Germline and somatic mutations in the *MTOR* gene in focal cortical dysplasia and epilepsy

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ABSTRACT

Objective: To assess the prevalence of somatic *MTOR* mutations in focal cortical dysplasia (FCD) and of germline *MTOR* mutations in a broad range of epilepsies.

Methods: We collected 20 blood-brain paired samples from patients with FCD and searched for somatic variants using deep-targeted gene panel sequencing. Germline mutations in *MTOR* were assessed in a French research cohort of 93 probands with focal epilepsies and in a diagnostic Danish cohort of 245 patients with a broad range of epilepsies. Data sharing among collaborators allowed us to ascertain additional germline variants in *MTOR*.

Results: We detected recurrent somatic variants (p.Ser2215Phe, p.Ser2215Tyr, and p.Leu1460Pro) in the *MTOR* gene in 37% of participants with FCD II and showed histologic evidence for activation of the mTORC1 signaling cascade in brain tissue. We further identified 5 novel de novo germline missense *MTOR* variants in 6 individuals with a variable phenotype from focal, and less frequently generalized, epilepsies without brain malformations, to macrocephaly, with or without moderate intellectual disability. In addition, an inherited variant was found in a mother–daughter pair with nonlesional autosomal dominant nocturnal frontal lobe epilepsy.

Conclusions: Our data illustrate the increasingly important role of somatic mutations of the *MTOR* gene in FCD and germline mutations in the pathogenesis of focal epilepsy syndromes with and without brain malformation or macrocephaly. *Neurol Genet* 2016;2:e118; doi: 10.1212/NXG.0000000000000118

GLOSSARY

ADHD = attention-deficit/hyperactivity disorder; **ADNFLE** = autosomal dominant nocturnal frontal lobe epilepsy; **CADD** = Combined Annotation Dependent Depletion; **DEPDC5** = Dishevelled, Egl-10, and Pleckstrin domain-containing protein 5; **DEPTOR** = domain-containing mTOR-interacting protein; **dPCR** = digital PCR; **ELM** = eukaryotic linear motif; **ExAC** = Exome Aggregation Consortium; **FAT** = FRAP, ATM, TRRAP; **FCD** = focal cortical dysplasia; **GATOR1** = GTPase-activating protein activity toward Rags complex 1; **HEAT** = huntingtin, elongation factor 3, protein phosphatase 2A, and TOR1; **H&E** = hematoxylin and eosin; **mTOR** = mammalian target of rapamycin; **NPRL** = nitrogen permease regulator–like.

A new class of genes encoding components of the mechanistic target of the rapamycin (mTOR) signal transduction pathway has recently been involved in familial focal epilepsies.[1] Dishevelled, Egl-10, and Pleckstrin domain-containing protein 5 (DEPDC5) together with nitrogen permease regulator–like 2 (NPRL2) and nitrogen permease regulator–like 3 (NPRL3) form the GTPase-activating

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protein activity toward Rags complex 1 (GATOR1), an amino acid–sensitive inhibitor of mTORC1.[2] Germline mutations in the GATOR1-encoding genes (*DEPDC5*, *NPRL2*, and *NPRL3*) have been reported in numerous familial and sporadic focal epilepsies.[3–9] In addition to nonlesional focal epilepsies, GATOR1 mutations have also been recognized in epilepsies associated with developmental cortical malformations such as focal cortical dysplasia (FCD), in particular FCD type IIb (with balloon cells) and IIa (without balloon cells).[10–14]

Recent studies reported brain somatic missense mutations in the *MTOR* gene itself in 3 patients with hemimegalencephaly,[12] in 12/77 (15%) with FCD II,[15] in 6/13 (46%) with FCD IIb,[16] in 1 with FCD IIa,[17] and in 10 with various developmental disorders from FCD IIa to megalencephaly.[18] Evidence for mTORC1 hyperactivity was shown in resected brain tissue after epilepsy surgery.[15,16,18]

Rare germline heterozygous missense mutations in *MTOR* have also been reported in a subset of patients with megalencephaly, intellectual disability, and seizures.[18–22]

In this study, we compared the prevalence and type of *MTOR* somatic mutations in a cohort of FCD patients, with *MTOR* germline mutations in cohorts of patients with epilepsy with or without brain malformations.

METHODS **Patients.** As a first step, 3 different cohorts were screened for pathogenic variants in *MTOR* (figure 1).

First, for mosaic mutation detection, we prospectively recruited a cohort of 20 participants with FCD who underwent surgery to treat drug-resistant focal epilepsy. The cohort consisted of 18 children operated at the Fondation Rothschild (Paris, France) between 2015 and 2016 and 2 adult participants

Figure 1   **Workflow of the study**
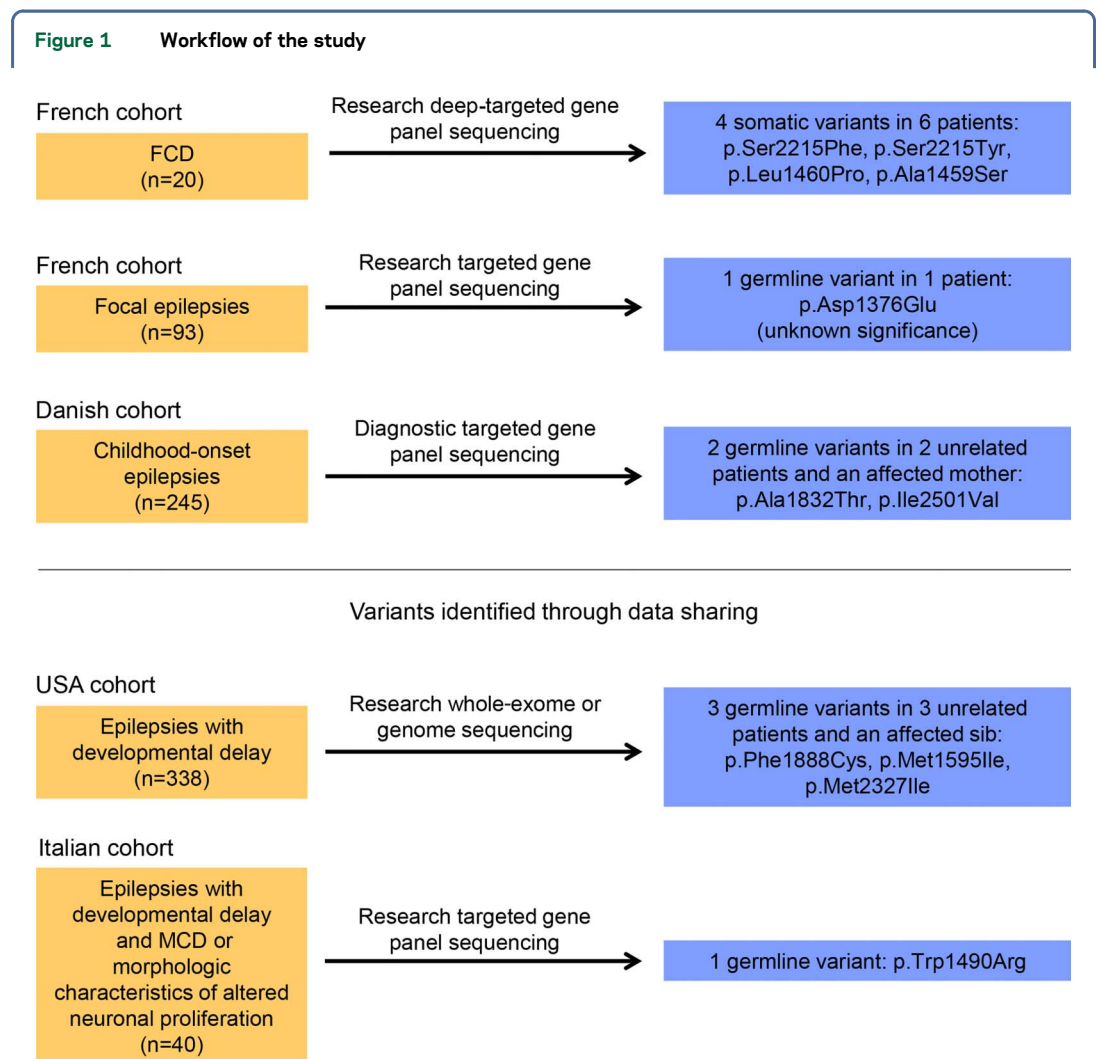
FCD = focal cortical dysplasia; MCD = malformation of cortical development.

operated at the Pitié-Salpêtrière hospital (Paris, France) and at the Swiss Epilepsy Center (Zurich, Switzerland).

Second, for germline mutation detection, we included a previously described French research cohort of 93 unrelated European patients with focal epilepsy, including 55 probands with familial focal epilepsy, 9 sporadic patients with nonlesional focal epilepsy, 14 patients with familial focal epilepsy, and at least one family member with FCD, and 15 sporadic patients with focal epilepsy and FCD.[8] In this cohort, 7 individuals were previously shown to have a mutation in one of the GATOR1-encoding genes.[8] We also screened a third Danish cohort of 245 unrelated patients with various forms of childhood-onset epilepsies referred for diagnostic gene panel testing. The phenotypic spectrum within this cohort was broad and ranged from benign familial neonatal/infantile epilepsy to generalized epilepsies, focal epilepsies, and severe epileptic encephalopathies.

In addition, we collected 5 previously unreported patients with *MTOR* mutations through data sharing with Italy and the United States. The Italian cohort consisted of 40 patients with epilepsy, intellectual disability, and malformations of cortical development or morphologic characteristics suggesting altered neuronal proliferation. The US cohort consisted of 338 probands (from 317 families) with developmental delay, intellectual disability, and/or other neurologic symptoms, including epilepsy.

All probands and affected family members underwent detailed clinical examination, including review of the medical files, MRIs, and EEG investigations.

Standard protocol approvals, registrations, and patient consents. The study was approved by the following local ethical committees: CPP Ile de France II, No ID-RCB/EUDRACT 2015-A00671-48, the Pediatric Ethical Committee of the Tuscany Region, and the Institutional Review Board at the University of Alabama at Birmingham (protocol #X130201001). Written informed consent was obtained from each participant or parents of minors. Parents of participants 10a, 10b, and 12 gave consent for publication of their children's picture. The Danish cohort was recruited in a diagnostic setting, and the patients or parents of minors gave written consent for publications of their findings.

Genetic testing. *Targeted gene panel for detection of somatic variants.* Genomic DNA was extracted from peripheral blood lymphocytes and from fresh frozen resected brain tissue of FCD participants. To search for mosaic mutations, we designed a custom gene panel of 25 genes belonging to the PI3K/AKT/mTOR pathway (SeqCapEZ custom design, Roche NimbleGen) including *MTOR* and the GATOR1-encoding genes (*DEPDC5*, *NPRL2*, and *NPRL3*).

Deep sequencing was performed on a NextSeq (2 × 75 nt). GenoSplice performed quality control, processing, and detection of the variants. FASTQ sequence files were mapped using hg19 reference with bwa-0.7.12 (mem) and converted to bam with samtools-1-1. Single nucleotide variant and insertion/deletion calling was performed with GATK-2014.3 to call heterozygous germline variants present either in the resected brain or lymphocyte-derived DNA.

Somatic variant calling was performed with samtools-1-1 (mpileup) to detect low frequency variants (i.e., <10%). The pileup was parsed with pileup2base which provides the count of reads for each strand and nucleotides by coordinates. A home-made Perl script was developed to filter variants according to the following criteria: 1/variants not retrieved by GATK; 2/≥ 10 reads with the alternate allele; 3/≥ 1% of allelic frequency; and 4/maximum strand bias value of 0.5.

Picoliter droplet-based digital PCR. Somatic p.Ser2215Phe and p.Ser2215Tyr mutations were confirmed by digital PCR (dPCR). Genomic DNA was extracted from a second piece of the same tissue block from participants 1–5 and was tested for the presence of specific *MTOR* mutations. No brain tissue of patient 6 was left. Custom LNA double-dye probes designed to detect both mutant alleles c.6644C>T (p.Ser2215Phe) and c.6644C>A (p.Ser2215Tyr) and wild-type were purchased from Eurogentec. RainDrop Digital PCR system (Raindance Technologies Billerica) was used as previously described[23] with the following modifications: dPCR was performed with Taqman Universal Master Mix (life Technologies), 125 nM final concentration of both probes and 250 nM final concentration of primers.

Targeted gene panels for detection of germline variants. In the French cohort of 93 patients with focal epilepsies, the search for heterozygous variants in genomic blood DNA was accomplished by a previously reported methodology and research epilepsy panel consisting of 76 genes (SeqCapEZ custom design, Roche NimbleGen).[8]

In the Danish cohort, the genomic blood DNA from 245 patients with various childhood-onset epilepsies was screened using a diagnostic sequencing panel (Sure Select, Agilent Technologies) consisting of 85 epilepsy genes.

The Italian cohort of 40 patients was screened using a gene panel consisting of 15 genes belonging to the PI3K/AKT/mTOR pathway (HaloPlex Target Enrichment System, Agilent Technologies).

MTOR variants were assumed to be pathogenic if they were nonsynonymous missense, seen less than 2 times in control samples (Exome Aggregation Consortium (ExAC) set of ~61,000 exomes—exac.broadinstitute.org [accessed August 2016]), had arisen de novo in patients without family history of epilepsy, or segregated with the disease in the family. Sanger sequencing was used to confirm all variants.

Exome/genome sequencing for detection of germline variants. The 338 USA probands were subject to trio-based exome or genome sequencing as part of a Clinical Sequencing Exploratory Research project (conducted at the HudsonAlpha Institute for Biotechnology).[24] Whole-exome (patients 10a, 10b, and 11) or genome (patient 12) sequencing was conducted to a median depth of 65X or 30X, respectively. Exome capture was completed using NimbleGen SeqCapEZ Exome version 3. Reads were aligned to reference hg19 using bwa (0.6.2). GATK best practice methods were used to identify variants. Maternity and paternity of each parent were confirmed by whole-exome/genome kinship coefficient estimation with KING. De novo variants were identified as heterozygous variant calls in the proband in which there were at least 10 reads in both parents and the proband, an alternate allele depth of at least 20% in the proband and less than 5% in each parent, and a minor allele frequency <1% in the 1000 Genomes Project and the ExAC. All candidate de novo variants that either affected a protein or had a scaled Combined Annotation Dependent Depletion (CADD) score >10 were manually reviewed.[25] In each patient described, no other variants were identified as potentially disease causing. All de novo variants were confirmed by Sanger sequencing to be heterozygous in the probands and absent from both of their parents.

Histopathology and immunostaining. Histopathologic diagnosis was made according to the classification of the International League Against Epilepsy Diagnostic Methods Commission[26] and included 3 FCD I, 9 FCD IIa, 7 FCD IIb, and 1 FCD III. Paraffin-embedded resected brain tissues of patients 1–5 were examined for the presence of cytoarchitectural features of FCD using hematoxylin and eosin (H&E) staining. Brain tissue of patient 6

was not available for further immunostaining. mTORC1 activity was assessed by visualizing the phosphorylation level of its downstream effector, the ribosomal protein S6, a commonly used biomarker in mTORC1 pathway research.[27] Sections (10 μm) were probed overnight at 4°C with an antibody against Ser235/236 phosphorylated S6 (#4856, 1:200 dilution; Cell Signaling, Danvers, MA) and visualized using avidin-biotin conjugation (Vectastain ABC Elite; Vector Laboratories, Burlingame, CA). Images were acquired using the Leica DM2500 microscope.

Protein modeling. Protein modeling was performed using YASARA structure (yasara.org), merging 29 models of multiple PDB files. Molecular dynamic simulations were performed using AMBER031, and structure statistics were calculated with the YASARA2 z score calculation. Eukaryotic linear motif (ELM) predictions were performed on the ELM server 3, and posttranslational modifications were predicted using multiple prediction tools.[28–36]

RESULTS Brain somatic mutations in *MTOR*. To identify low-level mosaic mutations, we designed a targeted sequencing panel comprising all 57 coding exons and flanking intronic regions of *MTOR* (NM_004958.3) and the GATOR1-complex encoding genes (*DEPDC5*, *NPRL2*, and *NPRL3*). We achieved a high sequencing coverage of all 4 genes (mean coverage: 2,653×, 2,527×, 2,666×, and 2,342×, respectively). We searched for mosaic variants with allele frequencies ≥1%. We detected neither germline nor mosaic coding pathogenic variants in *DEPDC5*, *NPRL2*, or *NPRL3* according to our filtering criteria. Brain mosaic mutations in *MTOR* were found in 2 patients with FCD IIa and 4 patients with FCD IIb (table 1, figure 1), representing 37% (6/16) of all FCD II individuals. A recurrent variant in *MTOR* (c.6644C>T/p.Ser2215Phe) was identified in patients 1–3 in 138/2,186 (6.31%), 79/2,504 (3.15%), and 28/2,481 (1.13%) reads, respectively. A second recurrent variant (c.6644C>A/p.Ser2215Tyr) affecting the same nucleotide was detected in patients 4–5 in 26/718 (3.62%) and 12/751 (1.6%) reads. Patient 6 with FCD IIb carried 2 *MTOR* variants located on the same allele: the recurrent variant c.4379T>C/p.Leu1460Pro was detected in 62/2,522 (2.46%) reads and c.4375G>T/p.Ala1459Ser (never reported) was detected in 60/2,493 (2.41%) alleles. To validate these findings, we performed picoliter droplet-based dPCR based on the single-molecule parallel amplification of *MTOR* wild-type and mutated alleles. dPCR confirmed both the presence and the allelic frequencies of the 2 c.6644C>T/p.Ser2215Phe and c.6644C>A/p.Ser2215Tyr variants in DNA sample replicates from the same tissue block to exclude sequencing artifacts (table 1).

Table 1	Clinical features of patients with somatic *MTOR* variants in FCD					
Participant (origin)	Patient 1 (French)	Patient 2 (French)	Patient 3 (French)	Patient 4 (French)	Patient 5 (French)	Patient 6 (French)
Sex/age, y	F/15	M/15	M/2	M/7	M/1	M/28
Variants: c.DNA/protein	c.6644C>T/p.Ser2215Phe	c.6644C>T/p.Ser2215Phe	c.6644C>T/p.Ser2215Phe	c.6644C>A/p.Ser2215Tyr	c.6644C>A/p.Ser2215Tyr	c.4379T>C/p.Leu1460Pro c.4375G>T/p.Ala1459Ser
Allele frequency in brain tissue: panel/digital PCR, %	6.31/6.8	3.15/2.62	1.13/0.93	3.62/3.67	1.6/1.06	2.46/not done; 2.41/not done
Allele frequency in blood, %	3.16	1.45	0.63	0.13	0.82	1.30
Age at onset	18 mo	16 mo	2 mo	2 mo	2 mo	6 y
Seizure types	Left head deviation then atonic and tonic seizures	Eye deviation, then head sensation, and right hand dystonia	Clonic sz, infantile spasms	Asymmetric infantile spasms, then clonic eye movements	Asymmetric infantile spasms, clonic eye movements	Drop attacks, nocturnal sz with psychic aura, loss of consciousness, gestural automatisms
EEG	Left fronto-parietal focus and sz	Left basal frontal focus and sz	Left anterior temporal focus and sz	Multifocal right frontal and parietal	Posterior right multiple foci	Right middle and posterior temporal discharges
MRI findings (age at investigation)	Left frontal FCD (3 y)	Left frontal FCD (18 mo)	Left anterior temporal FCD (23 mo)	Right parieto-occipital FCD (12 mo)	Right temporo-occipital FCD (9 mo)	Right posterior temporal FCD (23 y)
Histology	FCD IIb	FCD IIb	FCD IIa, with few balloon-like cells	FCD IIb	FCD IIa	FCD IIb
Cognition	Mild ID	Mild ID	Mild ID	Normal	Mild ID	Memory impairment
Neurologic examination	Normal	Attention deficit	Attention deficit and motor hyperactivity	Normal	Left strabismus	Normal
Postoperative outcome	Engel 1a at 8 mo	Engel 1 during 7 mo after surgery, then Engel 3	Engel 2 at 6 mo	Engel 1 during 4 mo after surgery, then Engel 3	Engel 1 at 10 mo	Engel 1b at 2 y

Abbreviations: FCD = focal cortical dysplasia; ID = intellectual disability; sz = seizures.

All recurrent mosaic *MTOR* variants were predicted deleterious by SIFT, disease-causing by Mutation Taster, and possibly damaging by PolyPhen-2, and were absent from the ExAC database. Allele frequencies of the mosaic variants were low in the genomic DNA extracted from the brain tissues (range 1.1%–6.3%) (table 1). We noticed that with the high sensitivity of the deep sequencing, mosaic variants could also be detected in the blood DNA, although with allele frequencies half lower than in the brain DNA (range 0.1%–3%). However, these variants were not present in the blood DNA of patients without detected *MTOR* brain somatic mutations. The recurrent variants p.Ser2215Phe, p.Ser2215Tyr, and p. Leu1460Pro were considered to be pathogenic as they have already been linked to FCD.[15,16,18] These mosaic variants were detected across several studies using distinct technologies and sequencing platforms, excluding that they might correspond to sequencing artifacts.

Germline mutations in *MTOR*. In the French cohort of 93 patients with focal epilepsy, we identified only one germline variant (c.4128T>G/p.Asp1376Glu) in a patient with nonlesional focal epilepsy with auditory features. This novel variant was predicted deleterious by all in silico prediction tools and was absent from the ExAC database. Sanger sequencing, however, revealed that the variant was inherited from the asymptomatic father and was thus considered to be a variant of unknown significance.

In the Danish cohort consisting of 245 patients, we detected 2 germline heterozygous variants in *MTOR* (c.5494G>A/p.Ala1832Thr and c.7501A>G/p. Ile2501Val) corresponding to ~1% of a diagnostic cohort of patients with childhood-onset epilepsy (figure 1, table 2). Variant c.5494G>A/p.Ala1832Thr occurred de novo in patient 7 with nonlesional occipital lobe epilepsy, macrocephaly, learning difficulties, and attention-deficit/hyperactivity disorder (ADHD). Based on the de novo occurrence, highly evolutionary conservation of the Ala1832 amino acid, and low frequency in the ExAC database (1/110,816 chromosomes), we considered the p.Ala1832Thr variant to be pathogenic. The c.7501A>G/p.Ile2501Val variant, identified in patient 8a, also affects a highly evolutionary conserved amino acid, was predicted to be disease causing by Mutation Taster (but was predicted to be benign by SIFT and PolyPhen-2) and was absent in the ~61,000 individuals from the ExAC database. The p.Ile2501Val variant was inherited from an affected mother (patient 8b). Both proband and mother had nonlesional nocturnal frontal lobe epilepsy, a phenotype compatible with autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE). Patients 7 and 8a did not carry additional pathogenic variants in one of the 84 epilepsy genes of the panel,

including genes known to be involved in ADNFLE such as *CHRNA4*, *CHRNA2*, *CHRNB2*, *DEPDC5*, or *KCNT1*, suggesting that this variant is likely to be pathogenic.

Further germline *MTOR* mutations were identified through data sharing with collaborators in the United States and Italy. We collected 4 additional de novo germline *MTOR* mutations as follows: c.4468T>C/p. p.Trp1490Arg (patient 9, Italian cohort); c.5663A>C/p.Phe1888Cys (identical twin pair, patients 10a and 10b, USA cohort); c.4785C>T/p.Met1595Ile (patient 11, USA cohort); and c.6981G>A/p.Met2327Ile (patient 12, USA cohort) (figure 1, table 2). All six variants were predicted to be disease-causing by different in silico prediction methods and were absent from the ExAC database. Sanger confirmation and segregation analysis revealed that all variants occurred de novo, suggesting that they are likely to be pathogenic.

Prediction of pathogenicity. We assessed the deleteriousness of the *MTOR* variants using different prediction tools. First, we calculated the CADD scores for all novel germline variants: 15.18 (p.Ile2501Val), 19.76 (p.Ala1832Thr), 26 (p.Met1595Ile), 29.2 (p.Phe1888Cys), and 34 (p.Met2327Ile), as well as the somatic variants: 26.1 (p.Leu1460Pro), 31 (p.Ala1459Ser), 32 (p.Ser2215Tyr), and 33 (p.Ser2215Phe). CADD scores were clearly consistent with them being pathogenic.

Second, we observed in ExAC that the number of missense variants expected in *MTOR* is 930, while those observed are only 438 (z score = 7.89), indicating that *MTOR* is intolerant to missense variants. For each variant identified in each cohort, we performed a χ^2 test with Yates correction (graphpad. com/quickcalcs/contingency1.cfm) using the ~61,000 ExAC individuals as a control cohort. All *MTOR* variants, either novel germline or recurrent somatic, reached extremely statistically significant values ($p < 0.0001$). In addition, with a 0.34% Residual Variation Intolerance Score (RVIS), *MTOR* ranks among the top most constrained human genes.[37]

Furthermore, we asked how the variants may affect the structure and/or function of the mTOR protein. Structural domains are composed of 2 HEAT (huntingtin, elongation factor 3, protein phosphatase 2A, and TOR1) domains, the FAT (FRAP, ATM, TRRAP) domain, the FKBP12-rapamycin binding domain, the kinase domain, and the FAT C-terminal domain. Most mutations reported here, either somatic or germline, were clustered in and around the FAT and the kinase domains (figure 2). The crystal structure of mTOR has revealed that the FAT domain interacts with the kinase domain[38] and DEP domain-containing mTOR-interacting protein

Table 2	Clinical features of patients with germline *MTOR* variants							
Participant (origin)	Patient 7 (Danish)	Patient 8a (Danish)	Patient 8b (Danish)	Patient 9 (Italian)	Patient 10a (USA)	Patient 10b (USA)	Patient 11 (USA)	Patient 12 (USA)
Sex/age, y	F/8	F/44	F/70	M/6	F/23	F/23	M/8	F/2.5
Variant: c.DNA/protein	c.5494G>A/ p.Ala1832Thr	c.7501A>G/ p.Ile2501Val	c.7501A>G/ p.Ile2501Val	c.4468T>C/ p.Trp1490Arg	c.5663A>C/ p.Phe1888Cys	c.5663A>C/ p.Phe1888Cys	c.4785C>T/ p.Met1595Ile	c.6981G>A/ p.Met2327Ile
Inheritance	Germline de novo	Germline maternal	Transmitted to daughter	Germline de novo	Germline de novo	Germline de novo	Germline de novo	Germline de novo
Family history of seizures	FS (father and sister)	Focal epilepsy (mother)	Focal epilepsy (daughter)	None	Identical twin sister same phenotype	Identical twin sister same phenotype	None	None
Age at seizure onset	Diagnosis at 2 y 3 mo. Retrospectively, onset at 8 mo	2 y	Diagnosis at 28 y. Retrospectively, onset in childhood (age 10 y)	2 y 5 mo	4 y	6 y	12 mo	No seizures
Seizure description	Irresponsive, cyanosis, generalized stiffening; visual aura, sensation of vomiting, irresponsive, elevation of the eyes	Nocturnal focal seizures; rising sensory aura, generalized stiffening with preserved consciousness; precipitated by fever	TCS, two episodes of convulsive SE, nocturnal focal seizures: rising sensory aura, generalized stiffening with preserved consciousness; precipitated by fever (also during wakefulness)	Focal motor seizures with rhythmic jerks of left arm, leg, and face, often during sleep	Irresponsive preceded or followed by detached laughing; convulsive status epilepticus	Giddy laughing with hyperactivity; TCS; convulsive status epilepticus (age 8)	Myoclonic; generalized TCS	No seizures
EEG	Right occipito-parietal spikes and slow activity	Theta activity intermixed with normal background activity	Polyspike wave with left-side predominance and slow wave activity with left temporal predominance	Slow and sharp waves during sleep, both diffuse and focal, mainly involving right hemisphere	Persistent diffuse sharp and slow wave activity, most prominent over right hemisphere	Mild slow background, diffuse and left centro-temporal epileptiform discharges	Irregular slowing of background rhythms and frequent bursts of generalized 1–2 Hz SWC	Normal
Epilepsy syndrome	Focal epilepsy, occipital lobe	Focal epilepsy, nocturnal frontal lobe	Focal epilepsy, nocturnal frontal lobe	Focal epilepsy, probably fronto-temporal lobe	Focal epilepsy, probably frontal or temporal lobe	Focal epilepsy, probably frontal or temporal lobe	Generalized epilepsy	No epilepsy
MRI findings	Aspecific bifrontal white matter signal changes at 3 y (1.5T)	Incomplete inversion of left hippocampus; pineal gland cyst; arachnoidal cyst at 42 y (1.5T)	Normal (1.5T)	Thin corpus callosum, ventricular dilatation at 2 y 8 mo and 5 y (3T)	Ventricular prominence with mild extra ventricular enlargement at 6 mo (1.5T)	Mild ventricular prominence at 6 mo (1.5T)	Normal at 18 mo	Mild ventricular dilatation and increased extra-axial spaces at 20 mo (1.5T)
Cognition before sz onset	Not available	Normal	Normal	Mild delay	Delayed	Delayed	Delayed	Delayed, moderate ID, behavioral outbursts
Cognition and behavior after sz onset	Learning difficulties, ADHD	Anxiety	Normal	Mild ID (IQ 64)	Moderate ID, aerophagia, autism	Moderate ID, autism	Moderate ID, autism	Not applicable
Neurologic examination	Normal	Normal	Normal	Normal	Hypotonia, nonverbal, shuffling gait	Hypotonia, nonverbal, shuffling gait	Ataxic gait, hypotonia	Normal
Treatment response	Drug resistant	LEV effective (seizures only with fever)	Seizure-free since age 36 y, on CBZ	CBZ effective; last seizure at 4 y 7 mo	No response to VPA; good response to TPM	Partial response to LEV, good response to TPM	TPM and VPA side effects, currently LEV, ZNS, and CLB	Not applicable
Dysmorphic features, including HC	Macrocephaly: HC 56 cm at 8 y (+3.31 SD); no dysmorphic features	HC 54.2 cm at 44 y (−0.11 SD); no dysmorphic features	HC 56 cm at last follow-up (+1.52 SD); no dysmorphic features	Macrocephaly: HC 54 cm at 3 y (+2.8 SD); no dysmorphic features	Macrocephaly: HC 61.3 cm at 12 y (+5.8 SD). Facial dysmorphy: prominent forehead, low-set ears, gingival hyperplasia, frontal bossing, kyphosis, micrognathia, long thin extremities	Macrocephaly: HC 60 cm at 12 y (+4.9 SD). Facial dysmorphy: prominent forehead, low-set ears, gingival hyperplasia, frontal bossing, kyphosis, micrognathia; long thin extremities	HC 49 cm at 18 mo (+0.95 SD); dysmorphology not done	Macrocephaly: HC 52.3 cm at 20 mo (+4.1 SD). Facial dysmorphy: frontal bossing, broad depressed nasal root, coarse facial features

Abbreviations: ADHD = attention-deficit/hyperactivity disorder; CBZ = carbamazepine; CLB = clobazam; FS = febrile seizures; HC = head circumference; ID = intellectual disability; LEV = levetiracetam; SWC = spike-and-wave complexes; TCS = tonic-clonic seizures; TPM = topiramate; VPA = valproic acid; ZNS = zonisamide.

Figure 2 Domain/structural organization of mTOR protein with the positions of the mutations indicated
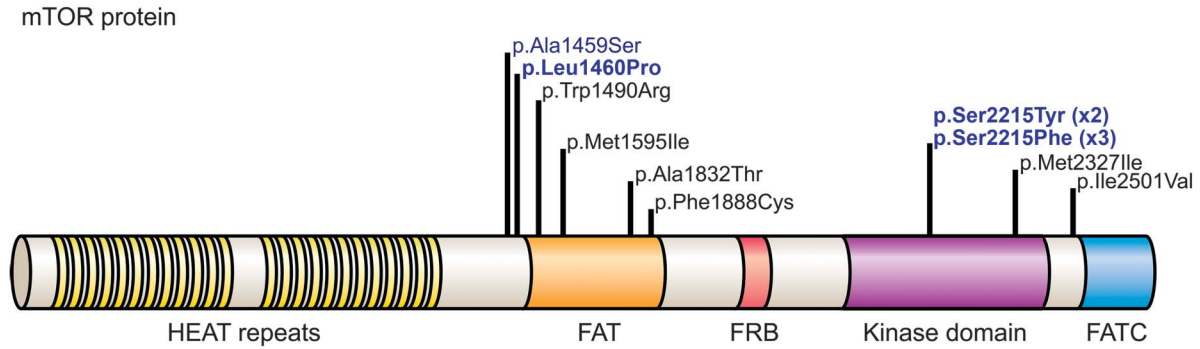
In black: germline mutations; in blue: somatic mutations; in bold: recurrent mutations. Structural domains are composed of HEAT (huntingtin, elongation factor 3, protein phosphatase 2A, and TOR1) repeats; FAT (FRAP, ATM, TRRAP) domain; FRB (FKBP12-rapamycin binding) domain; kinase domain; and FATC (FAT C-terminal) domain.

(DEPTOR),[39] a repressor of mTOR activity. We can speculate that mutations in the FAT domain may alter the conformational structure of protein or inhibit the binding with DEPTOR, maintaining the catalytic site exposed and active.

We further performed protein modeling of mTOR and showed that most of the variants seen have a high probability of altering protein structure and dynamics through either altered hydrophobic collapse (p.Met1595Ile, p.Met2327Ile, and p.Ile2501Val), helical packing (p.Ala1459Ser, p.Leu1460Pro, and p.Ser2215Phe/Tyr), or surface conserved sites likely involved in protein-protein interactions (p.Trp1490Arg) (table e-1, figure e-1 at Neurology.org/ng). While amino acid 1888 is not as strongly conserved, Phe1888Cys is predicted to elevate the probability for protein lipidation (myristoylation, palmitoylation, and farnesylation), a modification known to alter protein localization, and thus is also likely to deleteriously impact mTOR function. Altogether, these missense variants in *MTOR* are likely to be pathogenic.

Phenotypic analysis. Brain mosaic mutations in *MTOR* were found in 2 patients with FCD IIa and in 4 with FCD IIb (table 1), representing 37% (6/16) of all FCD II individuals. No *MTOR* variants were found in the 3 patients with FCD I nor in the only patient with FCD III. All patients had characteristic imaging features of FCD (figure 3) and drug-resistant focal epilepsy prior to surgery. Postoperative outcome ranged from Engel class 1 to 3. Histopathology of resected brain tissue showed an FCD IIa with dysmorphic neurons on H&E staining in patients 3 and 5, and an FCD IIb with dysmorphic neurons and balloon cells in patients 1, 2, and 4 (figure 3). We assessed mTORC1 activity by visualizing the phosphorylation level of its downstream effector S6 on Ser235/236 (pS6), a well-recognized marker of mTORC1 activity.[27]

Hyperactivation of the mTORC1 pathway as shown by S6 intense phosphorylation was observed in dysmorphic neurons of patients with FCD IIa and FCD IIb, in contrast to apparently normal adjacent neurons.

Germline mutations were found in patients with a variable phenotypic spectrum (table 2). Seven of the 8 individuals with germline variants had epilepsy. Six had focal epilepsy, and 1 had generalized epilepsy, with age at onset ranging from 8 months to 6 years. The presumptive lobe of seizure onset was variable from frontal, fronto-temporal to occipital. One mother–daughter pair (patients 8a and 8b) had ADNFLE, and 2 patients formed an identical twin pair (patients 10a and 10b) with gelastic seizures. Four patients did not have a family history of epilepsy. Five patients had macrocephaly, and 3 had dysmorphic facial features (figure 4). All patients with germline mutations had either normal imaging or unspecific MRI abnormalities including thin corpus callosum and/or ventricular dilatation (figure 4). Three patients had a normal or near normal development, 1 patient had mild and 4 had moderate intellectual disability. Behavioral and psychiatric disturbances including autistic features and ADHD were reported in 4 patients. No specific dermatological features were observed.

DISCUSSION Brain somatic *MTOR* mutations have recently been identified in patients with malformations of cortical development, in particular FCD (for review reference 40). In this study, we report recurrent somatic mutations in *MTOR* in 30% of patients with FCD and 37% of patients considering only patients with FCD II (2 FCD IIa and 4 FCD IIb). We also describe 8 patients with a spectrum of developmental disorders due to germline *MTOR* mutations. While most patients had focal (and less frequently generalized) epilepsy, mild-to-moderate intellectual

Figure 3 Imaging, histopathology, and mTORC1 pathway activity of patients with focal cortical dysplasia
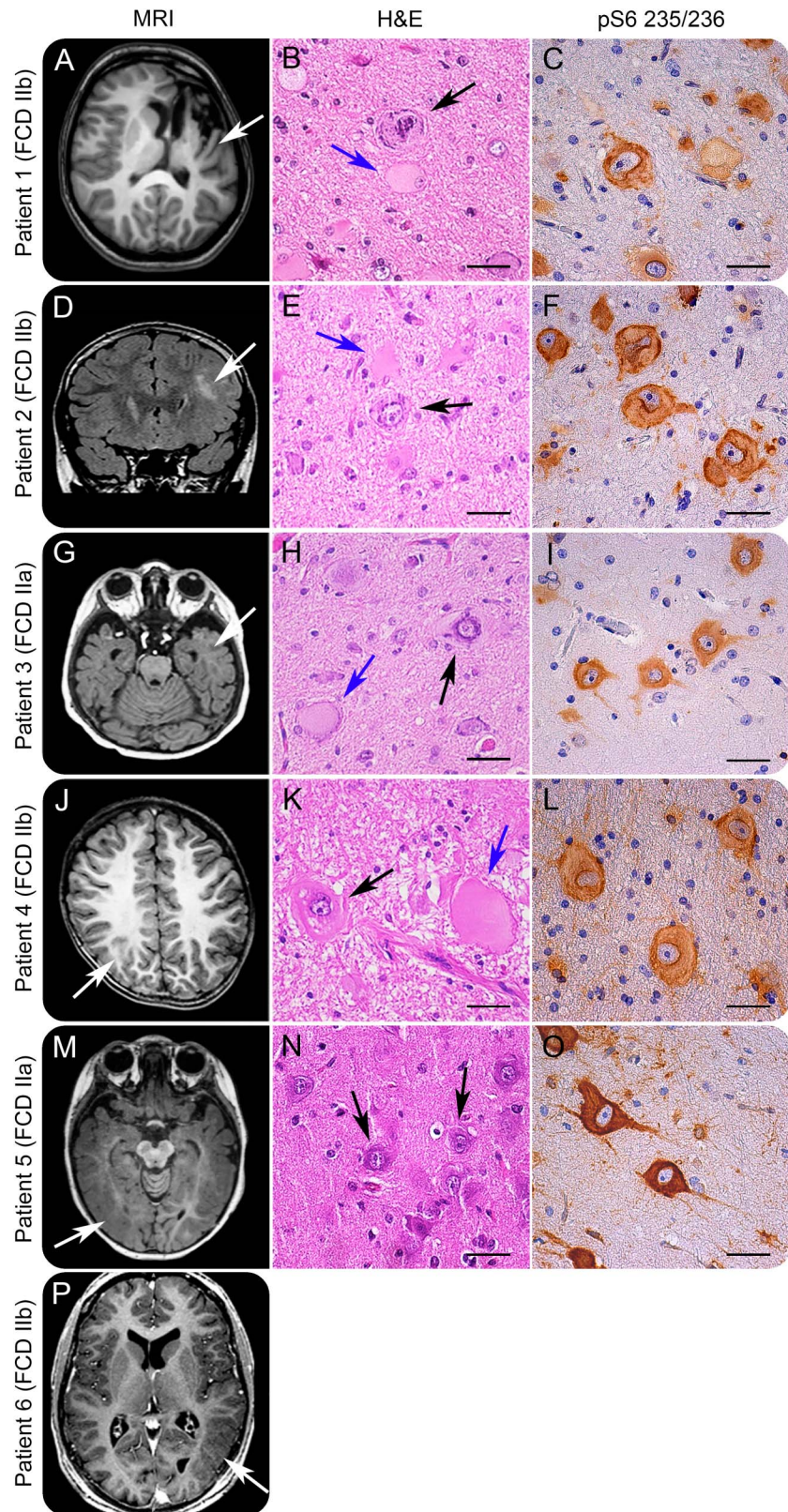
(A) MRI with residual lesion after the first and before the second operation because of incomplete initial resection (preoperative MRI unavailable). (D, G, J, M, P) Preoperative brain MRI; arrows indicate the dysplastic lesion. (B, E, H, K, N) Hematoxylin and eosin (H&E) staining; blue arrows indicate balloon cells, and black arrows indicate dysmorphic neurons. (C, F, I, L, O) Ser235/236 phosphorylated S6 (pS6 235/6) immunostaining counterstained with hematoxylin, showing labeling of dysmorphic neurons. Brain tissue of patient 6 was not available for further immunostaining. Scale bar: 20 μm. FCD = focal cortical dysplasia.

Figure 4 Unspecific MRI findings and facial dysmorphy in patients with germline variants
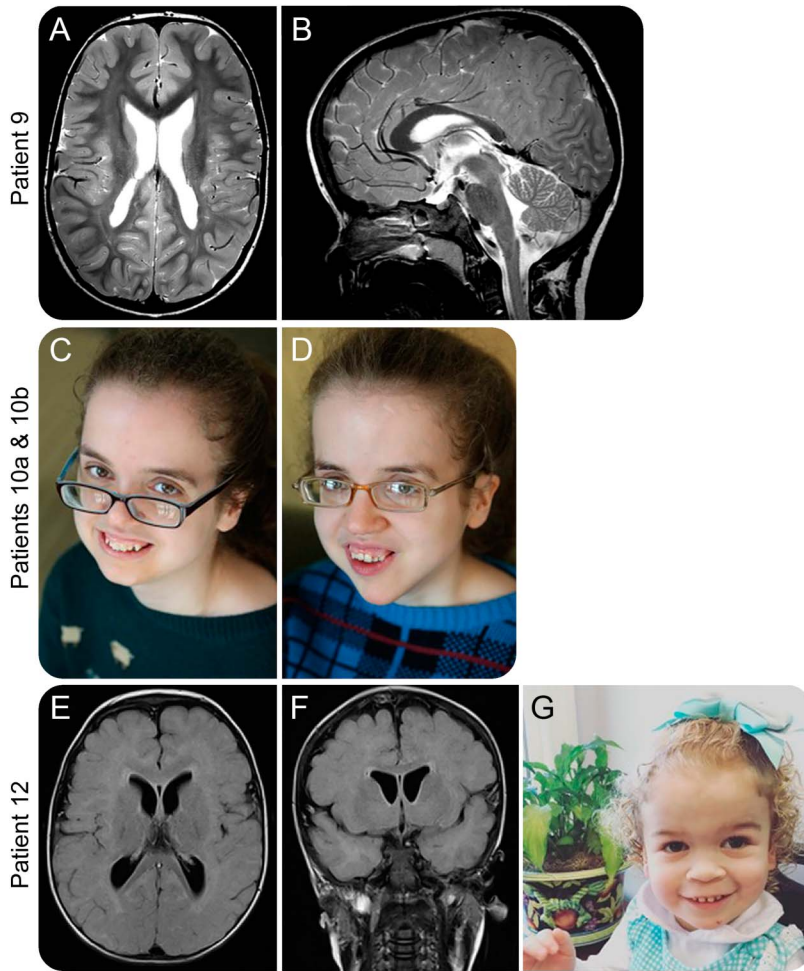
(A and B) T2-weighted 3T MRI images of patient 9. Axial image shows dilated lateral ventricles (A), and sagittal image shows thinning of the posterior half of the corpus callosum (B). (C and D) Pictures of identical twin pair 10a and 10b show prominent forehead, low-set ears, gingival hyperplasia, frontal bossing, and micrognathia (MRIs not available for publication). (E–G) Fluid-attenuated inversion recovery (FLAIR) weighted 1.5T MRIs of the brain, and the picture of patient 12. Axial and coronal images shows mild ventricular dilatation. Picture shows macrocephaly with frontal bossing, broad depressed nasal root, and coarse facial features.

disability, and/or dysmorphic features, we extended the phenotypic spectrum associated with germline variants to milder phenotypes than previously reported; none of the patients had a malformation of cortical development identified on MRI, and 3 patients had (near) normal cognitive outcome. At the mildest end of the spectrum, 1 mother–daughter pair had nonlesional treatment-responsive ADNFLE and normal intellectual development. In addition, we report 1 individual with macrocephaly and intellectual disability without seizures. Our results are consistent with previous studies reporting individuals with megalencephaly and seizures due to germline activating mutations in *MTOR*.[18–22]

In a previous report, the level of mosaicism of somatic mutations has been suggested to correlate with the extent of the brain malformation.[18] In our series of patients with FCD, we could not directly correlate the allele frequencies of somatic variants to the size of the lesion. Given the central and ubiquitous role of the mTORC1 transduction pathway in regulating cell functions, it was surprising to discover heterozygous germline *MTOR* mutations in the following: (1) patients with purely neurologic symptoms and (2) patients without brain malformations, in contrast to individuals with somatic mutations. Two possible hypotheses can be raised. First, it appears that the amino acids affected by germline mutations are different from the ones affected by somatic mutations. All variants were missense, assumed to enhance the activity of the mTORC1 pathway. However, while somatic mutations may be hyperactivating, and thus cause brain malformations of development, germline mutations could be less activating. A second hypothesis is the possible occurrence during brain development of multiple-hit mosaic mutations (missed with our targeted sequencing strategy) in different genes of the same pathway, explaining the apparition of a focal lesion as seen in FCD. This scenario is known as the multiple-hit or Knudson hypothesis in cancer[41] and is supported by our previous report of a patient with FCD with both a somatic and a germline *DEPDC5* mutation.[11] It is also supported by the finding that brain somatic mutations occur frequently.[42] In this FCD cohort, we did not identify an *MTOR* or a GATOR1 germline mutation in addition to the somatic *MTOR* variants.

While the somatic *MTOR* mutations were recurrent, all germline mutations identified in this study were novel. Recurrence of p.Ser2215Phe, p.Ser2215Tyr, and p.Leu1460Pro mutations that result in mTOR activation in multiple FCD cases[15,16,18] suggests mutational hotspots. Of interest, the same recurrent mutations were also found in various tumors (cancer.sanger.ac.uk/cancergenome/projects/cosmic).[43] Most of these oncogenic mutations, like FCD-causing mutations, cluster near the kinase domain and the FAT domain of mTOR protein. A recent study reported that the hyperactive kinase domain p.Met2327Ile variant confers drug resistance to mTOR second-generation inhibitors suggesting potential issues in the field of precision medicine.[44]

The role of genes of the GATOR1-mTORC1 pathway (*MTOR*, *DEPDC5*, *NPRL2*, and *NPRL3*) is being increasingly recognized in focal epilepsies, with and without structural abnormalities.[1] Most mutations in GATOR1-encoding genes are loss of function (nonsense, frameshift, splice variants), while all mutations in the *MTOR* gene are missense mutations predicted to be activating. Both GATOR1[8,13,14] and *MTOR* mutations[17] have been shown to lead to hyperactivation of the mTORC1 pathway in resected

epileptogenic brain tissue. How an increased activity of the intracellular signaling cascade alters network function and ultimately leads to seizures is one of the next challenging questions to be elucidated.

This study confirms the important role of somatic *MTOR* mutations in the pathogenesis of FCD, and jointly with previous reports shows that germline *MTOR* mutations may contribute to a broad phenotypic spectrum, ranging from focal epilepsy without MRI-detectable brain abnormalities and normal development, to hemimegalencephaly, intellectual disability, and epilepsy. The treatment effectiveness in these patients with mTORC1 pathway inhibitors needs further evaluation, but meanwhile, screening of *MTOR* in a broad range of focal epilepsies is warranted.

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