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.

ANN NEUROL 2015;77:720–725

Focal malformations of cortical development (MCDs), including focal cortical dysplasia (FCD) and hemimegalencephaly (HME), are important causes of intractable childhood epilepsy.[1] FCD is characterized by focal regions of abnormal cortex, whereas HME is characterized by enlargement of an entire cerebral hemisphere. HME and some subtypes of FCD share pathological features with tuberous sclerosis (TSC), which is caused by mutations in *TSC1* or *TSC2* that abnormally activate mammalian target of rapamycin (mTOR), suggesting that hyperactivation of the mTOR pathway may be a common mechanism underlying these disorders.[1]

Recently, somatic activating point mutations in *AKT3, PIK3CA,* and *MTOR* have been identified in HME, and germline and somatic point mutations in *AKT3, PIK3R2,* and *PIK3CA* have been identified in the related megalencephaly–capillary malformation syndrome and megalencephaly–polymicrogyria–polydactyly–hydrocephalus syndrome.[2–5] In addition, somatic chromosome 1q tetrasomy has been reported in HME, and de novo germline 1q43q44 trisomy in megalencephaly.[4,6,7] A genetic etiology for FCD has long been hypothesized, and recently germline mutations in *DEPDC5* have been associated with familial focal epilepsy, with some affected individuals showing FCD on imaging; additionally, somatic 1q21.1-q44 copy number increase has been associated with FCD.[8,9]

Here, we studied resected brain tissue and/or blood or buccal DNA from 53 patients with FCD or HME and report pathogenic mutations and additional likely pathogenic variants in *DEPDC5* in FCD, and in *DEPDC5, PIK3CA, MTOR,* and *TSC2* in HME. Our results confirm the role of *DEPDC5* in FCD and implicate it for the first time in HME. FCD, HME, and TSC

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Additional Supporting Information may be found in the online version of this article.

Received Oct 24, 2014, and in revised form Dec 19, 2014. Accepted for publication Dec 24, 2014.

View this article online at wileyonlinelibrary.com. DOI: 10.1002/ana.24357

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

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

[a]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.

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.[10] Molecular inversion probe sequencing (MIPS)[11] was performed for 44 samples (6 FCD, 38 HME). Twenty-four samples were analyzed using both techniques. Rare variants (minor allele frequency $\leq 1\%$) in genes in the PI3K/AKT3 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

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

All samples were screened for 5 *PIK3CA* mutations (p.E545K, p.E542K, p.H1047R, p.H1047L, and p.C420R) using ddPCR to validate mutations identified using WES/MIPS.[12] A mix of ddPCR Super Mix (Bio-Rad Laboratories, Hercules, CA), mutant and reference probes (0.25μM each), forward and reverse primers (0.9 μM each), and 30ng of sample DNA was emulsified into 20,000 droplets using a QX200 Droplet Generator (Bio-Rad Laboratories). PCR was performed using the following cycles: 10 minutes at 95°C, 40 cycles of 30 seconds at 94°C, and 60 seconds at 60°C, 10 minutes at 98°C. Samples were analyzed using a QX100 Droplet Reader and QuantaSoft software (Bio-Rad Laboratories).

Results

To investigate the role of the PI3K/AKT pathway in focal malformations of cortical development, 14 patients with FCD and 39 patients with HME were studied using WES and/or MIPS. In total, we identified and validated 20 rare and protein-altering variants in the PI3K/AKT pathway genes *DEPDC5*, *MTOR*, *PIK3CA*, *PIK3C2B*, *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,[13] which we conservatively classified as a VUS given recent preliminary functional studies suggesting the variant may not be pathogenic.[14]

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.[15] 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.[16,17] 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.[2,5]

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[9]; 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[18] (Fig. 2). 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).[18,19] 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

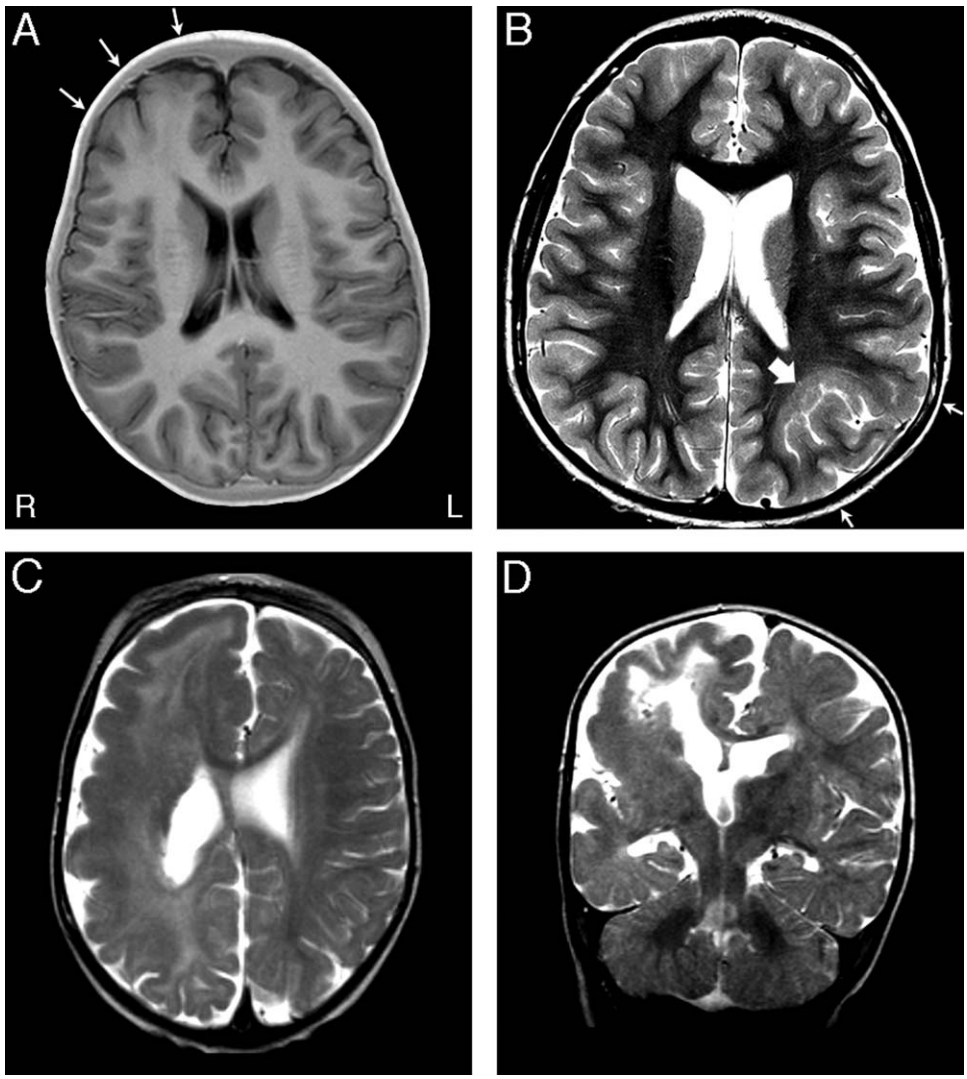

FIGURE 1: Magnetic resonance imaging (MRI) of focal cortical dysplasia (FCD) and hemimegalencephaly (HME) mutation-positive patients. (A) This axial inverted T2 image from the MRI of Patient FCD-1, with the germline *DEPDC5* p.N261Kfs*11 frameshift mutation, shows a right frontal FCD II *(arrows)*, seen 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).

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.[18] Similarly, the GATOR1 complex provides critical regulation of mTORC1 through its GAP activity on the Rag GTPases.[9] 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 germline, 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.[20] Given the

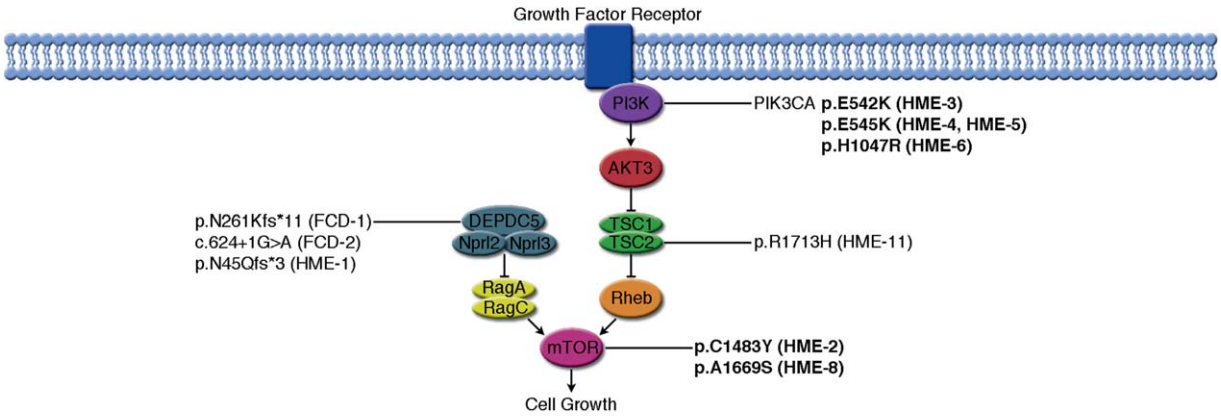

FIGURE 2: Schematic of the mammalian target of rapamycin (mTOR) pathway annotated with pathogenic mutations and likely pathogenic variants identified in this study; mosaic mutations are shown in boldface. FCD = focal cortical dysplasia; HME = hemimegalencephaly.

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 nonpenetrance 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 *TSC1* or *TSC2* mutation,[21] 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.[22] 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.[19]

Acknowledgment

A.M.D. was supported by the NIH National Institute of General Medical Sciences (T32GM007753) and NIH National Research Service Award (5T32 GM007226-39). D.J.K. was supported by the European Commission (602391-2). J.S. was supported by the National Cancer Institute (1R21CA160080). G.W.M. was supported by the NIH National Institute of Neurological Disorders and Stroke (R01NS083823 and R01NS038992). A.P. was supported by the NIH National Institute of Neurological Disorders and Stroke (K23 NS069784). C.A.W. was supported by the NIH National Institute of Mental Health (R01MH083565 and 1RC2MH089952), NIH National Institute of Neurological Disorders and Stroke (R01NS032457, R01NS079277, and R01NS035129), Simons Foundation, Paul G. Allen Family Foundation, and Manton Center for Orphan Disease Research. C.A.W. is an Investigator of the Howard Hughes Medical Institute.

We thank J. Goto for sample preparation; M. Warman for reagents and helpful discussion; and P. Black, J. Madsen, and E. Engle for patient recruitment.

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

E.A.B.: patent pending, molecular inversion probe design. D.J.K.: personal fees, Novartis. H.V.V.: stock,

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

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