Toward a better definition of focal cortical dysplasia: An iterative histopathological and genetic agreement trial

Ingmar Blümcke1,2 | Roland Coras1 | Robyn M. Busch2,3,4 | Marcia Morita-Sherman2 | Dennis Lal2,4 | Richard Prayson5 | Fernando Cendes6,7 | Iscia Lopes-Cendes7,8 | Fabio Rogerio7,9 | Vanessa S. Almeida7,8 | Cristiane S. Rocha7,8 | Nam Suk Sim10 | Jeong Ho Lee10,11 | Se Hoon Kim12 | Stephanie Baulac13 | Sara Baldassari13 | Homa Adle-Biassette14,15 | Christopher A. Walsh16,17 | Sara Bizzotto16,17 | Ryan N. Doan16,17 | Katherine S. Morillo16,17 | Eleonora Aronica18,19 | Angelika Mühlebner18,20 | Albert Becker21 | Jesus Cienfuegos22,23 | Rita Garbelli24 | Caterina Giannini25,26 | Mrinalini Honavar27 | Thomas S. Jacques28,29 | Maria Thom30 | Figen Söylemezoglu37 | Imad Najm2,3

1Department of Neuropathology, University Hospital, Erlangen, Germany | 2Epilepsy Center, Cleveland Clinic, Cleveland, OH, USA | 3Department of Neurology, Cleveland Clinic, Cleveland, OH, USA | 4Genomic Medicine Institute, Cleveland Clinic, Cleveland, OH, USA | 5Department of Anatomic Pathology, Cleveland Clinic, Cleveland, OH, USA | 6Department of Neurology, University of Campinas, Sao Paulo, Brazil | 7Brazilian Institute of Neuroscience and Neurotechnology, Sao Paulo, Brazil | 8Department of Medical Genetics and Genomic Medicine, University of Campinas, Sao Paulo, Brazil | 9Department of Pathology, University of Campinas, Sao Paulo, Brazil | 10Graduate School of Medical Science and Engineering, Korea Advanced Institute of Science and Technology, Daejeon, South Korea | 11SoVarGen, Inc., Daejeon, Korea | 12Department of Pathology, College of Medicine, Yonsei University, Seoul, South Korea | 13Sorbonne Université, Institut du Cerveau - Paris Brain Institute - ICM, Inserm, CNRS, Paris, France | 14Pathological Anatomy Service, Public Hospital Network of Paris, Paris, France | 15NeuroDiderot, Inserm U1141, University of Paris, Paris, France | 16Division of Genetics and Genomics, Manton Center for Orphan Disease Research, Department of Pediatrics, and Howard Hughes Medical Institute, Boston Children’s Hospital, Boston, MA, USA | 17Departments of Pediatrics and Neurology, Harvard Medical School, Boston, MA, USA

Ingmar Blümcke, Fernando Cendes, Jeong Ho Lee, Stephanie Baulac, Christopher A. Walsh, Eleonora Aronica, Rita Garbelli, Maria Thom, Hajime Miyata, Harvey B. Sarnat, and Imad Najm are members of the International League Against Epilepsy Task Force on Focal Cortical Dysplasia.

Dennis Lal, Iscia Lopes-Cendes, Vanessa S. Almeida, Cristiane S. Rocha, Nam Suk Sim, Jeong Ho Lee, Stephanie Baulac, Sara Baldassari, Christopher A. Walsh, Sara Bizzotto, Ryan N. Doan, and Katherine S. Morillo are members of the Focal Cortical Dysplasia Gene Panel Analysis Group.

Ingmar Blümcke, Roland Coras, Richard Prayson, Fabio Rogerio, Se Hoon Kim, Homa Adle-Biassette, Eleonora Aronica, Angelika Mühlebner, Albert Becker, Jesus Cienfuegos, Rita Garbelli, Caterina Giannini, Mrinalini Honavar, Thomas S. Jacques, Maria Thom, Anita Mahadevan, Hajime Miyata, Pitt Niehusmann, Harvey B. Sarnat, and Figen Söylemezoglu are members of the Histopathology Review Group.

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Correspondence
Ingmar Blümcke, Department of Neuropathology, University Hospitals Erlangen, Schwabachanlage 6, D – 91054 Germany.
Email: bluemcke@uk-erlangen.de

Abstract

Objective: Focal cortical dysplasia (FCD) is a major cause of difficult-to-treat epilepsy in children and young adults, and the diagnosis is currently based on microscopic review of surgical brain tissue using the International League Against Epilepsy classification scheme of 2011. We developed an iterative histopathological agreement trial with genetic testing to identify areas of diagnostic challenges in this widely used classification scheme.

Methods: Four web-based digital pathology trials were completed by 20 neuropathologists from 15 countries using a consecutive series of 196 surgical tissue blocks obtained from 22 epilepsy patients at a single center. Five independent genetic laboratories performed screening or validation sequencing of FCD-relevant genes in paired brain and blood samples from the same 22 epilepsy patients.

Results: Histopathology agreement based solely on hematoxylin and eosin stainings was low in Round 1, and gradually increased by adding a panel of immunostainings in Round 2 and the Delphi consensus method in Round 3. Interobserver agreement was good in Round 4 (kappa = .65), when the results of genetic tests were disclosed, namely, MTOR, AKT3, and SLC35A2 brain somatic mutations in five cases and germline mutations in DEPDC5 and NPRL3 in two cases.

Significance: The diagnoses of FCD 1 and 3 subtypes remained most challenging and were often difficult to differentiate from a normal homotypic or heterotypic cortical architecture. Immunohistochemistry was helpful, however, to confirm the diagnosis of FCD or no lesion. We observed a genotype-phenotype association for brain somatic mutations in SLC35A2 in two cases with mild malformation of cortical
1 | INTRODUCTION

Etiology matters in the clinical management of patients with difficult-to-treat focal epilepsies. Epilepsy surgery has been established as a reliable treatment option in many of these patients, and the spectrum of structural brain lesions amenable for surgical treatment is well described. Malformations of cortical development (MCDs) account for the most common surgical pathologies in children and the third most common in adults, with focal cortical dysplasia (FCD) representing almost 75% of all MCD cases. Postsurgical seizure outcomes vary considerably between various FCD subtypes. In a recent European multicenter study of 9147 epilepsy surgery patients, FCD Type 1 and mild MCD (mMCD) showed the poorest outcome, whereas Type 2 FCDs were associated with much higher rates of long-term seizure freedom on and off antiseizure medications up to 5 years after surgery. FCD was first described in 1971, but its clinicopathological classification scheme remains a topic of ongoing discussion. FCD originally contained only cytoarchitectural dysplasia including cytomegalic neurons and balloon cells in an architecturally abnormal neocortex, for example, FCD 2A or 2B in the current terminology. It was then extended to add a new category, frequently encountered in patients with epilepsy, that involves architectural abnormalities of the neocortex but lacking cytopathologic features, termed FCD Type 1 by the Palmini classification in 2004. Microscopic hallmarks of a reliable histopathological diagnosis of FCD Type 1 remained suboptimal and were revised in the international FCD consensus classification scheme of 2011.

This widely used International League Against Epilepsy (ILAE) classification further introduced FCD Type 3, wherein architectural abnormalities were associated with and adjacent to another principal lesion, for example, hippocampal sclerosis (HS; FCD Type 3A), low-grade developmental brain tumors (FCD Type 3B), vascular malformations (FCD Type 3C), or any other lesion acquired during early life (FCD Type 3D). The foundation for this classification was laid by observations that patients with isolated (pure) FCD Type 1 have a more severe clinical phenotype and may require more extensive resection or hemispheric disconnection procedures. In contrast, clinical phenotypes and postsurgical seizure outcome were not significantly different between patients with HS or other principal brain lesions with or without accompanying FCD. Microscopic intra- and interobserver agreement was tested for the 2011 ILAE classification addressing different levels of professional experience. Overall kappa values reached good levels, but the study also highlighted the need for continuous training and that FCD Type 1 and 3 subtypes required further attention.

Challenges in interobserver agreement with respect to histopathological diagnosis are long-standing and occur not only in the arena of epilepsy surgery. In 2016, the World Health Organization Classification of Tumors of the Central Nervous System proposed a multilayered diagnostic scheme to address this issue by integrating genetic testing with immunohistochemical stainings for a better histopathological workup and definition of FCD subtypes. We also propose adding the level of genetic findings to obtain a comprehensive, reliable, and integrative genotype-phenotype diagnosis in the near future.

KEY POINTS

- We performed an iterative histopathological agreement trial with genetic testing to challenge the widely used ILAE classification scheme for FCD
- Twenty neuropathologists from 15 countries completed four digital pathology trials of 196 surgical tissue blocks obtained from 22 epilepsy patients
- Five independent laboratories performed genetic screening or validation sequencing of FCD-relevant genes in paired brain and blood samples from the same 22 patients
- Interobserver agreement increased when immunohistochemistry was available and results of genetic tests were disclosed
- We propose adding the level of genetic findings to the ILAE classification scheme to obtain an integrative genotype-phenotype diagnosis in the near future

Keywords: brain, classification, epilepsy, genes, neuropathology, seizure
The integrated genotype–phenotype classification of brain tumors significantly moved the field forward with more targeted therapies becoming available, despite the lack of resources for genetic testing in many regions of the world. A similar progress in FCD genetics emerged, revealing that somatic and/or germline mutations activating the mTOR–GATOR signaling pathway cause FCD Type 2.\textsuperscript{17–19} Also, somatic mutations in \textit{SCL35A2} leading to aberrant N-glycosylation were reported in FCD or mMCD,\textsuperscript{20,21} which has recently been confirmed and assigned histopathologically to mild malformation of cortical development with oligodendrogial hyperplasia in epilepsy (MOGHE)\textsuperscript{22} in a collaborative series of 26 cases.\textsuperscript{23}

To identify the challenges in clinical practice and improve the validity of FCD diagnoses we selected a single center (Cleveland Clinic, Cleveland, Ohio, USA) to access brain samples surgically resected from a group of highly characterized patients with medically intractable focal epilepsies. These samples were histologically and immunohistochemically reviewed by a group of neuropathologists with experience in the diagnosis of epilepsy-related specimens through a web-based digital pathology platform. DNA extracted from resected brain tissue and peripheral blood from the same patients were genetically studied at five centers to assess the impact of a genotype–phenotype integration for the classification of FCD.\textsuperscript{24,25}

**MATERIALS AND METHODS**

Twenty-two consecutive cases were selected from the Cleveland Clinic Epilepsy Center Biorepository according to

| Case | Sex | Age at seizure onset, years | Age at surgery, years | Localization\textsuperscript{a} | MRI | RF | iEEG | Outcome, Engel\textsuperscript{b} | FU, years |
|------|-----|----------------------------|----------------------|-----------------|-----|----|-----|----------------|--------|
| 1    | F   | 13                         | 28                   | L Fr            | pos | No | SDG, icEEG | IA     | 1.25       |
| 2    | F   | 4                          | 14                   | R T C           | pos | DD | icEEG    | IC     | 4.17       |
| 3    | M   | 8                          | 50                   | R Fr T          | pos | FAM| SDG, icEEG | IA     | 4          |
| 4    | F   | 13                         | 25                   | L Fr            | pos | TBI| SDG, icEEG | IA     | 1.17       |
| 5    | M   | 9                          | 19                   | R P             | pos | FAM| icEEG    | II     | 2.5        |
| 6    | F   | 17                         | 27                   | L T             | neg | TBI| icEEG    | IV     | 2.25       |
| 7    | M   | 5                          | 16                   | L Fr            | pos | FAM| icEEG + ECoG | II     | 1.58       |
| 8    | M   | 49                         | 53                   | L T             | pos | Npl| No       | IA     | 1.33       |
| 9    | M   | 36                         | 49                   | L T             | pos | No | No       | III    | 1          |
| 10   | F   | 22                         | 44                   | L T             | pos | No | No       | IA     | 1.08       |
| 11   | F   | 4                          | 26                   | R T C           | pos | TBI| No       | IA     | 1.08       |
| 12   | M   | .8                         | 8                    | L Fr            | pos | DD | icEEG + ECoG | n.a.  | .17        |
| 13   | F   | 0                          | 37                   | R P             | pos | BIRTH| icEEG   | III    | 4.75       |
| 14   | F   | 2                          | 39                   | L T             | pos | DD | icEEG    | IA     | .25        |
| 15   | F   | 23                         | 34                   | R T C           | neg | FS | No       | IA     | .5         |
| 16   | F   | 6                          | 28                   | L Fr            | pos | No | icEEG    | IA     | .58        |
| 17   | F   | 20                         | 28                   | R Fr T          | pos | TBI| icEEG    | IA     | .75        |
| 18   | F   | 5                          | 24                   | R P O           | pos | No | SDG + ECoG | II     | 1          |
| 19   | F   | 25                         | 42                   | R T C           | pos | FS | No       | IA     | 1.08       |
| 20   | F   | 5                          | 17                   | R Fr T          | pos | BIRTH| icEEG + ECoG | IB    | .58        |
| 21   | M   | 2.5                        | 19                   | L Fr            | pos | No | ECoG    | IA     | .42        |
| 22   | F   | .3                         | 19                   | R T P O         | pos | DD | SEEG    | IV\textsuperscript{c} | n.a.\textsuperscript{d} |

**Table 1** The 22 patients included in the study

*Note:* Overview of 22 consecutive patients, who met the inclusion criteria.

Abbreviations: BIRTH, perinatal stroke; C, central; DD, developmental delay; ECoG, electrocorticography; EEG, electroencephalography; F, female; FAM, family history of seizures; Fr, frontal; FS, febrile seizure; FU, postsurgical follow-up period; icEEG, intracerebral EEG; iEEG, invasive EEG; L, left; M, male; MRI, magnetic resonance imaging; n.a., not available; neg, negative; Npl, neoplasia; O, occipital; P, parietal; pos, positive; R, right; RF, risk factors; SDG, subdural grids; SEEG, stereo-EEG; T, temporal; TBI, traumatic brain injury.

\textsuperscript{a}Brain localization of surgery.

\textsuperscript{b}Postsurgical outcome using Engel scale.

\textsuperscript{c}Engel Class IV outcome with elimination of drop attacks after previous surgery in outside hospital.

\textsuperscript{d}No follow-up data available after last surgery at the Cleveland Clinic Epilepsy Center.
the following inclusion criteria: (1) presurgical evaluation and patient management conference protocols suggested a disease etiology within the spectrum of FCD; (2) formalin-fixed and paraffin-embedded (FFPE) surgical specimens were available for microscopic re-evaluation and immunohistochemistry, if requested (see below); (3) fresh frozen surgical brain samples and a matched blood sample would be available for DNA extraction and genetic testing; (4) the selection period included years 2018 and earlier to allow for postsurgical follow-up; and (5) all patients had provided informed consent for the scientific use of their biomaterials (Table 1). The use of biorepository tissues and the study protocol were approved by the Cleveland Clinic Institutional Review Board (IRB#18-1134), the institutional review boards of Boston Children’s Hospital, the Committee for the Protection of Persons Ile de France II (No. ID-RCB/ EUDRACT-2015-A00671-48) registered on ClinicalTrials.gov (No. NCT02890641), and the Korea Advanced Institute of Science and Technology Institutional Review Board and Committee on Human Research.

### Table 2 Requested immunostains

| Case | NeuN | GFAP | MAP2 | NFL | SYN | VIM | αBC | CDs | O2 | Other |
|------|------|------|------|-----|-----|-----|-----|-----|----|-------|
| 1    | x    | x    | x    | x   | x   | x   | x   | CV-LFB |
| 2    | x    | x    | x    | x   | x   | x   | CV-LFB |
| 3    | x    | x    | x    | x   | x   | x   | CV-LFB |
| 4    | x    | x    | x    | x   | x   | x   | CV-LFB |
| 5    | x    | x    | x    | x   | x   | x   | CV-LFB |
| 6a   | x    | x    | x    | x   | x   | CV-LFB |
| 7a   | x    | x    | x    | x   | x   | x   | Ki67 |
| 8    | x    | x    | x    | x   | x   | x   | 68  | Ki67 |
| 9a   | x    | x    | x    | x   | x   | CV-LFB |
| 10a  | x    | x    | x    | x   | x   | CV-LFB |
| 11a  | x    | x    | x    | x   | x   | CV-LFB |
| 12   | x    | x    | x    | x   | x   | x   | x   | CV-LFB |
| 13   | x    | x    | x    | x   | x   | CV-LFB |
| 14   | x    | x    | x    | x   | x   | CV-LFB |
| 15a  | x    | x    | x    | x   | x   | 3   | CV-LFB |
| 16   | x    | x    | x    | x   | x   | CV-LFB |
| 17a  | x    | x    | x    | x   | x   | CV-LFB |
| 18   | x    | x    | x    | x   | x   | CV-LFB |
| 19a  | x    | x    | x    | x   | x   | CV-LFB |
| 20   | x    | x    | x    | x   | x   | 68  | PB  |
| 21   | x    | x    | x    | x   | x   | 68  | Ki67 |
| 22   | x    | x    | x    | x   | x   | 68  | PB  |

Note: Case identification numbers are the same as used in Table 1. Specific stainings were prepared as indicated (x).

Abbreviations: CDs, T-cell marker CD3 (MRQ-39; Cell Marque); CV-LFB, cresyl violet–Luxol fast blue (Sigma); GFAP, glial fibrillary acidic protein (Dako Cytomation); IDH1, R132H point mutation-specific antibody (clone H09; Dianova); Ki67, proliferation marker (clone MIB-1; Cell Marque); MAP2, microtubule-associated protein 2 (clone HM2; Dako); NeuN, neuronal nuclei (clone A60; Millipore); NFL, nonphosphorylated neurofilament protein (clone SMI32; BioLegend); O2, oligodendroglial marker protein Olig2 (JP18953; IBL International); PB, Prussian blue (Merck); SYN, synaptophysin (SP11; Thermo Fisher Scientific); VIM, vimentin (SP20; Thermo Fisher Scientific); αBC, heat shock protein αB-crystallin (ADI-SPA-223; Enzo).

*In these eight cases, more than one formalin-fixed and paraffin-embedded block was requested for special stainings.

### 2.1 First and second agreement trials (hematoxylin and eosin vs. immunohistochemistry)

Thirty-five neuropathologists from 18 countries were invited to review the series of 22 cases. A total of 196 hematoxylin and eosin (H&E)-stained FFPE tissue blocks and their H&E-stained sections were retrieved from the pathology archives of the Cleveland Clinic. Whole slide images (WSIs) were digitally scanned using Aperio ScanScope (Leica). All WSIs were uploaded into the open microscopy environment platform for secured web-based access (OMERO.web, University of Dundee). Each case included a short description of the patient’s age at surgery, age at seizure onset, whether a lesion was visible by magnetic resonance imaging (MRI), and lobe localization for brain surgery. All reviewers were asked in an open text format to (1) identify the FFPE tissue block with best visible abnormalities; (2) indicate which, if any, additional...
immunostainings they would need; and (3) submit a provisional diagnosis. Twenty-four neuropathologists submitted their reports within the given time limit of 6 weeks. None of the reviewers had access to the results of other reviewers. The OMERO.web administrator, who is a senior pathologist neither experienced in epilepsy surgery nor enrolled in this survey, retrieved and forwarded all answers pseudonymously to the study coordinator. Requested immunostainings (see Table 2) were prepared from blocks with most votes using a semiautomated immunostainer (Ventana Ultra), digitally scanned and uploaded onto the OMERO.web platform. After a 3-month interval, the same group of 24 participants was invited to rereview the case series including requested immunostainings. Twenty reviewers submitted their final diagnosis within a 6-week time period. Interobserver agreement was calculated by kappa coefficient analysis.26 Kappa values are always equal to 1 or less. In our study, kappa values were interpreted as follows: <.2, slight; .2–<.4, fair; .4–<.6, moderate; .6–<.8, substantial/good; .8–<1.0, very good/perfect.27

2.2 Third and fourth agreement trials (Delphi consensus and gene panel analysis)

In a third agreement trial, we applied the Delphi method to build consensus.28,29 All answers of the first and second agreement trials were pseudonymously disclosed to the 20 colleagues who completed both previous rounds. Answers were assembled with (1) histopathological landmark images from selected FFPE blocks and requested immunostainings; and (2) a summary of clinical information including preoperative MRI findings, postsurgical outcome, information about invasive diagnostic evaluation, and potential epilepsy risk factors (Table 1). All reviewers were invited to answer an online survey evaluation form (the open access LimeSurvey application) and choose a final diagnosis from the list of all previously submitted diagnoses. The OMERO.web platform remained open for microscopy review during the entire period. All 20 collaborators submitted their Delphi consensus survey within 6 weeks, and interobserver agreement was calculated by kappa coefficient analysis. In December 2019, the Task Force convened at a group meeting to discuss all previous results. Histopathology patterns were reviewed for all cases, regardless of whether consensus agreement had been achieved. Commentaries were prepared to inform the reviewers when anatomical landmarks or clinical histories were perceived as important for the differential diagnosis (Figure S1). The same group of 20 participants was invited to a second, and final, Delphi consensus trial using a similar LimeSurvey questionnaire, which included all previous answers from the third round, assembled with histology images and the glossary mentioned above. FCD gene panel results collected from four different laboratories became available at this time and were also disclosed to the reviewers. All reviewers submitted their final diagnosis within 8 weeks. Interobserver agreement was calculated by kappa coefficient analysis. Please see Figure 1 for an overview of the study design.

2.3 Deep targeted sequencing analysis of FCD-relevant genes

DNA extracted from snap-frozen surgical brain tissue and matched blood samples of 22 patients were sent to five genetic laboratories. Each group performed an independent analysis with hybrid capture sequencing targeting their own set of FCD-relevant genes (Table S1). Genetic results from four of the five laboratories were available for the final round of reviews. In Lab A (Daejeon), technical replication of deep sequencing targeting of 13 genes was performed (SoVarGen). Analysis for replicated data was performed in accordance with a previous study.18 The RePlow variant caller was applied for somatic mutations.20 After a filtering process, all candidate variants were validated using targeted amplicon sequencing (Miseq Dx sequencer; Illumina). Germline mutations were analyzed in a similar pipeline as Genome Analysis Toolkit (GATK)’s best practices. In Lab B (Paris Brain Institute), libraries were prepared and sequenced as previously described,19 using a targeted panel of 57 genes (Twist Bioscience). Brain somatic variants at low variant allele frequency were validated by droplet digital polymerase chain reaction (ddPCR) as previously described; ddPCR Mutation Detection Assays (FAM + HEX) were purchased from Bio-Rad Laboratories to detect the MTOR variant p.Thr1977Lys and AKT3 variant p.Glu17Lys. For Lab C (Campinas), a hybrid capture panel was used. Four bioinformatics algorithms were used (Mutect231 from GATK, Strelka2,32 VarScan,33 and Somatic Sniper34; see codes used for bioinformatic analysis in Appendix S1). In Lab D (Boston) a custom double-stranded molecular inversion probe (MIP) panel was used covering 18 FCD-relevant genes (see Table S1). For the MIP design, custom scripts incorporating the MipGen tool were generated that allowed for dense tiling of all exons and up to 60 bp of flanking intronic regions, and putative splicing regulators for all genes. All 33 183 unique MIPs were designed to include a custom backbone consisting of primer binding sites and a dual 8-nt unique molecular index (UMI). MIP were rebalanced in the pool based on the percent of GC content within the regions. MIP pool was amplified with low cycles (17 cycles), high-fidelity polymerase, and custom common primers, allowing for large quantities to be generated.
Captured products were amplified using 15 cycles of PCR with dual 8-nt indexing primers and sequenced on the Illumina HiSeq platform with 1 × 150-bp paired end reads. All regions were sequenced to a minimum of ×1000 coverage, allowing for accurate assessment of somatic alleles down to .5% alternate allele frequency. Raw sequencing data were analyzed using existing in-house pipelines that, briefly, include quality assessment, mapping using BWA-mem, UMI-collapsing, additional trimming, and local realignment. Finally, variants were called using multiple calling algorithms including Mutect2 and CLC Genomics’ Low-Frequency algorithm. Lab E (Amsterdam) carried out additional validation of somatic single nucleotide variants (SNVs) using a dedicated custom Ion AmpliSeq FCD next generation sequencing (NGS) panel and ddPCR for MTOR and AKT3 variants in Cases 3, 12, 14, 16, 21, and 22 (Table 3; results from individual centers are summarized in Tables S2–S6).

3 | RESULTS

Twenty reviewers completed the four histopathological agreement trials, and 1760 answers were collected for the analysis (Figure S2). In the first round, which was based on 196 H&E stainings, the interobserver agreement was low, with a kappa value of .16. Two cases of FCD 2B were agreed upon by more than 75% of reviewers (2/22 = 9%). An additional 11 cases only reached a majority vote (defined as simple majority) for the diagnosis, as finally agreed upon after the fourth round.
We added 10 different antibody stainings to the second round (Table 2), which yielded >75% agreement in two additional cases of FCD 2B and two cases of FCD 2A (6/22 = 27%). This resulted in an improvement of the interobserver agreement to “fair” (kappa value of .35). The Delphi consensus method was applied in the third round and reached a moderate interobserver agreement, with a kappa value of .5. Diagnosis for nine cases was agreed upon by more than 75% of reviewers (41%), seven of which were FCD Type 2A or 2B. An agreement was reached by more than 75% of reviewers for 14 cases in the fourth round (64%). An agreement was reached for all subtypes of FCD 2, polymicrogyria, FCD 3 associated with perinatal stroke (FCD Type 3D), MOGHE, and a case associated FCD (three with HS and one with a tumor).

Eight cases finally achieved a consensus agreement for FCD Type 2A and 2B (n = 4 each); 86.7% of all FCD Type 2 diagnostic labels were already assigned to these eight cases in the first round, and this increased to 97.5% in the fourth round. Results differed in FCD Type 1. Only 20% of all FCD Type 1 labels were assigned to the first round to Case 5, which received the final majority vote for FCD Type 1A (Figure 2H). FCD Type 1 labels were scattered among all other cases and represented a common differential diagnosis for mMCD.

FCD Type 3 labels represented 14.3% of all answers in the first round, which increased to 17.5% in Round 2 and 20.5% in Round 3 (Figure 1, Figure S2). FCD 3A was the most frequent differential diagnosis in all cases with HS, representing 33% of all answers in the first, 50% in the second, and 56% in the third round. A Task Force group meeting was organized to clarify common patterns of FCD Type 3A recognized by the current ILAE classification scheme and provided as a commentary to the reviewers. In the fourth round, only 28% of answers selected FCD 3A in cases with HS and all cases reached a majority vote for “HS without FCD Type 3A” (Figure S2). Instead, 61.6% of all FCD 3 labels in Round 4 were assigned to both cases with the final diagnosis of FCD 3D (Figure 2J).

Only 4.7%–8.8% of all answers specified mMCD, which is defined as excessive (heterotopic) neurons in the white matter. Another lesion preferentially affecting the white matter was mMCD with oligodendroglial hyperplasia, for example, MOGHE, which also showed heterotopic neurons in the white matter, and collected 3.5%–7% of all answers. Based on consensus diagnosis, two cases reached the final agreement of MOGHE (Figure 2D). Interestingly, both cases revealed a somatic brain mutation for the SLC35A2 gene (Table 3).

The widespread distribution of diagnostic MOGHE labels observed during Rounds 1–3 collated to the two SLC35A2 mutant cases after disclosure of the genetic information in the fourth round. The same holds true for one case with polymicrogyria, which was difficult to identify in the oblique cutting plane of the neocortical specimen. A germline NPRL3 mutation was detected in blood and tissue and the diagnosis reached agreement status in the fourth round, as already suggested by a majority after the second round.

### TABLE 3 Summary of consensus genetic findings validated by independent laboratories

| Case | Diagnosisa | Geneb | Amino acid change | Lab A (hybrid capture, brain/blood)c | Lab B (hybrid capture, brain/blood)d | Lab C (hybrid capture, brain/blood)d | Lab D (MIP), brain/bloodd | Lab E (validation), brain/bloodd |
|------|------------|-------|-------------------|--------------------------------------|--------------------------------------|--------------------------------------|---------------------------------|---------------------------------|
| 3    | FCD 2A     | DEPDC5| p.Arg874*         | 46.6%/53.3%                          | 48%/47%                              | 51%/51.1%                            | 19%/39.5%                       | 49%/54%                         |
| 14   | FCD 2B     | MTOR  | p.Thr1977Lys      | .3%/1.01%                            | .4%/0%                               | .4%/0%                               | 2.8%/0.02%                      | .3%/nd                          |
| 22   | FCD 2A     | MTOR  | p.Ser2215Tyr      | .6%/0.02%                            | 1.1%/0%                              | 1.1%/1.3%                            | nd                              | .6%/nd                          |
| 21   | FCD 2A     | AKT3  | p.Glu17Lys        | 1%/0%                                | .2%/0%                               | .5%/0%                               | nd                              | 9%/nd                           |
| 16   | PMG        | NPRL3 | p.Ala384fs        | 47.6%/50%                            | 50%/50%                              | 57.7%/42.8%                         | nd                              | 47.2%/49.2%                     |
| 12   | MOGHE      | SLC35A2| p.Cys210Tyr       | 51.4%/0%                             | 37%/0%                               | nd                                   | nd                              | nd                              |
| 17   | MOGHE      | SLC35A2| p.Pro15Thr        | 5.1%/0%                              | nd                                   | nd                                   | 3.3%/0%                         | nd                               |

Note: Case identification numbers are the same as used in Table 1.

Abbreviations: FCD, focal cortical dysplasia; MIP, molecular inversion probe; MOGHE, mild malformation of cortical development with oligodendroglial hyperplasia in epilepsy; nd, not detected due to low coverage or absence of the gene in the targeted panel; PMG, polymicrogyria.

1Final histopathology diagnosis after the fourth round.
2DEPDC5 transcript ID: NM_001242896, MTOR transcript ID: NM_004958, AKT3 transcript ID: NM_005465, NPRL3 transcript ID: NM_001077350, SLC35A2 transcript ID: NM_001242896.
3Variant allele frequency detected in DNA from fresh frozen surgical brain sample.
4Variant allele frequency detected in DNA from a peripheral blood draw.
5Mutation-negative in bulk frozen tissue, but mutation-positive in pathologically reviewed formalin-fixed and paraffin-embedded tissue samples.
6Mutation-negative in bulk frozen tissue, but mutation-positive in pathologically reviewed formalin-fixed and paraffin-embedded tissue samples.
All genetic laboratories independently performed genetic analysis of somatic and germline mutations in FCD-relevant genes (Table S1). To detect low-frequency somatic mutations, high-depth hybrid capture sequencing of FCD-relevant genes was performed (>×1000 read depth), followed by comprehensive bioinformatic analysis as described in the Materials and Methods section. All somatic and germline mutations were validated by alternative sequencing methods including target-specific amplicon sequencing, ddPCR, and Sanger sequencing. Most laboratories reproducibly found somatic brain SNVs in MTOR, AKT3, and SLC35A2 in four patients and germline mutations in DEPDC5 and NPRL3 in two patients. Six panel-negative cases with a histopathologically verified lesion after the third histopathology review (Cases 1, 4, and 18 with FCD 2B, Case 2 with FCD 2A, Case 5 with FCD 1A, and Case 17 with MOGHE) were subjected to a second round of genetic testing using DNA extracted from FFPE slides of a paraffin block containing the lesion. In this study, a new somatic brain mutation was identified in SLC35A2 in Case 17 with MOGHE. As a result, genetic tests of FCD-relevant genes performed by independent laboratories identified genetic causes in seven of 22 (31.8%) patients including five of 22 (22.7%) patients with somatic brain mutations and two of 22 (9%) patients with germline mutations.

4 | DISCUSSION

Despite the ubiquitous use of the international consensus classification of FCD in research and clinical practice, the histopathological characterization of FCD subtypes remains a matter of ongoing debate. The presented results show...
that the diagnosis of (1) FCD Type 2 subtypes can be improved using a selected protocol of immunostains, for example, NeuN, nonphosphorylated neurofilament, vimentin, and microtubule-associated protein 2 (Table 2) and genetic tests

Interestingly, we observed a pathogenic germline mutation in one case of FCD Type 2A. 17–19 Genomic somatic and germline mutations in mTOR or GATOR pathway genes were identified only in FCD Type 2. 17–19 Neun immunoreactivity also helped to anticipate the regional variability of the laminar neuroanatomical organization (Figure S1). Antibodies directed against nonphosphorylated neurofilaments (SMI32) are sensitive markers to identify dysmorphic neurons in all FCD 2 subtypes, and dense accumulation of neurofilaments in large cell bodies of up to 50 µm diameter must be considered pathognomonic. Of note, SMI32 labeling remains weak in the normal neocortex of children and young adults but increases with age. 37 Vimentin or αB-crystallin were frequently requested to confirm the presence of balloon cells in FCD Type 2B. However, both antibodies also label reactive astrocytes. Their distinction from vimentin- or αB-crystallin-positive balloon cells can be challenging, therefore, whereas balloon cells do not exhibit cellular processes or end feet extending toward capillaries.

Consistent with previous genetic studies of FCD, pathogenic somatic and germline mutations in mTOR or GATOR pathway genes were identified only in FCD Type 2. 17–19 Interestingly, we observed a pathogenic germline DEPDC5 mutation in one case of FCD Type 2A. DEPDC5 mutations have been assigned mostly to FCD Type 2A, 19,38–40 but were also reported in other FCD subtypes. 31 In the case of DEPDC5, a repressor of the mTOR pathway, a second-hit somatic mutation has been shown to lead to the development of dysmorphic neurons in the dysplastic lesion. 20,42 This second hit is yet to be identified in Cases 3 and 16, however. Regarding the laboratory requirements for the reliable detection of low-level somatic mutations implicated in FCD, our result suggested that FCD genetic tests require (1) hybrid capture and high-depth NGS sequencing (≥x1000 read depth) of FCD-relevant genes; (2) the use of somatic mutation callers, for example, MuTect2, Replow, and Strelka2; and (3) the validation sequencing of candidate variations using orthogonal technology, for example, ddPCR or target site-specific amplicon sequencing. Although genetic testing of somatic and germline mutations for FCD is not yet available in most pathology laboratories, it is important information for the genetic consultation whether FCD patients carry pathogenic somatic (not inherited, not transmissible) or germline (inherited and transmissible) variants. A hyperactivated mTOR kinase due to somatic or germline mutations in FCD Type 2 is also a promising candidate for targeted drug treatment of mTOR inhibitor (ClinicalTrials.gov identifier NCT03198949). 43 Thus, genetic diagnosis will be helpful and add an objective measure for an integrated diagnosis of FCD, thereby better stratifying the patient cohort, advancing our knowledge about the underlying disease pathomechanism, and promoting personalized medicine in epileptology.

MOGHE is a recently described new entity observed in young children with early onset and difficult-to-treat frontal lobe epilepsy, and histopathologically defined by oligodendroglial hyperplasia and heterotopic neurons in the shallow subcortical white matter. 22,44 There was a majority vote from only 23% of reviewers in the first and second rounds for Cases 12 and 17. The Delphi process assisted most reviewers in recognizing this entity. Surprisingly, SLC35A2 somatic variants were identified in both MOGHE cases. MOGHE neuropathology has only recently been associated with SLC35A2 mutations in an unprecedented cohort of 26 cases including those SLC35A2 mutated cases previously published in the literature as mMCD or FCD Type 1. 19–21,45 Both differentials were also commonly assigned to MOGHE cases in our study. We suggest, however, recognizing MOGHE diagnosis by the ILAE consensus classification scheme as a specific disease entity preferentially affecting the white matter (Table 4).

FCD 1 subtypes pose a major challenge in clinical practice. 7,13,46 Their histopathological hallmarks comprise a large spectrum of horizontal or vertical abnormalities of the six-layered neocortex. 31 The human neocortex reveals different architectural patterns across the cortical ribbon (Figure S1), which are cytologically classified into 52 Brodmann areas. 47 However, it is almost impossible to recognize these Brodmann areas on a thin H&E-stained section obtained from an epilepsy surgery tissue sample. Clinical data, as well as knowledge of the cortical region resected, are essential when considering FCD Type 1 diagnoses. We finally agreed, therefore, that FCD Type 1 was unlikely in Patient 8, in whom seizures started after a motor vehicle accident at age 49 years (Table 1), and who received 15%–20% of all FCD Type 1 votes in Rounds 1, 2, and 3. In contrast, histopathologically well-documented patients with FCD Type 1A were reported to have early seizure onset, daily seizures, and a developmental delay. 13,46

The FCD Type 3 subgroup presented another diagnostic challenge during this agreement trial. The ILAE classification has specified several histopathology patterns and conditions
for this FCD category. A pattern of temporal lobe sclerosis as defined by neuronal cell loss and astrogliosis in neocortical Layers 2 and 3 in patients with HS has been assigned to FCD Type 3A.\textsuperscript{11} Lentiform heterotopias in the white matter of patients with HS\textsuperscript{49} was also assigned to FCD 3A. It came as no surprise, therefore, that 65\% of reviewers recognized these lentiform heterotopias in Patient 11 as FCD Type 3A (Figure S1). However, neuroanatomists would classify lentiform heterotopias as part of the ventral claustrum, fragmented by fibers of the fasciculus uncinatus,\textsuperscript{54} which frequently extend into the whiter matter of the superior temporal gyrus, an area often included in the surgical resection for epilepsy. This information was disclosed to our reviewers in Round 4, and the final majority vote was HS, no FCD.

In addition, this agreement study highlighted cases where no FCD was reported despite focal epilepsy and subtle features on MRI; "no FCD" was used in 10\% in Round 1, 13\% in Round 2, and 12\% in Round 3, and was finally assigned by 70\% of the reviewers to Case 8 and 85\% of the reviewers to Case 15. We propose that "no FCD" should be incorporated into the diagnostic framework of the international FCD classification scheme (Table 4). It needs to be recognized that even where dysplastic cortical lesions are suspected on MRI (or quantitative MRI) there may not be a confident FCD diagnosis possible within the current limitations of histology and genetic analysis. Acknowledging this option may reduce any tendency to overdiagnose, particularly the FCD 1 and FCD 3 subtypes.

In conclusion, the international FCD classification scheme is widely accepted in research and clinical practice and is helpful in stratifying clinicopathological disease conditions. Inclusion of newly recognized disease categories, a better definition of histopathological features, and genotype integration will help to improve the reliability of diagnosing FCD. This may also stimulate our research efforts into elucidating disease pathomechanisms and the development of targeted therapies.

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CONFLICT OF INTEREST

None of the authors has any conflict of interest to disclose, with the exception of J.H.L., who is a cofounder and chief technology officer of SoVarGen, which seeks to develop new diagnostics and therapeutics for brain disorders.

ORCID

Ingmar Blümcke https://orcid.org/0000-0001-8676-0788
Robyn M. Busch https://orcid.org/0000-0002-5442-4912
Fernando Cendes https://orcid.org/0000-0001-9336-9568
Iscia Lopes-Cendes https://orcid.org/0000-0002-6221-6822
Stephanie Baulac https://orcid.org/0000-0001-6430-4693
Sara Baldassari https://orcid.org/0000-0003-4851-2796
Angelika Mühlebner https://orcid.org/0000-0001-9102-7353
Albert Becker https://orcid.org/0000-0003-2661-3705
Rita Garbelli https://orcid.org/0000-0002-6475-4974
Maria Thom https://orcid.org/0000-0001-7712-2629
Anita Mahadevan https://orcid.org/0000-0002-4837-1463
Hajime Miyata https://orcid.org/0000-0003-2551-5978

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