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Genetic Testing for Malformations of Cortical Development

A Clinical Diagnostic Study

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Abstract

Background and Objectives

Malformations of cortical development (MCD), though individually rare, constitute a significant burden of disease. The diagnostic yield of next-generation sequencing (NGS) in these patients varies across studies and methods, and novel genes and variants continue to emerge.

Methods

Patients (n = 123) with a definite radiologic or histopathologic diagnosis of MCD, with or without epilepsy were included in this study. They underwent NGS-based targeted gene panel (TGP) testing, whole-exome sequencing (WES), or WES-based virtual panel testing. Selected patients who underwent epilepsy surgery (n = 69) also had somatic gene testing of brain tissue–derived DNA. We analyzed predictors of positive germline genetic finding and diagnostic yield of respective methods.

Results

Pathogenic or likely pathogenic germline genetic variants were detected in 21% of patients (26/123). In the surgical subgroup (69/123), we performed somatic sequencing in 40% of cases (28/69) and detected causal variants in 18% (5/28). Diagnostic yield did not differ between TGP, WES-based virtual gene panel, and open WES (p = 0.69). Diagnosis of focal cortical dysplasia type 2A, epilepsy, and intellectual disability were associated with positive results of germline testing. We report previously unpublished variants in 16/26 patients and 4 cases of MCD with likely pathogenic variants in non-MCD genes.

Discussion

In this study, we are reporting genetic findings of a large cohort of MCD patients with epilepsy or potentially epileptogenic MCD. We determine predictors of successful ascertainment of a genetic diagnosis in real-life setting and report novel, likely pathogenic variants in MCD and non-MCD genes alike.

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MCD Prague Study Group coinvestigators are listed in the appendix at the end of the article.

Glossary

aCGH = array comparative genomic hybridization; **ADHD** = attention deficit hyperactivity disorder; **CNV** = copy-number variants; **FCD** = focal cortical dysplasia; **HPO** = human phenotype ontology; **ILAE** = International League Against Epilepsy; **MCD** = malformations of cortical development; **NGS** = next-generation sequencing; **OMIM** = Online Mendelian Inheritance in Man; **PCR** = polymerase chain reaction; **TGP** = targeted gene panel; **VAF** = variant allele frequency; **WES** = whole-exome sequencing.

Malformations of cortical development (MCD) represent a significant cause of neurologic morbidity and mortality in both children and adults. The exact prevalence of all MCD is difficult to ascertain; however, in epilepsy surgery series, patients with MCD account for the most numerous etiologic groups.[1-3] Patients usually present in infancy or childhood with epileptic seizures, often drug-resistant, developmental delay, varying levels of intellectual disability, sensory deficits, and other symptoms.[4]

Despite advances in next-generation sequencing (NGS) and diagnostic efforts in general, the etiology of MCD remains unknown in 63%–85% of patients.[4] Why is it so that in such a significant proportion of patients with MCD we cannot ascertain a definite genetic diagnosis? First, it is highly likely that yet unknown genes might contribute to MCD formation. Second, a lack of detailed phenotypic information may limit genetic analyses only to genes "strictly" associated with MCD, and variants in "non-MCD" genes may escape evaluation. We can assume that there might be more genes previously unknown to be involved in MCD formation; however, pathogenic variants in these genes may be lost in gene panel–based testing or if the analysis is targeted specifically on MCD phenotype only.

Up until now, most of the studies that analyzed diagnostic yield of genetic testing in patients with MCD have resulted from collaborative efforts of multiple centers[4] or they reported the use of a single methodology, e.g., targeted gene panel (TGP) testing, in a single center.[5] Multicentric studies provide the most precise estimate of the proportion of genetic etiology in patients with MCD; however, they often do not compare diagnostic yield between multiple NGS-based methods. Single-center studies, on the other hand, that use a single methodology correctly assesses the diagnostic yield of a specific method; however, this approach limits the number of patients included.

Before the advent of NGS and discovery of genetic origins of focal cortical dysplasia (FCD), epilepsy surgery candidates were not referred for genetic consultation and germline genetic testing. In large series, somatic TGP testing in patients with surgically amenable MCD achieves diagnostic yield of 31.9%.[6] However, the studies published so far report either individual patients or rather small patient cohorts with limited number of individuals carrying germline pathogenic variants who underwent germline genetic testing.[7,8]

In this study, we are reporting the largest cohort of patients with MCD with epilepsy or epileptogenic MCD from a single center, including a subgroup analysis of patients with MCD who underwent epilepsy surgery. Our study compares 3 major NGS-based diagnostic methods (TGP sequencing, WES-based virtual panel, open WES) in a large cohort of patients with MCD over time.

Methods

Patient Identification and Inclusion Criteria

Patients who fulfilled the inclusion criterion were identified over the period of 2017–2021. The inclusion criterion was a definite diagnosis of MCD ascertained through neuroimaging or histopathologic examination of the brain tissue. Clinical data were obtained from electronic medical records of Motol University Hospital. Most of the patients also had the diagnosis of epilepsy as defined by the International League Against Epilepsy (ILAE)[9] or another neurologic deficit that prompted clinical investigation, including MRI and EEG. Patients with genetic syndromes and inherited errors of metabolism in which MCD does not represent the main diagnostic feature were excluded, as well as patients with electro-clinically suspected "MRI-negative" FCD without histopathologic confirmation. Most of the patients were referred to the Department of Paediatric Neurology and to the Department of Neurology, Second Faculty of Medicine, Charles University and Motol University Hospital by local neurologists and child neurologists. The lesser proportion of patients were referred for genetic diagnostic testing by medical geneticists (M.V., P.T., M.B.). Patients were enrolled in the study in a prospective manner, and the selected method of genetic testing reflects an overall shift in methodology from TGP resequencing to virtual gene panel testing based on whole-exome sequencing (WES) data to open WES analysis based on detailed phenotyping using human phenotype ontology (HPO) terms (for temporal distribution, see below).

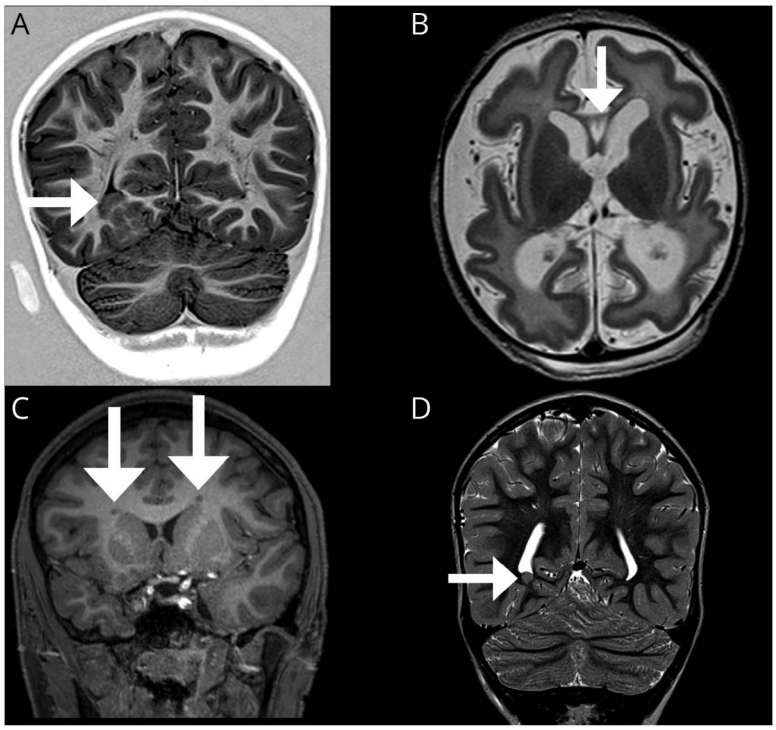
Standard Protocol Approvals, Registrations, and Patient Consents

The study was approved by the Institutional Ethics Committee. The participants or their legal guardians provided informed consent with the study.

Neuroimaging and Histopathology

All 123 patients underwent high-resolution brain MRI of 1.5T or 3T field strength. Most of the patients underwent a

Figure 1 Overview of the Germline Diagnostic Workflow



MCD = malformations of cortical development; TGP = targeted gene panel; WES = whole-exome sequencing; WES_VP = WES-based virtual panel.

dedicated epilepsy MRI protocol in Motol University Hospital; in selected pediatric cases, MRI was performed in a local hospital, and if the MRI was of a sufficient quality, it was not repeated in our center because these children require general anesthesia for MRI. All MRIs were evaluated by specialists with experience in MCD neuroimaging (M.K., P.K., B.B.), in accordance with the published recommendations for diagnosing MCD[10] and FCD.[11] The term "complex MCD" was used in cases when patients displayed multiple abnormalities consistent with more than 1 specific MCD.

In patients who underwent epilepsy surgery for drug-resistant epilepsy,[12] the expert neuropathologist (J.Z.) performed histopathologic evaluation of the resected brain tissue samples in accordance with the ILAE diagnostic guidelines.[13]

Germline Gene Testing

Fifty patients who were initially investigated over the period of 2017–2019 underwent TGP sequencing for detection of single-nucleotide variants and small insertions and deletions and copy-number variants (CNV) as described previously[14]; the respective gene panels included 63, 104, and 113 genes associated with MCD. In the period 2019–2021, 73 patients underwent WES for detection of single-nucleotide variants and small insertions and deletions with subsequent data filtration for variants solely in genes associated with MCD (virtual panels of 240, 285, 382, and 366 genes); 5 patients underwent both TGP and WES (Figure 1). Open WES analysis was performed in patients with MRI-negative FCD (with subsequent histopathologic confirmation of FCD) and in those with complex MCD in whom no putative variants were detected in virtual gene panel. In cases when patients underwent multiple germline testing (e.g., TGP and WES), we report the one that led to diagnosis or the most comprehensive one performed.

For WES, we used SureSelect V6-Post Library Kit (Agilent, Santa Clara, CA) with ×200 raw coverage and Illumina sequencing platform (NovaSeq and HiSeq, Illumina). Library preparation and sequencing took place at Macrogen, Inc.; raw data analysis was performed in-house as reported elsewhere,[14] using standard bioinformatics tools for sequence alignment and variant identification, including SureCall (Agilent) and analysis pipeline based on GATK Best Practices workflow (BroadInstitute, gatk.broadinstitute.org/hc/en-us). For variant visualization, we used Alamut Visual (Sophia Genetics, Saint-Sulpice, Switzerland) software tool. Additional software tools for WES analysis included Qiagen Digital Insights (Qiagen, Hilden, Germany) and Exomiser (Sanger Institute, Hinxton, UK). For the purpose of open WES analysis, the patients' phenotypes were described using HPO terms (hpo.jax.org/app/) based on the most recent release available, according to HPO coding methodology published.[15] Variants were filtered and prioritized by comparison with genes listed in Online Mendelian Inheritance in Man (OMIM) and by their frequencies listed in gnomAD (BroadInstitute) and HGMD Professional (Qiagen).

Table 1 Summary of Clinical Findings

Variable	Yes	No	N/A	Yes (%)	No (%)	N/A (%)
Family history of epilepsy	25	92	6	20.3	74.8	4.9
Perinatal risks	32	90	1	26.0	73.2	0.8
Developmental delay (younger than 1 y)	52	69	2	42.3	56.1	1.6
Drug-resistant epilepsy	100	16	7	81.3	13.0	5.7
Associated syndrome	10	113	0	8.1	91.9	0.0

	No ID	ID	Severe ID	N/A	No ID (%)	ID (%)	Severe ID (%)	N/A (%)
ID	65	39	17	2	52.8	31.7	13.8	1.6

	Yes	No	VNS	N/A	Yes (%)	No (%)	VNS (%)	N/A (%)
Epilepsy surgery	63	47	7	7	51.2	38.2	5.7	5.7

	SF	Non-SF	N/A	SF (%)	Non-SF (%)	N/A (%)
Epilepsy surgery outcome[a]	50	19	54	72.5	27.5	78.3

	ASM-free	Non–ASM-free	N/A	ASM-free (%)	Non–ASM-free (%)	N/A (%)
Epilepsy surgery outcome[a]	16	53	54	23.2	76.8	78.3

Abbreviations: ASM = antiseizure medication; DF = drug-free; ID = intellectual disability; N/A = not applicable; SF = seizure-free; VNS = vagus nerve stimulation (IQ/DQ < 70); SF = severe intellectual disability (IQ/DQ < 35).
[a] Percentages apply to the cohort of surgical patients (n = 69).

Putative variants were confirmed by Sanger sequencing, and segregation analysis was performed to validate the presence (or absence) of a given variant in a patient's parents and siblings where available. Detected variants were interpreted in accordance with the American College for Medical Genetics guidelines.[16] All patients included in germline genetic analyses underwent genetic counseling with a geneticist (M.V., P.T., M.B.).

Somatic Gene Testing

Somatic gene testing has gradually been implemented since 2019. Sample processing, including DNA isolation, library preparation, and sequencing took place at the Department of Pathology and Molecular Medicine, Motol University Hospital and Second Faculty of Medicine, Charles University, using the MiSeq system (Illumina) and in selected patients at the Graduate School of Medical Science and Engineering, KAIST, Daejeon, Republic of Korea, as reported elsewhere.[6] Brain tissue samples of focal MCD were obtained during epilepsy surgery and when possible, procured in accordance with the ILAE guidelines.[17] Specimen for DNA isolation was first evaluated by the neuropathologists (J.Z.) to ensure the presence of dysplastic features in the tissue samples used for sequencing. Brain tissue samples were stored as fresh frozen at −80°. DNA isolation from frozen tissues was performed using QIAamp DNA Mini and Micro Kits, Qiagen AllPrep DNA/RNA Micro Kit as per manufacturer's protocol. For somatic gene panel analysis, we performed targeted enrichment of coding regions of 43 selected genes associated with MCD, using SureSelectXT HS system (average read depth ×1,500–×2,000). For downstream bioinformatics analysis, we used a SureCall (Agilent, Santa Clara) software with settings modified specifically for somatic variant analysis (variant detection threshold of variant allele frequency equal and above 1%). Variant analysis and filtering followed similar steps as described above. The presence of variants with variant allele frequency above 10% was confirmed by Sanger sequencing.

Array CGH Analysis

Patients who showed signs of facial dysmorphism and those with MCD associated with chromosomal aberrations and CNV also underwent array comparative genomic hybridization (aCGH). aCGH was performed using the commercially available oligonucleotide microarray platforms ISCA 4 × 180 K, 8 × 60 K (SurePrint CGH 4 × 180 K G3 ISCA; Agilent Technologies; SurePrint CGH 8 × 60 K G3 ISCA; Agilent Technologies; CytoChip Oligo 8 × 60 K, BlueGnome, UK), also according to the manufacturer's instructions. Data were analyzed using CytoGenomics software (Agilent Technologies) and BlueFuse Multi software. The analysis of the clinical impact of deletions and duplications used the UCSC Genome Browser, DECIPHER, International Standards for Cytogenomic Arrays (ISCA) (ClinVar), ClinGen, OMIM, and DGV databases.

Statistical Analysis

For descriptive statistics, we used the SPSS Statistics software (IBM, NY), and for analytical statistical calculations, we used the MatLab version 2017b and its statistical computing toolbox. First, we selected the positive genetic finding of germline testing, declared formally as a dependent variable in the statistical analyses, and performed univariate analysis to detect which of the selected phenotype-related variables (declared as "independent" in the analysis) reach statistically

Table 2 Summary of Likely Pathogenic and Pathogenic Germline Variants

Gene	c_variant	p_variant	Mutation type	Segregation	Method	method_specified	Reported
DXC	NM_000555.3(DCX):c.503T>G	p.Leu168Arg	Missense	De novo	TGP	TGP63	Here
PAFAH1B1	NM_000430.3(PAFAH1B1):c.232G>T	p.Glu78*	Stop gain	De novo	TGP	TGP63	Here
NPRL2	NM_006545.4(NPRL2):c.100C>T	p.Arg34*	Stop gain	Inherited from affected parent	TGP	TGP63	Baldassari S, et al. 2016
DYNC1H1	NM_001376.4(DYNC1H1):c.10030C>T	p.Arg3344Trp	Missense	De novo	TGP	TGP63	Di donato N, et al. 2018
COL4A1	NM_001845.5(COL4A1):c.1685G>A	p.Gly562Glu	Missense	De novo	TGP	TGP104	Gould DB, et al. 2006
DEPDC5	NM_001242896.1(DEPDC5):c.2843T>C	p.Leu948Pro	Missense	Inherited from affected parent	TGP	TGP104	Benova B, et al. 2021
DEPDC5	NM_001242896.1(DEPDC5):c.2294del	p.Gly765Alafs*29	Frameshift	Inherited from affected parent	TGP	TGP104	Benova B, et al. 2021
OCLN	NM_001205254.1(OCLN):c.639T>G	p.Tyr213*	Stop gain	Inherited from unaffected parent	TGP	TGP104	Here
OCLN	NM_001205254.1(OCLN):c.1218C>T	p.Gly406	Splicing effect	Inherited from unaffected parent	TGP	TGP104	Jenkinson EM, et al. 2018
NPRL3	ENST00000399953.3:c.318 + 1G>C	p?	Splicing effect	Inherited from affected parent	TGP	TGP113	Here
DYNC1H1	NM_001376.4(DYNC1H1):c.3010T>G	p.Tyr1004Asp	Missense	De novo	TGP	TGP113	Here
NPRL3	ENST00000399953:c.924 + 1G>A	p?	Splicing effect	Inherited from unaffected parent	WES-based VP	WES-based VP240	Here
PAFAH1B1	NM_000430.3(PAFAH1B1):c.1108A>T	p.(Lys370*)	Stop gain	De novo	WES-based VP	WES-based VP240	Here
NPRL3	ENST00000399953.3:c.189-1G>A	p?	Splicing effect	Inherited from unaffected parent	WES-based VP	WES-based VP240	Here
PIK3R2	NM_005027.3(PIK3R2):c.1117G>A	p.(Gly373Arg)	Missense	De novo	WES-based VP	WES-based VP240	Mirzaa GM, et al. 2013
PTEN	NM_000314.8(PTEN):c.207_208insGT	p.(Leu70Valfs*30)	Frameshift	De novo	WES-based VP	WES-based VP382	Here
GDI1	NM_001493.2(GDI1):c.895C>T	p.(Arg299Cys)	Missense	Inherited from unaffected parent	Open WES	Open WES	Here
IRF2BPL	NM_024496.3(IRF2BPL):c.1072_1073del	p.(Ser358Argfs*64)	Frameshift	De novo	Open WES	Open WES	Here
NPRL3	ENST00000399953.3:c.1469C>A	p.(Ser490*)	Stop gain	Inherited from unaffected parent	WES-based VP	WES-based VP382	Here
GLI3	NM_000168.5(GLI3):c.2737dup	p.(Asp913Glyfs*171)	Missense	De novo	TGP	TGP113	Here
PIK3R2	NM_005027.3(PIK3R2):c.1117G>A	p.(Gly373Arg)	Missense	De novo	TGP	TGP113	Rivière JB, et al. 2012
TET3	NM_001287491.2(TET3):c.1052del	p.(Asn351Thrfs*190)	Frameshift	De novo	Open WES	Open WES	Here
PORCN	NM_203475.2(PORCN):c.727C>T	p.(Arg243*)	Stop gain	De novo?	Open WES	Open WES	Grzeschik KH, et al. 2007
KIAA0586	NM_001244189.1(KIAA0586):c.428del	p.(Arg143Lysfs*4)	Frameshift	Inherited from unaffected parents	Open WES	Open WES	Stephen LA, et al. 2015
COL4A1	NM_001845.5(COL4A1):c.4688_4711del	p.(Gln1563_Cys1570del)	In-frame	De novo	WES-based VP	WES-based VP366	Here
SETD1B	NM_001353345.2(SETD1B):c.3049dup	p.(Val1017Glyfs*88)	Frameshift	De novo	Open WES	Open WES	Here

Abbreviations: TGP = targeted gene panel testing; WES = whole-exome sequencing; WES-based VP = WES-based virtual panel.

Table 3 Summary of Likely Pathogenic and Pathogenic Somatic Variants

Gene	c_variant	p_variant	Mutation type	VAF in brain (%)	VAF in blood
PIK3CA	NM_006218.2(PIK3CA):c.1633G>A	p.(Glu545Lys)	Missense	22.4	Not present in blood
MTOR	NM_004958.3(MTOR):c.5930C>A	p.(Thr1977Lys)	Missense	5.5	Not present in blood
MTOR	NM_004958.3(MTOR):c.6644C>T	p.(Ser2215Phe)	Missense	3	Not present in blood
MTOR	NM_004958.3(MTOR):c.4379T>C	p.(Leu1460Pro)	Missense	4	Not present in blood
SLC35A2	NM_001032289.2(SLC35A2):c.265C>T	p.(Gln89*)	Stop gain	6	Not present in blood

Abbreviation: VAF = variant allele frequency.

significant association. The selected independent variables included age at epilepsy onset, family history of epilepsy, perinatal risks, developmental delay before age 1 year, intellectual disability, diagnosis of epilepsy, diagnosis of drug-resistant epilepsy, MRI diagnosis (for the full list, Table 1), and histopathologic diagnosis. In the multivariate analysis, we calculated a multiple regression based on the general linear model by stepwise regression algorithm with the variables that reached statistical significance in univariate testing; the algorithm already accounts for correction of multiple testing. The beta coefficients with p-values of <0.05 were considered statistically significant. We performed the Fisher exact test to analyze whether there exists a statistically significant difference between the 3 methods of germline testing (TGP vs WES-VP vs WES).

Data Availability

Anonymized data will be shared by request from any qualified investigator.

Results

Overall Characterization of the Cohort

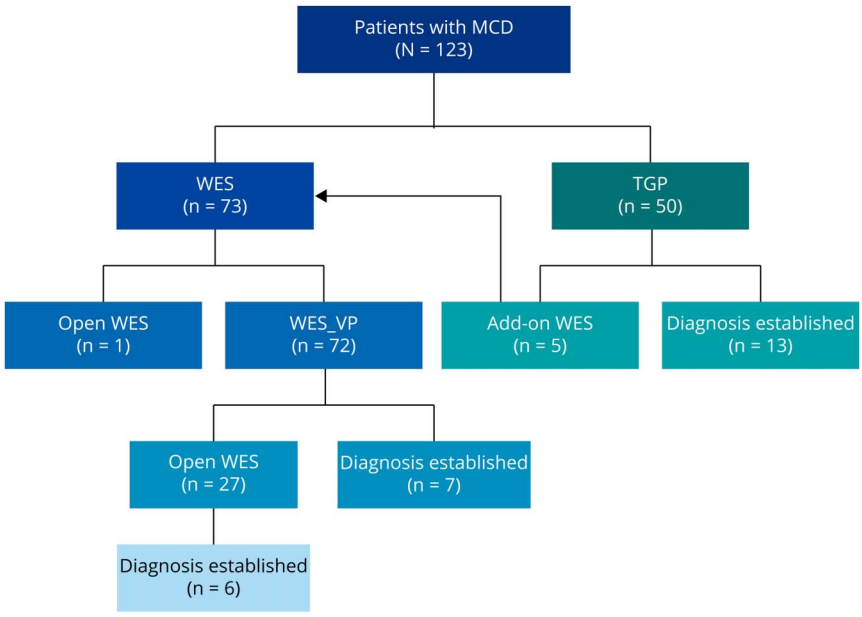
We included 123 patients with the diagnosis of MCD who underwent germline genetic testing; the surgical subgroup comprised 69/123 patients who were evaluated in the epilepsy surgery program and eventually underwent epilepsy surgery. MRI diagnoses were represented as follows: FCD (n = 50), MRI-negative FCD (n = 20), polymicrogyria (n = 5), lissencephaly spectrum (n = 10), hemimegalencephaly (n = 4), periventricular nodular heterotopia (PVNH) (n = 6), and complex and other MCD (n = 28). Histopathologic diagnoses were represented as follows: hemimegalencephaly (n = 4), FCD type I (n = 19), FCD type IIa (n = 5), FCD type IIb (n = 29), complex MCD without a clear diagnosis of FCD (n = 3), gliosis (n = 1), and no definite FCD on histopathology[18] (n = 1). One patient underwent a stereo EEG study with electrocoagulation of the heterotopia; therefore, no sample for histopathology was available. We were able to ascertain germline genetic diagnosis in 21% (26/123) of the entire cohort. One patient was diagnosed with a chromosomal aberration dup15q11.2q13.1; this result was ascertained only after the patient underwent WES. In the surgical subgroup (69/123), we performed somatic sequencing in 40% of cases (28/69) because of later introduction of somatic testing (in 2019) and detected causal variants in 18% (5/28); in addition, 9% of surgical patients (6/69) carried germline pathogenic and likely pathogenic variants in genes of GATOR1 complex or *PTEN*. In 5 patients, germline genetic testing was negative (TGP in 4 and WES-based VP in one), and genetic diagnosis was established afterward by somatic gene testing—3 patients carried pathogenic variants in *MTOR*, 1 in *PIK3CA*, and 1 in *SLC35A2*.

Clinical data for the entire cohort are summarized in Table 1. Table 2 and Table 3 display specific germline and somatic genetic variants, respectively, observed in the patients, including novel, previously unpublished variants. All clinical and genetic data are available in eTable 1, links.lww.com/NXG/A546.

Predictors of Successful Ascertainment of Genetic Diagnosis in Patients With MCD

Univariate analysis showed that positive results of germline genetic testing were associated with developmental delay before age 1 year, intellectual disability, diagnosis of epilepsy, MRI, and histopathologic diagnosis ($p < 0.05$). However, multivariate testing using generalized linear regression model showed that the only variable significantly associated with positive results of germline genetic testing was the histopathologic diagnosis of FCD 2A ($p < 0.01$; p value of the model 0.04, 61 observations, 54 error degrees of freedom). Multivariate model without the histopathologic diagnosis (data not available for nonsurgical patients) showed that a positive result of germline genetic testing was associated with the diagnosis of epilepsy and intellectual disability ($p < 0.01$ and $p < 0.05$ respectively; p value of the model <0.001, 121 observations, 117 error degrees of freedom). Complete calculations and data for the multivariate model are available in the Supplement. Thirteen of 50 patients who underwent TGP had their diagnosis established (26%), as compared with 7/72 (10%) who underwent WES-based virtual panel testing and 6/28 who underwent open WES (21%) (Figure 1). We observed no statistically significant difference between the diagnostic yields of the respective methods ($p = 0.38$).

Figure 2 Patient With Periventricular Nodular Heterotopia and *GDI1* Variant



(A) MRI, coronal T1-IR. Subependymal nodular heterotopia around occipital horn of the right lateral ventricle (arrow). (B) Patient with periventricular nodular heterotopia and *IRF2BPL* variant. MRI, axial T2-weighted. Microcephaly, simplified gyral pattern, delayed myelination of white matter, periventricular atrophy, and hypoplasia of corpus callosum (arrow). (C) Patient with periventricular nodular heterotopia and *TET3* variant. MRI, coronal T2-weighted. Subependymal nodular heterotopia around occipital horn of the right lateral ventricle (arrows). (D) Patient with periventricular nodular heterotopia and *SETD1B* variant. MRI, coronal T1-weighted 3D turbo field echo (reformatted view). Periventricular gray matter heterotopia bifrontally (arrow). IR = inversion recovery.

Novel, Likely Pathogenic Variants in Non-MCD Genes Detected in Patients With MCD

GDI1 Variant in a Patient With PVNH

The patient was referred to us with the MRI finding of an extensive PVNH (Figure 2A) around temporal and occipital horn of the right lateral ventricle. The patient displayed features of moderate intellectual disability and attention deficit hyperactivity disorder (ADHD), and he displayed specific facial dysmorphic features (bilateral epicanthi and open mouth appearance). He never experienced an epileptic seizure, despite the finding of potentially epileptogenic PVNH. Long-term video EEG monitoring showed abnormal baseline activity and almost continuous focal slowing over the right occipital region. Open WES analysis led to the detection of a missense variant in *GDI1* (Guanosine Diphosphate Dissociation Inhibitor 1), associated with X-linked intellectual development disorder 41 (OMIM #300849), NM_001493.2(GDI1):c.895C>T p.(Arg299Cys); the variant is located in a functional protein domain (GDP-dissociation inhibitor), absent from controls in large databases (e.g., gnomAD, 1000Genomes, dbSNP), and multiple lines of computational evidence support a deleterious effect of the variant on gene or gene product (SIFT v6.2.0: deleterious; MutationTaster v2021: deleterious, moderately conserved nucleotide, highly conserved amino acid). The patient's phenotype corresponds to those described previously—the common features include cognitive impairment, facial dysmorphism, and ADHD reported in selected female carriers. However, he is the first to our knowledge with the diagnosis of PVNH. The variant was inherited from the seemingly unaffected patient's mother. Female carriers reported by Strobl-Wildemann had normal intelligence, learning disability, or ADHD, suggestive of a semidominant inheritance pattern.[19,20] In addition, female carriers, reported in earlier publications, of missense variants tended to be unaffected in contrast to affected female carriers of nonsense variants.[21,22]

IRF2BPL Variant in a Patient With Microcephaly With Simplified Gyral Pattern

The patient came to our attention for neonatal seizures that first occurred in the 6th postnatal hour that were temporarily controlled by antiseizure medication but became drug-resistant at age 2 months. MRI showed microcephaly with simplified gyral pattern, cortical and periventricular atrophy, diffuse periventricular leukomalacia, and delayed myelination and hypoplasia of corpus callosum (Figure 2B). WES analysis revealed a de novo variant, NM_024496.3(IRF2BPL):c.1072_1073del p.(Ser358Argfs*64) in *IRF2BPL* (Interferon Regulatory Factor 2-Binding Protein-Like). Pathogenic variants in *IRF2BPL* cause autosomal dominant developmental and epileptic encephalopathy (OMIM #618088); patients present with epileptic seizures (myoclonic, tonic-clonic, spasms, and other) with variable age of epilepsy onset, global developmental regression associated with seizure onset, movement abnormalities, spasticity, and other neurologic abnormalities.[23] Compared with the individuals reported, our patient had the earliest seizure onset, along with the most severe structural CNS involvement. CNS involvement in previous cases was limited to diffuse or focal brain atrophy, cerebellar atrophy, and "bulky" corpus callosum.[23,24]

TET3 Variant in a Patient With PVNH

The patient was referred to genetic investigation for mild-to-moderate cognitive impairment, muscle hypotonia, and joint hypermobility; the MRI finding of bilateral occipital PVNH (Figure 2C) prompted investigation with WES-based virtual panel of genes associated with MCD that did not reveal a putative causal variant. Open WES analysis based on HPO

terms describing all phenotypic features of the patient led to detection of a de novo variant NM_001287491.2(TET3): c.1052del p.(Asn351Thrfs*190). *TET3* (Tet Methylcytosine Dioxygenase 3) is associated with Beck-Fahrner syndrome (OMIM #618798); truncating variants were only observed in heterozygous state.[25] Our patient's phenotype corresponds well to the phenotype of Beck-Fahrner syndrome, including muscle hypotonia, joint hypermobility, dysmorphic facial features (long narrow face, narrow gothic palate in our case), and cognitive impairment; however, Beck reported no patient with PVNH.[25]

SETD1B Variant in a Patient With PVNH

The patient was referred to us for frequent epileptic seizures classified as myoclonic absences and for video EEG monitoring that showed generalized spike-wave complexes with 3.5 Hz frequency. Her MRI showed foci of gray matter heterotopia in left dorsal temporal area and bifrontally that prompted indication for virtual gene panel testing of genes associated with MCD (Figure 2D). The patient also showed dysmorphic features and lower-average cognitive skills probably related to drug-resistant epilepsy. Virtual gene panel analysis detected no putative pathogenic variants, but open WES analysis revealed a de novo variant NM_001353345.2(SETD1B [SET Domain-Containing Protein 1B]):c.3049dup p.(Val1017Glyfs*88), a gene associated with autosomal dominant intellectual developmental disorder with seizures and language delay (OMIM #619000). The patient's phenotype corresponded to recent reports[26,27]; however, the reported MRI findings were either normal or nonspecific, in contrast to our patient who had distinct PVNH.

Novel, Previously Unpublished Variants Were Detected in Genes Associated With MCD

Novel, previously unpublished variants are reported in 16/26 patients with a definite germline genetic diagnosis (Table 2). We detected novel variants in gene coding for cytoskeletal proteins (e.g., *DCX*, *PAFAH1B1*, *DYNC1H1*) in patients with double cortex, lissencephaly, and pachygyria, respectively, genes of mTOR signaling cascade (e.g., *NPRL3*, *PTEN*) in patients with FCD, and other genes associated with specific MCD (e.g., *OCLN*–pseudo-TORCH syndrome).

Discussion

In this article, we summarize clinical and genetic findings from a large cohort of patients with MCD. We were able to ascertain germline genetic diagnosis in one-fifth of the cohort, and we detected somatic causal variants in 18% of surgical patients who underwent somatic gene panel testing. Apart from reports of novel, previously unpublished genetic variants, we detected pathogenic and likely pathogenic variants also in genes previously not associated with MCD.

The most striking findings in our cohort included cases of patients with likely pathogenic variants in genes not previously known to be involved in MCD formation. In these cases, MCD might have either been a coincidental finding or a previously unknown part of the phenotype. The first analysis of MCD virtual gene panel was negative; therefore, we proceeded with open WES analysis based on HPO terms describing all known features of the patients' phenotypes, including developmental delay, intellectual disability, epilepsy, muscle hypotonia, and others. The importance of detailed phenotypic analysis has been highlighted by the analysis of HPO terms among 314 individuals with developmental and epileptic encephalopathy of unknown cause and discovered a recurrent pathogenic missense variant in *AP2M1* gene.[28] Other recent works reported patients with polymicrogyria harboring homozygous truncating variants in *ATP1A2*, a gene previously associated with alternating hemiplegia of childhood, familial hemiplegic migraine, and severe epilepsy, among others.[29-31] A detailed phenotype-based analysis led to the detection of novel likely pathogenic variants in genes previously unrelated to MCD. In fact, this is the first report of PVNH in patients with conditions related to pathogenic variants in *TET3*, *GDI1*, and *SETD1B*. *TET3* encodes for one of the family of ten-eleven translocases that initiates DNA demethylation; it is highly expressed in oocytes, zygotes, and neurons, and inhibition of its function in a mouse model was shown to impair synaptic function.[25] GDI1 protein, GDP-dissociation inhibitor alpha, plays role in synaptic vesicle biogenesis and recycling in mice hippocampi.[20,32] *SETD1B* encodes for an enzyme involved in histone methylation, SET domain-containing protein 1B, and thereby participates in regulation of gene expression.[27] *IRF2BPL*, a transcriptional regulator interferon regulatory factor 2-binding protein-like, is an intronless gene, and its role in CNS development remained elusive until certain features of human neurologic phenotypes were recapitulated in drosophila model—the model provided evidence for the role of *IRF2BPL* in neuronal function and maintenance.[24]

All 4 genes are expressed in the brain, and therefore, their mutations might have contributed to the formation of cortical structural anomalies. However, because PVNH is a relatively common condition, we cannot definitely infer whether the variants are indeed the cause of PVNH without functional studies. From a clinical diagnostic perspective, our findings highlight the importance of complex WES analysis that includes detailed patient phenotyping using HPO terms. Many patients with less extensive MCD, e.g., PVNH, may remain undiagnosed through investigation of MCD-specific gene panels; open WES analysis provides a viable approach, especially in patients with complex phenotypes.

In our study, we devoted special attention to patients with focal MCD who underwent epilepsy surgery. We found that almost one-tenth of these patients carried germline pathogenic and likely pathogenic variants, most frequently in genes of GATOR1 complex. Of 5 GATOR1 patients, 3 were seizure-free at the last follow-up (October 2021); all 5 continued using antiseizure medication. Based on such small numbers, one cannot infer whether GATOR1 patients are good surgical candidates; in our previous work, we showed that patients with GATOR1 pathogenic variants and a family history of epilepsy represent especially

challenging surgical candidates, and despite extensive diagnostic and surgical efforts, epilepsy surgery may fail.[14] However, based on a longer follow-up and more experience with GATOR1 patients, we can now conclude that these patients should be referred to epilepsy surgery centers because those with well-defined epileptogenic zone (by means of MRI, EEG, or invasive stereo EEG study) may significantly benefit from resective epilepsy surgery. Based on our findings, we advocate for routine germline and somatic genetic testing in all eligible and consenting surgical candidates and patients undergoing epilepsy surgery.

In some patients, however, the risks of resective epilepsy surgery outweigh its benefits or reasonable surgical resection is not feasible (e.g., patients with epileptogenic zone located in eloquent cortical areas, those with bilateral inoperable MCD). These patients with a definite genetic diagnosis might in future profit from targeted pharmacologic therapy, such as patients with TSC benefitted from adjunctive treatment with everolimus leading to decrease in seizure frequency.[33] Another mTOR inhibitor, sirolimus, temporarily aborted seizures in a 3.5-month-old infant with deletion of *NPRL3* and hemispheric cortical dysplasia. However, because of side effects, sirolimus was later completely discontinued and seizures reoccurred.[34] Targeted therapy could also be used in patients with PROS (PIK3CA-related overgrowth spectrum)—a compound BYL719 administered to a cohort of 19 patients with severe complications of PROS reduced the size of vascular tumors and hemihypertrophy, alleviated heart failure, and attenuated scoliosis. In addition, parents of 2 patients with PROS reported improvement in behavior and cognitive functions, although none of the patients was reported to have epilepsy, and the potential effect of BYL719 on seizures could not have been evaluated. In summary, targeted therapy could in future significantly modify disease trajectory of patients with mTORpathies; however, we still have a long way to go.

There are 2 tertiary centers for complex and severe epilepsies and epilepsy surgery in the Czech Republic, Motol University Hospital in Prague, being one of them. Therefore, we preferentially see patients with MCD associated with drug-resistant epilepsies, and this might have introduced a bias toward more severe cases. In our cohort, we achieved a diagnostic yield of 21%. Given that the cohort is a selected one, we cannot infer a true incidence, prevalence, and an actual diagnostic yield of genetic testing in MCD. However, the diagnostic yield we observed corresponds to that of the total cohort of 737 patients investigated by the international Neuro-MIG consortium—they report diagnostic yield of 15%–37% among all the centers involved.[4] Regarding somatic testing, the proportion of diagnosed cases is lower than reported by Sim et al. who detected causal somatic variants in 31.9% of 446 tissue samples from 232 patients with various histopathologic diagnoses. However, in their cohort, they also included patients with epilepsy-related tumors, hippocampal sclerosis, and other etiologies; 160 patients were diagnosed with MCD.[6] By contrast, our surgical cohort comprised a smaller number of patients with MCD only. The proportion of mutation-positive samples varies with the diagnosis and with the methodology applied—in a most recent report, somatic WES led to detection of PIK3CA-AKT-mTOR pathway pathogenic variants in 75% of patients with hemimegalencephaly, 29% with FCD type II, and 8% of MOGHE samples carried likely pathogenic variants in *SLC35A2*. In addition, pathogenic or likely pathogenic variants in *CASK*, *KRAS*, *NF1*, and *NIPBL* were detected in patients with FCD type I.[35] In our center, we began to introduce somatic testing in diagnostic workup in 2019, and the numbers of patients examined continue to increase. In addition, somatic sequencing of brain tissue samples obtained in epilepsy surgery involves certain technical caveats. First, variant allele frequency (VAF) of mutated allele ranges from <1% (mTOR pathway genes) to 32% (*SLC35A2*) in bulk tissue, and the highest VAF (>40%) was observed in dysmorphic neurons and balloon cells dissected from tissue samples by laser microdissection.[36] Therefore, bulk tissue sequencing may result in a lower detection rate of pathogenic variants if the sequenced tissue sample contains only few affected cells.

Our analyses also found no significant difference between the diagnostic yield of TGP testing, WES-based virtual panel, and open WES. Intuitively, one would expect a higher sensitivity of the more comprehensive approaches, such as WES-based virtual panel or open WES. We presume this result reflects an important, albeit clinically inevitable, limitation of our study. Initially, patients were investigated with TGP testing, and because of its limited availability, we included predominantly well-selected patients in whom we expected genetic etiology. As genetic testing, especially WES, became more available, we started including a wider spectrum of patients, to eliminate selection bias. The selection bias might have accounted for the fact that TGP achieved a relatively higher diagnostic yield (26% compared with open WES–21% and WES-based VP–10%), and therefore, we observed no statistically significant difference between TGP and open WES and/or WES-based VP. In addition, some patients underwent more than 1 method of genetic testing; the results account for the method that led to the final result. On the other hand, lower price and increased availability of WES effectively eliminated NGS-based TGP testing from clinical practice, and WES-based virtual panels with open WES analysis are now indicated as the first-choice diagnostic method.[4]

Our study is based on data obtained in real-life clinical setting; therefore, it reflects the limitations we face in diagnostic process of patients with MCD. Currently available bioinformatic pipelines may not provide reliable results of CNV analysis from WES data. We aimed to minimize this potential diagnostic gap by aCGH testing in all patients in whom large CNVs might explain their phenotype (especially those with PVNH and polymicrogyria[37,38]). In addition, standard bioinformatic pipelines reliably detect small indels. For somatic gene testing, no additional validation method was available for patients with VAF below 10%, e.g., digital droplet PCR or amplicon sequencing. Variants with VAF>10% were

confirmed by standard Sanger sequencing. However, all reported variants were clearly visible in BAM files that showed high sequencing depth and no strand bias.

In summary, our study provides evidence that NGS-based genetic testing represents a viable and irreplaceable diagnostic tool for patients with MCD, surgical and nonsurgical alike. We need a more standardized and streamlined approach to increase diagnostic yield and to narrow time gap from the first presentation to diagnosis. For somatic sequencing, introduction of somatic WES, in combination with detailed histopathologic characterization, could lead to discovery of novel genes, previously unknown in MCD, as has been the case with germline WES and HPO analysis.[15] From a clinical perspective, detection of somatic variants from cell-free DNA circulating in cerebrospinal fluid would significantly contribute to presurgical diagnostic process of patients with focal MCD and drug-resistant epilepsy.[39] Overall, a more comprehensive understanding of genetic and epigenetic causes of MCD could take us on a journey toward gene therapy for these extremely complex and fascinating patients.

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Disclosure

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Appendix 1 *(continued)*

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