Molecular diagnostics in drug-resistant focal epilepsy define new disease entities

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Abstract
Structural brain lesions, including the broad range of malformations of cortical development (MCD) and glioneuronal tumors, are among the most common causes of drug-resistant focal epilepsy. Epilepsy surgery can provide a curative treatment option in respective patients. The currently available pre-surgical multi-modal diagnostic armamentarium includes high- and ultra-high resolution magnetic resonance imaging (MRI) and intracerebral EEG to identify a focal structural brain lesion as epilepsy underlying etiology. However, specificity and accuracy in diagnosing the type of lesion have proven to be limited. Moreover, the diagnostic process does not stop with the decision for surgery. The neuropathological diagnosis remains the gold standard for disease classification and patient stratification, but is particularly complex with high inter-observer variability. Here, the identification of lesion-specific mosaic variants together with epigenetic profiling of lesional brain tissue became new tools to more reliably identify disease entities. In this review, we will discuss how the paradigm shifts from histopathology toward an integrated diagnostic approach in cancer and the more recent development of the DNA methylation-based brain tumor classifier have started to influence epilepsy diagnostics. Some examples will be highlighted showing how the diagnosis and our mechanistic understanding of difficult to classify structural brain lesions associated with focal epilepsy has improved with molecular genetic data being considered in decision making.

KEY WORDS
DNA methylation, focal epilepsy, malformation of cortical development (MCD), somatic mutation, structural brain lesion

1 | MOLECULAR DIAGNOSTICS—LESSONS LEARNED FROM BRAIN TUMORS

Histopathological diagnosis of brain tumors is robust for many entities. Examples of clearly recognizable primary intracranial tumors through standard staining and immunohistochemistry are the very most frequent meningioma and glioblastoma. However, recent developments have taught that other entities believed to be well established needed significant adjustments. Upon in-depth molecular analysis, the so-called primitive
neuroectodermal tumors (PNET) proved a mixed bag (1) so that this entity was entirely purged from the WHO classification. Medulloblastomas (2) and ependymomas (3) received an overhaul and by now are classified by their genetic features in many places.

A major driving force behind the current dynamic was the recognition of epigenetic profiling as a powerful tool for classification, ultimately leading to the development of the brain tumor classifier (4). This tool employs a fraction of CpG sites encoded on a commercially available chip which exhibits best discrimination between predefined reference groups of tumors. The approach is assumed to favor a positive bias for differentiation-specific over tumor-specific methylation statuses explaining why the tool’s strength is in classification and only to a lesser degree in grading. Major impact has been exerted by the novel options of analyzing large sets of methylation profiles generated in different sites. Therefore, the pool of comparable cases has dramatically increased by now reaching over 50,000 cases, resulting in the detection of many novel tumor entities. In contrast to previous approaches, usually starting with morphological parameters, an increasing number of novel tumor entities are now identified by unique genetic features with the detection of potential pathological hallmarks and clinical characterization to follow. Examples of novel tumor entities or variants detected on grounds of DNA methylation patterns are diffuse glioneuronal tumors with oligodendroglioma-like features and nuclear clusters (DGONC; (5)), primary mismatch repair-deficient IDH-mutant astrocytoma (IDG; (6)), primary intracranial spindle cell sarcoma with rhabdomyosarcoma-like features DICER1-mutant (SCS-DICER1; (8)), and others (Figure 1). Noteworthy is that these novel entities frequently are morphologically quite heterogeneous and, therefore, evaded detection in the pre-molecular era.

The other development in brain tumor classification is emphasizing pathognomonic entity-specific mutations. This process started with WHO defining astrocytoma IDH-mutant and oligodendroglioma IDH-mutant and 1p/19q co-deleted. More recently added entities include solitary fibrous tumor with the NAB2-STAT6 translocation (9), supratentorial ependymoma with either RELA- or YAP fusions (3), medulloblastomas with mutations in the WNT or SHH pathways and high-grade gliomas with H3 mutations (10). Interestingly, this list now also includes low grade glial, glioneuronal, and neuronal tumors such as pilocytic astrocytoma exhibiting KIAA1549-BRAF fusions (11), papillary glioneuronal tumor with PKRCA fusions (PGNT; (12)), polymorphous low-grade neuroepithelial tumor of the young with FGFR2/3 fusions (PLNTY; (13)), and extraventricular neurocytoma with FGFR1-TACC fusions (14).

Focal lesions underlying epilepsy have extensively been studied by histology. However, morphological hallmarks for the distinction of malformations or low-grade glioneuronal tumors proved difficult to establish due to the frequently subtle nature of the changes. Therefore, the recent advances in the molecular characterization of brain tumors and especially lower grade lesions will exert great influence on the research of brain malformations. By now, both developments, methylation-based analysis, and DNA- or RNA sequencing, are actively employed in the research of epilepsy-associated brain lesions.

2 | GENETIC VARIANTS IN EPILEPSY-ASSOCIATED GLIONEURONAL TUMORS

Low-grade epilepsy-associated tumors (LEATs) represent mostly benign brain tumors associated with drug-resistant epilepsy. They consist primarily of neuronal and mixed neuronal-glial variants. Characteristic entities comprise the ganglioglioma (GG), the dysembryoplastic neuroepithelial tumor (DNET), the gangliocytoma (GC), the astrocytoma IDH-mutant, the oligodendroglioma IDH-mutant, the ependymoma, the low-grade glioma, and the meningioma. These tumors are characterized by a complex genetic landscape with chromosomal abnormalities, copy number changes, and somatic mutations.

The most common genetic alteration in LEATs is the IDH1/2 mutation, which is found in approximately 70% of cases. The IDH1/2 mutation leads to the conversion of isocitrate to 2-hydroxyglutarate, an epigenetic modifier that can accumulate in cells and lead to the hypermethylation of DNA and the inhibition of histone deacetylases, contributing to the development and progression of tumor cells. Other genetic alterations include amplifications of the FGFR1 and FGFR2 genes, which are involved in the MAPK and hedgehog signaling pathways, respectively, and are related to the development of low-grade gliomas.

Another important genetic variant in LEATs is the 1p/19q codeletation, which is found in approximately 20% of cases. The 1p/19q codeletation leads to the loss of function of the tumor suppressor genes PTEN and CDKN2A, respectively, which are involved in the regulation of cell proliferation and apoptosis. The loss of these genes is associated with an increased risk of tumor development and progression.

In addition to these genetic alterations, LEATs also show microRNA (miRNA) dysregulation, which is related to the development of tumor cells. MiRNAs are small non-coding RNA molecules that regulate gene expression by binding to the 3′-untranslated region of messenger RNA (mRNA), leading to the degradation of the mRNA or inhibition of its translation. The dysregulation of miRNAs is associated with the development of tumor cells and can be used as biomarkers for the diagnosis and prognosis of tumors.

The identification of genetic variants in LEATs is important for the development of targeted therapies. For example, the use of inhibitors of the MAPK and hedgehog signaling pathways can be effective in the treatment of tumors with amplifications of the FGFR1 and FGFR2 genes. The use of inhibitors of the PTEN/mTOR and CDKN2A/p16 pathways can be effective in the treatment of tumors with loss of function of these genes.

In conclusion, LEATs are complex tumors with a diverse genetic landscape. The identification of genetic variants in LEATs is important for the development of targeted therapies and personalized medicine. The understanding of the genetic landscape of LEATs is also important for the development of biomarkers and the identification of novel therapeutic targets.
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these patients (16). Another characteristic feature is their frequent localization in the temporal lobe (17, 18). While for common glial entities, the diagnostic criteria and parameters determining the clinical outcome are well established, and the situation is more difficult for LEAT. For GG and DNET, the neuropathological stratification is particularly complex with high inter-observer variability (19). This is resembled by inconsistent diagnose frequencies, showing variations from 6% to 49% for GG and 7% to 80% for DNT in large series. Striving for more reliable diagnostic approaches, recent studies started investigating the molecular background of LEAT. The molecular-genetic landscape of LEAT is different from other tumor types. They lack the IDH1/2 mutations as well as 1p/19q co-deletions commonly found in high-grade gliomas. Instead, genetic drivers include BRAF V600E mutations mainly in GG and FGFR1 mutations in DNET (17, 20). Gene expression studies identified alterations in the RAS–RAF–MAPK, and PI3K–AKT–mTOR signaling pathways, which affect cell proliferation and differentiation, autophagy, protein synthesis, and cell survival. BRAF V600E mutations have been negatively associated with recurrence-free survival in WHO grade I pediatric GG showing the high relevance of molecular-genetic markers for patient management and disease prognosis (21). However, the pathophysiological mechanisms that give rise to epileptic seizures in brain tumor patients remain to be fully elucidated. Koh et al. recently developed the first mouse model of GG, in which somatic BrafV637E mutations (orthologous to human oncogenic BRAF V600E) in neural progenitors during embryonic brain development recapitulated histopathological features of GG and lead to epileptic seizures 4 weeks postnatally, independent of mTOR signaling (22). Consistent with this finding, Goz et al. showed that BRAFV600E expression in neural progenitor cells resulted in hyperexcitable phenotypes in neocortical pyramidal neurons (Figure 2; (23)). Epilepsy models of DNET are yet missing and need to be developed based on their known germline and somatic FGFR1 mutations.

3 | EPILEPSY GENETICS AND THE BROAD SPECTRUM OF CORTEXAL MALFORMATIONS

MCD is the most frequent causes of focal refractory childhood epilepsies, carrying a lifelong disability perspective and reduced quality of life (15, 16). They represent a wide range of cortical lesions resulting from derangements of normal intrauterine developmental processes and
involving cells implicated in forming the cortical mantle. The pathological features depend mainly on the defect’s timing in the developmental processes and the cause, for example, abnormal proliferation or apoptosis, neuronal migration, or layering, or differentiation. Classification of MCD has proven challenging as sometimes two or more forms of MCD may coexist (e.g., polymicrogyria/PMG may be next to nodular heterotopia/NH (44)). Also, a particular defect in corticogenesis may give rise to more than one morphological subtype of MCD (e.g., migration defects may cause lissencephaly, focal cortical dysplasia/FCD, PMG, and heterotopia/HET). Conversely, a morphological subtype of MCD may have more than one mechanism for its formation (e.g., PMG may be a consequence of proliferation and migration defects (45)).

Identifying specific gene defects has provided a deeper understanding of the mechanisms involved in brain malformations and epileptogenesis (Table 1). With the ever-increasing sensitivity of sequencing and new analysis tools available, the epilepsy-associated genome has been rapidly expanding with the identification of de novo germline variants and, more recently, somatic variants occurring during development (31, 46). In particular, many recent studies using advanced sequencing technologies have revealed that germline and low-level somatic mutations activating the GATOR1 or mTORC1 pathway account for up to ~60% of hemimegalencephaly (HME) or FCD type 2 characterized by cortical dyslamination plus dysmorphic neurons (FCD type 2A) or balloon cells (FCD type 2B) (29, 47, 48). Hyperactivation of mTOR kinase in a small subset of mouse neocortical excitatory neurons seems sufficient to cause focal seizures as well as cortical dyslamination and dysmorphic neurons that fully recapitulate the phenotypes of FCD type 2 (29, 49-51).

4 | SLC35A2 BRAIN SOMATIC MOSAICISM IN MOGHE

Beyond mTOR signaling, somatic mutations in UDP-galactose transporter SLC35A2 leading to aberrant
N-glycosylation were reported in FCD1, mMCD, and non-lesional focal epilepsy (37, 47, 52). Loss of SLC35A2 protein function abolishes the transport of UDP-galactose from the cytosol into the Golgi apparatus, resulting in the synthesis of truncated glycans in human resected tissues (37). For long only pathogenic de novo germline variants in this X-linked gene were reported causing a rare type of congenital disorder of glycosylation (CDG) where the majority of affected individuals are primarily characterized by varying degrees of neurologic impairments including intellectual disability and epilepsy, and dysmorphisms (53). Recently, somatic pathogenic SLC35A2 mutations were also identified in 9 out of 20 (45%) cases with variable mosaic rates from 7 to 52% with a mild malformation of cortical development with oligodendrogial hyperplasia (MOGHE) phenotype (38). MOGHE is a focal MCD causing childhood-onset seizures, often refractory to anti-seizure medication and therefore subjected to epilepsy surgery. It is characterized by the clusters of increased oligodendroglial cell densities, patchy zones of hypomyelination, and heterotopic neurons in the white matter (54). Because of the high frequency of SLC35A2 mutations among the MOGHE population, Bonduelle et al. hypothesized that previously published SLC35A2 cases might also belong to the MOGHE spectrum (Table 1). Histopathological reassessment using NeuN and OLIG2 staining confirmed that 17 previously reported SLC35A2 cases, indeed, showed heterotopic neurons and oligodendroglial cell clusters of high density, the two main features characteristic of MOGHE. In addition, analysis by droplet digital PCR of pools of microdissected cells from MOGHE tissue revealed a variant enrichment in clustered oligodendroglial cells and heterotopic neurons, indicating that variants likely occurred in a neuroglial progenitor cell during brain development. The histopathological enrichment of OLIG2-positive oligodendroglial cells in the white matter and identification of brain somatic SLC35A2 variants are of high specificity to MOGHE and distinguish it from mMCD, FCD1, and non-lesional focal epilepsy.

5 | BRAIN SOMATIC 1q TRISOMY

PMG is a group of highly heterogeneous and difficult to classify MCD (55, 56). Their description is primarily based on their localization (by MRI; e.g., focal, multifocal, or generalized, unilateral or bilateral symmetric/asymmetric) and correlation with clinical aspects including developmental course, growth anomalies, and dysmorphism, seizure history, family history, and genetic testing of blood for PMG-associated genes (24). Over 40 genes with germline mutations have so far been linked to PMG types and syndromes, but only very recently also brain somatic events were reported. In a series of 26 PMG patients, Kobow et al. identified a group of seven patients exhibiting a unique molecular fingerprint, including the invariable detection of a brain mosaic duplication of the entire long arm of chromosome 1 and a specific genomic DNA methylation signature. This molecular profile was associated with a combination of characteristic clinical features, including a unilateral rather than focal and isolated PMG lesion, early-onset epilepsy in the first months of life, and severe combinatorial developmental delay, thereby defining a new distinct PMG entity (35). In general, copy number variants are thought to contribute up to 5% of epilepsy cases. Smaller deletion syndromes (e.g., 22q11) had also been identified in PMG, but this was the first report of a brain somatic 1q duplication in PMG. Despite the large size of the chromosomal imbalance, none of the PMG 1q patients displayed HME in contrast to PMG patients without this genetic variant. This may be explained by the gradient distribution of 1q trisomy nuclei. About 50% of all cells in the center of the PMG lesion displayed 1q trisomy in FISH analysis, but not in adjacent architecturally normal-appearing perilesional tissue supporting mosaicism with a clear association to the malformed cortex. Intriguingly, Poduri et al. had previously reported two HME patients without PMG and also brain somatic trisomy of chromosome 1q.(34) The authors argued that the activation of AKT3, either by duplication or by a point mutation (as identified in another HME patient), contributed to hemispheric brain overgrowth. The big structural differences between the described PMG 1q and HME 1q patients in the respective studies (i.e., lesion size, cortical thickness, lamination and gyration, histomorphology) may be related to differences in mutation load and timing of appearance during development together with complex and manifold functions mediated by the vast number of genes linked to chromosome 1q.

6 | DNA METHYLATION-BASED DISEASE CLASSIFICATION IN EPILEPSY

DNA methylation profiling of lesional brain tissue became a promising new option to reliably identify disease entities, particularly for human brain tumors (4). While epigenetic research in developmental biology and cancer has been at the forefront for decades, it was not before early into the millennium that epigenetics became a research focus in epilepsy. In fact, it is only about now that epigenetics is stepping out of a niche with accumulating evidence for epigenetic pathomechanisms in epilepsy and utility of epigenetic marks for disease diagnosis and prediction of disease progression or treatment response. Just about a decade passed since the first report of gene-specific altered DNA methylation in human TLE associated with distinct histopathology, that is, hippocampal sclerosis and granule cell dispersion (57). Since then, evidence was provided for a targetable key gene regulatory
role of DNA methylation in the process of epileptogenesis and chronification of seizures (58) as well as for the etiology-dependence of such molecular findings in different animal models of epilepsy (59). This finding was validated in structural brain lesions associated with human focal epilepsy, mainly MCDs including major FCD subtypes and PMG (35, 60). These data support the development of a DNA methylation-based disease classification system also for focal epilepsy, and the high level of standardization of such an approach has great promise to reduce the substantial inter-observer variability observed in current MCD histopathological diagnostics (61). Significant limitations at this point include conceptual barriers (the field of epigenetics in epilepsy is still in its infancy), the availability of technical infrastructure at epilepsy centers around the globe, and consequently, the restricted number of samples that have yet been analyzed for DNA methylation. Collaborative research studies will be needed to address these issues and to be able to capture rare and extremely rare disease entities (62) as well as to evaluate the predictive value of DNA methylation for, for example, genotype, disease progression, or treatment response.

7 | A NEW APPROACH TO MOLECULAR DISEASE DIAGNOSIS AND FUTURE CHALLENGES

In 2016, the World Health Organization provided guidelines for making an integrated diagnosis in a subset of brain tumors. Since then, there have been periodic updates and clarifications reflecting the expanding knowledge on the molecular pathology of brain tumors, but at the same time raising a challenge in rapidly incorporating new molecular findings into diagnostic practice (45, 63). Although in epilepsyology, we are only about to start accumulating such molecular data, and the precision of identified markers has already been demonstrated, supporting an integrated diagnostic approach in structural brain lesions associated with epilepsy. Our current genetic knowledge of MCD shows some mechanistic overlap with LEAT and other cancers, which is not only limited to the mTOR pathway regulating proliferation, migration, autophagy, metabolism, and cellular functions but also points at a previously unanticipated role for RTK/MAP kinase signaling. This is in line with other findings showing that some candidate genes or genetic variants may be shared between different diseases and phenotypes (64).

Unlike the higher mutational burden often found in brain tumors (VAF thresholds usually >5% (65)), the level of somatic mosaicism in MCD can be too low to be detected by conventional sequencing technology. Accurate detection of low-level somatic mosaicism requires high-depth sequencing and advanced bioinformatic analysis followed by independent validation sequencing (e.g., digital droplet PCR or site-specific amplicon sequencing, see the review of KhoshkOOO et al. in this minisymposium), which might be challenging in most of the current clinical laboratories (66). However, similar to the benefits from the advances in cancer genomics, precise genetic diagnosis of germline and somatic mutations in FCD and MOGHE can provide better genetic consultation of childhood intractable epilepsy and potential therapeutic alternatives based on their molecular genetic profiles (67).

In addition to the sensitivity problem in detecting brain somatic mutations at low allele frequencies, there remains a limited understanding of the consequences of such mutations, particularly their interpretation concerning relevance for seizure development. In the currently accepted pathogenic mechanism for FCD2 and HME, early occurring mutations affecting a progenitor cell in the ventricular zone are associated with HME, whereas late occurring mutations are considered to cause a more focal lesion. Seizures may persist or relapse in up to 40% of patients treated with focal resections for FCD and could originate from the incompletely removed neighboring dysplastic cortex. It has been suggested that MTOR mutations originating before hemispheric cleavage could favor its passing asymmetrically to daughter cells on either side, but only one hemisphere receiving enough mutated cells to develop a macroscopically visible dysplasia (68). Alternatively, a second somatic mutational hit may have occurred, involving a different mTOR pathway gene or any of many other epilepsy genes. This second hit, not present in the resected dysplastic tissue, would be sufficient to activate epileptogenesis but not to determine an MRI-visible malformation (68). Pelorosso et al. recently showed how a somatic MTOR mutation in the dysplastic hemisphere and a systemic mosaic RPS6 mutation synergistically concurred in determining an HME/epilepsy phenotype in which seizure onset had occurred in the seemingly intact hemisphere after hemispherectomy (69).

To date, a large fraction of FCD2 and MOGHE is caused by somatic mutations that are found exclusively in the brain, restricting the molecular diagnosis to surgical or autopsy specimens. Recently two studies independently provided the proof of principle that somatic mutations may be detected in the cell-free DNA present in the CSF collected by dural puncture during surgery of patients with FCD, GG, or lissencephaly (70,71). It remains to be proven that even low-level somatic mutations can be reliably detected from CSF obtained by lumbar puncture, but—if reveals to be true—offers tremendous opportunities to obtain a genetic diagnosis before surgery and in patients that are not eligible to surgery (70). This will also allow envisioning targeted therapies acting on the affected neurobiology pathways, that is, galactose supplementation in SLC35A2 cases or mTOR inhibitors in respective FCD cases. Similarly, the identification of epigenetic
diagnostic markers, including DNA methylation profiles from peripheral blood, could promote epilepsy diagnosis and prognosis.

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Figure 2 was created with BioRender.com. It has been adapted from Koh et al. 2018 (22).

DATA AVAILABILITY STATEMENT
The original contributions referred to in Figure 1 of this review have been provided by AvD and are publicly available. This data can be found in GEO under accession numbers GSE136361 and GSE109381.

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