affected brain tissue, given that the mutation may be present in only a small fraction of the cells. Both WES and MIPS analysis are also not sensitive to genic deletions, which would be a plausible cause of such second hits. Moving forward, it will be critical to perform such ultradeep sequencing, ideally using a targeted list of known and candidate genes, for FCD, HME, TSC, and related disorders.

Finally, the growing evidence that the shared pathology of FCD, HME, and TSC reflects shared genetic etiology suggests that modulators of the mTOR pathway, currently in clinical trials for patients with TSC, may also apply to the refractory epilepsy associated with FCD and HME, for which patients currently rely on surgical resection to alleviate seizures.

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**Authorship**
A.M.D., Y.G., C.A.W., and A.P. designed the study. C.A.W. and A.P. supervised the study. A.M.D., Y.G., J.A.C, B.M., E.A.B, C.M.L., A.H., and N.E.H. performed experiments and analyzed data. B.B. coordinated sample collection and phenotyping. D.J.K., H.V.V., and G.W.M. recruited patients and collected and prepared tissue samples. J.S. designed and supervised MIPS experiments. Y.G., A.J.B., G.W.M., C.A.W., and A.P. interpreted brain imaging data. A.M.D., C.A.W., and A.P. wrote the manuscript. All coauthors edited the manuscript. C.A.W. and A.P. are co-senior authors.

**Potential Conflicts of Interest**
dividends, 3M, GE, Becton Dickinson, Teva Pharma, SmithKline Beecham, Pfizer; royalties, Mosby; speaking fees, CME lectures (unrelated to current subject).

References


