DIAGNOSTIC BIOMARKERS OF EPILEPSY

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ABSTRACT

Background: diagnostic biomarkers of epilepsy are objectively measurable variables associated to the development of epilepsy or the propensity to generate seizures. Identification of biomarkers could be helpful for differential diagnosis and for tailored therapeutic approaches.

Objective: this review focuses on diagnostic biomarkers of epilepsy, including genetic, serological, neuroimaging and electrophysiological variables.

Methods: references were mainly identified through PubMed search until December 2017 and backtracking of references in pertinent studies.

Results: several promising diagnostic biomarkers of epilepsy exist, with causative value or predicting liability to develop seizures after acquired brain injuries. Short non-coding RNAs are deregulated in serum and cerebral tissue of epilepsy subjects: these molecules are promising diagnostic biomarkers, being easy to assess and reproducible. Advanced imaging techniques may allow identification of subtle epileptogenic lesions, often with prognostic value. Novel electrophysiological biomarkers of epilepsy include perturbed cortical connectivity and excitability induced by transcranial magnetic stimulation, as well as high-frequency oscillations detected by intracranial and scalp electroencephalographic recordings. Finally, serological biomarkers may support the differential diagnosis between epileptic seizures and non-epileptic events.

Conclusions: ongoing research on diagnostic biomarkers of epilepsy is promising and future pre-clinical and clinical studies are warranted.

KEYWORDS: genetic, miRNA, HFO, TMS-EEG, prolactin, MRI, seizures.

1. INTRODUCTION

Epilepsy is a highly heterogeneous and multifactorial condition, for which there is a lack of reliable and validated biomarkers. Biomarkers are defined as objectively measurable variables of a biologic process, either physiologic or pathologic, that provide reliable information on the status of that specific process in a specific moment [1]. Since their presence or level correlates with a specific aspect of the process, biomarkers can be artificially divided according to their prevalent diagnostic, prognostic or therapeutic value. Diagnostic biomarkers of epilepsy are aimed to identify the existence and the entity of cerebral tissue propensity to generate seizures or epilepsy and to support differential diagnosis of epileptic seizures [2]. In the current review, we focus on diagnostic biomarkers of epilepsy, including genetic, serological, neuroimaging and electrophysiological variables, and define current unmet needs and future perspectives.

2. SEARCH STRATEGY

Publications on diagnostic biomarkers of epilepsy were reviewed. References were identified by PubMed and Scopus search until December 2017, with various combinations of the terms “epilepsy”, “seizures”, “epileptogenesis", “ictogenesis”, “genetics”, “genes”, “miRNA”, “neuroimaging”, “MR”, “hippocampal sclerosis”, “focal cortical dysplasias”, “serological”, “prolactine”, “inflammation”, “EEG”, “TMS”, “electrophysiological”, “HFO”, “biomarker”. Articles were also identified through searches of the authors’ own files. Only articles published in English were reviewed. Selection criteria were novelty, importance, originality, quality, and relevance to the scope of this review.

3. GENETIC BIOMARKERS

Genetic contribution to epilepsy is increasingly recognized, and consists of a range of different and complex mechanisms [3]. Genotype-phenotype correlation is often not straightforward, with both gain- and loss-of-function variants causing very similar phenotypes but presumably different

response to treatment [4,5]. Each person with epilepsy (PWE) has a complex genetic architecture where genetic variation may contribute to the epileptic phenotype and to develop epileptic seizures (ES) after acquired brain injuries. Some epilepsies have an established genetic etiology, either Mendelian or polygenic. Well-recognized Mendelian inheritance causes numerous focal and generalized syndromes [6]. *De novo* pathogenic single gene mutations are identified in 30–50% of patients with different epileptic encephalopathies [7]. Epilepsies with polygenic inheritance imply the involvement of multiple genes. The most common epilepsy syndromes with recognized or suspected polygenic etiology are "idiopathic generalized epilepsies" and "self-limited focal epilepsies" [8]. One of the most intriguing topic of ongoing research is the study of the influence of multiple genes on epilepsy and their interaction with brain lesions (i.e hippocampal sclerosis, HS, focal cortical dysplasia, FCD, etc) or environment (i.e. brain inflammation, trauma, etc). HS is the main cause of mesial temporal lobe epilepsy (TLE) [9]. Genetic susceptibility seems associated with the occurrence of TLE independent of underlying HS. TLE-susceptibility genetic abnormalities include single-nucleotide polymorphisms in the aquaporin-4 gene (Heuser et al, 2010), potassium channel Kir4.1 gene [10], gamma-amino-butyric acid A and B receptor subunit genes [11,12], acid-sensing ion channel 1a gene [13], serotonin-related genes [14-16], calcium homeostasis modulator 1 gene [17], and prodynorphin gene promotor [18]. Genetic variants sometimes appear to influence susceptibility to TLE depending on gender, as for the prion protein gene in males [19], or the neuregulin [20] gene in females. However, none of these findings has been replicated in larger studies [21]. Another field of increasing interest is the involvement of genes influencing inflammation pathways in epileptogenesis. Functional variants in the promoter of the complement C3 gene have been associated with susceptibility to human mesial TLE and febrile seizures [22], and a gene-regulatory network analysis has shown that sestrin 3, a stress responsive protein, acts as positive regulator of a pro-inflammatory transcriptional program in the human epileptic hippocampus [23]. Polymorphisms in pro-inflammatory cytokines genes, such as interleukin (IL) 1 α and 1 β, have been associated with TLE, HS, or prolonged febrile convulsions

[24,25]. A polymorphism of the tissue inhibitor of metalloproteinase 4 gene, encoding for an inflammation-induced apoptosis and matrix turnover factor, has been associated with susceptibility to focal epilepsy in Asian subjects [26]. Also polymorphisms in kelch-like ECH-associated protein 1 and nuclear erythroid 2-related factor 2, implicated in neuroprotection due to induction of antioxidant enzymes, have been linked to susceptibility to TLE and drug-resistant epilepsy [27]. Other genetic variants have been associated to the liability to develop ES or post-traumatic epilepsy. Both ex vivo and in vivo models have shown the role of adenosine and its A1 receptor (A1R) in modulating the severity of status epilepticus [28] and the development of post-traumatic epilepsy [29,30]. Genetic variants in apolipoprotein E [31], glutamic acid decarboxylase 1 [32], neuronal high-affinity excitatory amino acid transporter [33], IL-1β [34], and methylenetetrahydrofolate reductase [35] genes are associated with increased risk of developing post-traumatic ES or epilepsy after traumatic brain injury. Lastly,-functional single-nucleotide polymorphisms in the Cluster of Differentiation 40 [36] and in the mitochondrial aldehyde dehydrogenase 2 genes have been associated with the susceptibility to develop post-stroke epilepsy [37] in single studies. Unfortunately, most of these genetic polymorphisms are not yet of proven value as epilepsy biomarkers since some results have not been replicated and might be specific to certain populations.

4. SEROLOGICAL BIOMARKERS

Serological biomarkers of epilepsy include inflammatory proteins, hormones, enzymes and micro-RNAs (miRNAs). These biomarkers are appealing, since blood, serum, and plasma are easy to obtain.

4.1 Inflammatory proteins

Several circulating inflammatory proteins have shown to contribute to ictogenesis in preclinical models of epilepsy [2,38]. Although their utility needs to be better clarified, also in consideration of their short half-life and low specificity [2], inflammation molecules have been proposed as serological biomarkers of epilepsy. In particular, IL-6 levels influence neuromodulation and may

contribute to neuronal network excitability [39]. IL-6 levels are not only a promising biomarker of epilepsy, but may also vary as a function of seizure type and frequency. Indeed, IL-6 blood concentrations are chronically increased in epilepsy patients, especially in TLE subjects, compared with healthy controls [40,41]. Serum levels of IL-6 are significantly increased (compared to baseline) between 3 and 24h after a seizure [42]. Post-ictal peak blood concentration of IL-6 is significantly higher after tonic-clonic seizures than after focal seizures, independently from seizure duration [42,43]. Among subjects with focal epilepsies, TLE subjects show significantly higher post-ictal peak levels of IL-6. In particular, long seizure duration (i.e., >100s), low seizure-frequency (i.e., < 10 seizures/month), and low baseline (under 5 pg/ml) IL-6 blood levels have been associated to higher IL-6 serum concentration within 24-hours after a seizure, in subjects with TLE [42]. Significantly increased blood levels of IL-6 and other cytokines (i.e., IL-8, IL-1β) have been shown in subjects with drug-resistant focal epilepsy, independently from the time of their last seizure, compared to healthy controls [44]. Similarly, altered levels of other cytokines (increased IL-8 and epidermal growth factor, lower ratios of IL-1 receptor antagonist (IL-1RA)/IL-1β and IL-1RA/IL-8) have been shown in children with febrile status epilepticus compared to children with fever but not ES [45]. A lower ratio of IL-1RA/IL-6 was a strong predictor (OR 21.5, 95% CI: 1.17–393) of acute hippocampal injury in children with febrile status epilepticus [45]. A 5-fold reduction of blood levels of the anti-inflammatory molecule “telencephalin” in refractory focal epilepsy subjects has also been shown [44].

4.2 Hormones

Serum levels of various hormones have been suggested as candidates in the identification of ES. Prolactin (PRL) is the most studied and the most promising hormonal diagnostic biomarker, although its assessment should be performed very soon after a seizure. Its serum levels rise 10-20 minutes after ES and remain high for up to 2 hours, as shown by studies on serial post-ictal PRL measurements in PWE as compared to patients with psychogenic non-epileptic seizures (PNES),

and healthy controls [46]. Capillary measurement of PRL supports the differential diagnosis between all types of ES and PNES, with an approximate diagnostic sensitivity of 96% [47] and up to 100% positive predictive value for ES [48]. It is noteworthy that high PRL values, up to 3-times the baseline level, has also been found within 1 hour after vaso-vagal syncope [49,50] and that its role as diagnostic biomarker of ES has been questioned [51,52].

The role of other hormones (such as cortisol, adrenocorticotropic hormone, growth hormone, and thyrotropin-releasing hormone) has been described in methodologically heterogeneous studies leading to inconsistent results [53-58].

4.3 Enzymes

Enzymes have also been suggested as candidate biomarkers of epilepsy. In particular, creatine kinase (CK) blood levels commonly rise after generalized tonic-clonic ES, although elevated CK levels should always be interpreted cautiously, after exclusion of other clinical conditions [59,60]. Increased CK levels have 75% sensitivity, 86% specificity, 63% positive predictive value, and 91% negative predictive value for convulsive ES versus PNES [61-64]. Neuron-specific enolase (NSE) can be elevated after different types of febrile and afebrile seizures [65-68], but not after PNES [61,69]. Noteworthy, increased NSE levels may be found in other conditions (e.g. hemolytic processes) [70,71].

4.4 Micro-RNAs

MiRNAs represent an endogenous class of short noncoding RNA molecules, of about 22 nucleotides, which may play a key role in epileptogenesis and ictogenesis by regulating neuronal excitability, morphology, apoptosis and inflammation [72,73]. MiRNAs negatively control gene expression (post-transcriptional gene repression) of target mRNAs [74,75]. They are detected in both biological fluids and brain tissue, bound to proteins or encapsulated into extracellular vesicles. Circulating miRNAs can also be actively secreted from pathological tissues during a disease, and a

strong relationship between circulating and tissutal miRNAs does exist. Therefore, miRNAs represent non-invasive biomarkers, also in virtue of their stability and simple assessment [74,76-78]. Up to now, over 100 different miRNAs have been identified in animal models of epilepsy and in PWE [73,77-79]. Table 1 summarises recent studies on miRNAs as diagnostic biomarkers of epilepsy. Most studies are based on a two-phase approach. The first phase consists of the identification of the expression profile of different miRNAs in animal models of epilepsy or in small cohorts of PWE in comparison with a control group. Then, a subsequent validation phase on larger cohorts of PWE is performed. Wang et al. [80] found serum up-regulation of miR-106b-5p, -130a-3p and -146a-5p and down-regulation of miR-15a-5p and -194-5p in 117 TLE patients (regardless epilepsy etiology), compared to 112 healthy controls, with miR-106b-5p showing the highest sensitivity (80.3%) and specificity (81.2%). Sun et al. [81] found significantly higher expression of miRNA-129-2-3p in plasma samples from refractory TLE subjects, in comparison to healthy controls. Combined serum increased expression of miR-146a and miR-106b has shown a higher sensitivity and specificity in comparison to miR-146a or miR-106b alone [82]. Another study [83] found significant up-regulation of hsa-miR-4521 in serum samples of patients with refractory ES or with FCDs. Despite miRNAs are promising biomarkers of epilepsy, some issues need to be further assessed, such as their specificity for epilepsy and their association (causative vs. consequential) with seizures.

4.5 Other serological findings

The role of neuropeptides (i.e., ghrelin and nesfatin-1) is still questioned. One study [84] reported increased serum and salivary nesfatin levels after ES but not after PNES, and lower serum ghrelin levels after ES as compared to PNES. However, the levels of these neuropeptides were not assessed in healthy subjects.

5. IMAGING BIOMARKERS

Since 1990s, brain imaging techniques have been routinely applied in the evaluation of PWE [85]. In the last twenty years, a mass of abnormalities has been described in patients with epilepsy, in particular, using routine Magnetic Resonance (MRI) with specific epilepsy protocols as well as morphometric analysis, magnetic resonance relaxometry, diffusion-weighted imaging, MR spectroscopy, volumetry, voxel-based analysis and PET imaging [85-87]. Many of these abnormalities could serve as biomarkers of epilepsy [2]. The use of an optimal worldwide imaging protocol for PWE represents the basis to look for potential and specific biomarkers. MR scanning protocol for PWE must include T1-weighted imaging (for the initial definition of brain anatomy), T2-weighted imaging and fluid-attenuated inversion recovery (FLAIR) imaging for the detection of specific brain pathologies such as hippocampal sclerosis (HS), and 3-D volume acquisition sequences to allow identification of subtle abnormalities, such as malformations of cortical development [85-87]. At present, there are no pathognomonic neuroimaging markers of epileptogenicity. As an example, HS is not only a diagnostic biomarker of mesial TLE, since it may be found in elderly individuals without epilepsy, particularly in those with Alzheimer's disease [88]. The identification of neuroimaging biomarkers might have a high impact on both diagnostic and therapeutic work-up. Engel et al. [1] suggested that a first step to identify potential biomarkers for pharmacoresistance may be to classify several well-defined epilepsy syndromes that are associated with drug resistance but in which there are also patients that are well controlled. In this way, the cohort of patients with mild mesial temporal lobe epilepsy (MTLE), a common and often unrecognized clinical entity with onset in adulthood and good response to the medications [89], symbolizes an ideal epileptic syndrome to be studied with imaging as potential diagnostic/prognostic biomarker. We recently showed that mild MTLE remained drug-responsive in about three-fourths of patients and became refractory in the remaining one-fourth during a mean follow-up > 11 years [90]. In this population, earlier age at onset, history of febrile convulsions and the presence of HS on MRI, represented early prognostic biomarkers of drug-refractoriness [89,91]. Using advanced MRI technique [92-94], we further showed a significant reduction of fractional

anisotropy along the white matter of the temporal lobes in drug-resistant MTLE, implying that it as a valuable biological marker of refractoriness [95]. Afterwards, we extended these findings and showed diffusion abnormalities and reduced cortical thickness of the corpus callosum only in patients with refractory MTLE, suggesting that differences in the distribution of such alterations might represent a biomarker of refractoriness [96]. Advance of ?Brain imaging has dramatically helped to identify subtle and occult epileptogenic lesions and, thus, to define the etiology of otherwise "cryptogenic" epilepsies. In particular, MRI has contributed significantly to identify cortical malformations and encephaloceles [97-100]. Focal cortical dysplasias (FCDs) are the most common developmental pathologies in children with extratemporal ES and MRI can help to differentiate among FCD subtypes with diagnostic and prognostic implications [97,99,101]. Small encephalocele, which may remain occult without careful investigations, is an increasingly recognized cause of epilepsy [98,102]. In a case-control study [100], occult temporal encephalocele was found with targeted MRI in 5% of TLE patients and in none of 151 healthy controls, therefore representing a promising biomarker of epileptogenicity.

6. ELECTROPHYSIOLOGICAL BIOMARKERS

The role of electroencephalogram (EEG) in the diagnosis of ES is well-known [103]. The use of transcranial magnetic stimulation (TMS) combined with electromyography as diagnostic biomarker of epilepsy has been already described [104-106]. In the following sections, the role of advanced electrophysiological analysis (i.e., combined TMS-EEG recordings and identification of high-frequency oscillations, HFOs) in the diagnostic workup of PWE will be detailed.

6.1 TMS/EEG

EEG is an established tool in PWE that can provide useful information on cortical excitability: its diagnostic application mainly relies on visual inspection and interpretation. TMS is a non-invasive brain stimulation technique that is able to induce local cortical excitation by electromagnetic induction at specified locations properly targeted with an integrated navigation system. TMS has

initially been applied to the primary motor cortex, thus evoking motor-evoked potentials and, consequently, the appearance of a stereotyped movement. The development of TMS-compatible EEG amplifiers has allowed to record TMS-evoked cortico-cortical potentials, i.e. the electrical brain responses to direct cortical stimulation [107]. Although this approach is technically challenging [108], it allows to investigate the reactivity (i.e. excitability and connectivity) of the whole brain to a focal stimulation delivered over an arbitrary cortical site which can be located outside the primary motor cortex [109-112]. A few studies have performed TMS-EEG in patients with focal [113,114] or generalized [115,116] epilepsies. Valentin et al. [113] have explored the appearance as well as the lateralizing and localizing value of EEG responses to single-pulse TMS applied on different scalp regions in 15 patients with focal epilepsies compared to 15 healthy volunteers. These authors found that late EEG responses, in terms of single epileptiform abnormalities or changes in baseline activity, were evoked in 11/15 PWE and none of the controls and were localizing in most subjects, even in those with normal baseline EEG. TMS-EEG was also applied to the study of patients with periventricular nodular heterotopia. In those patients, late cortical responses were evoked not only in the proximity of lesions, but also in functionally connected regions [114]. These data suggest that late responses to magnetic pulses, typically registered 100-1000 msec after stimulus, may represent a useful biomarker of increased cortical excitability and connectivity in patients with focal epilepsy. TMS-EEG may significantly contribute to epilepsy diagnosis and to the localization of epileptogenic focus also in pre-surgical evaluations. With regard to generalized epilepsies, a study [115] explored the effect of sleep deprivation on EEG activity after TMS in patients with Juvenile Myoclonic Epilepsy and healthy controls. A significant increase in late peak amplitudes (100-190 ms after stimulus) in response to single TMS pulses over motor areas was observed in patients and controls during the sleep-deprived condition, with different topographical distribution (anterior spread) and higher amplitude potentials in patients as compared to controls. In another phase II study [116] a paired-pulse TMS-EEG protocol was applied at rest, during and after hyperventilation and tested for diagnostic accuracy in 25 patients

with various idiopathic generalized epilepsies (both drug-responsive and drug-resistant) and 11 controls. Features extracted from multi-level analyses of EEG allowed a global diagnostic accuracy of 0.84 for the classification “patients vs. controls” and 0.76 for the classification “resistant vs. non-resistant epilepsy”. These studies highlight that TMS-EEG is able to discriminate between healthy controls and PWE, so that the role of this technique as a diagnostic biomarker in epilepsy seems promising. The main limitations of TMS-EEG are the necessity of a dedicated device combining high-density EEG and TMS, technical issues mainly due to handling of artifacts (stimulus artifacts and sensory evoked potentials), long duration of experiments and difficulties in elaboration and interpretation of EEG responses. So far, these limitations have confined TMS-EEG to research laboratories only, preventing its use in clinical practice. Recently, open source software using MATLAB language have been developed and are helpful both in artifact removal and in signal processing and analysis [117]. The reduction in machinery costs and the overcoming of technical issues will hopefully lead to standardization and larger use of this promising technique in the near future.

6.2 HFOs

HFOs are defined as EEG events characterized by at least four oscillations, which undoubtedly stand out from the background activity and having frequency ranging between 80 and 500 Hz. HFOs are classified as ripples (from 80 to 250 Hz) and fast ripples (> 250 Hz), depending on the HFO frequency range. HFO marking is time consuming and nowadays several automatic detection programs are available. To have a complete view on how to record HFOs in epilepsy, refer to Zijlmans et al. [118]. The applications of HFOs detection are expanding over the years, ranging from the identification of seizure onset zone (SOZ) to the assessment of epilepsy severity and monitoring of antiepileptic treatment. According to Jacobs et al. [119], sensitivity in the identification of SOZ (using a pre-set threshold of specificity of 95%) is 52% for fast ripples, 37% for ripples and 33% for spikes in sleep invasive EEG recordings. Ripples co-occurring with a spike

may be even more strictly related to the SOZ than ripples without a spike [120,121]. HFOs increase just immediately prior or at the onset of a seizure [122,123]. Whereas HFOs are confined to the same epileptogenic area during ictal and interictal periods, spikes are more widespread during seizures than interictally [124]. Resection of cortical areas with pre-surgical high rate of HFO is linked to a better post-surgical outcome than resection of area with low HFO rate [125-129]. HFO rate increases after medication reduction suggesting that it is tightly linked to seizure occurrence [130]. The role of HFOs in differentiating epileptogenic lesions including HS, FCD, nodular heterotopia, polymicrogyria and tuberous sclerosis complex is controversial [131-133]. HFOs can also be recorded from scalp EEG. HFOs activity on scalp recordings was described at seizures onset in epileptic encephalopathies, such as epileptic spasms in children [134,135] and tonic seizures in Lennox–Gastaut syndrome [136]. HFOs were also recorded in children with electrical status epilepticus during slow wave sleep [136] and in adults with focal epilepsy [137]. Finally, HFOs recorded from scalp EEG may be helpful in the lateralization of the epileptic focus in focal to bilateral convulsive seizures, although they do not differentiate between “primary” and “secondary” bilateral synchrony [138].

7. CONCLUSIONS

Numerous studies demonstrate the existence of promising biomarkers in epilepsy. Susceptibility genes are related to polygenic predisposition to epilepsy and represent a new research field in genetics of epilepsy. Promising serological biomarkers of epileptogenicity include inflammation molecules and miRNAs. Hormones, enzymes and neuropeptides serum levels are easy to assess and represent reproducible biomarkers supporting the differential diagnosis between ES and non-epileptic events. Neuroimaging techniques may allow identification of subtle epileptogenic lesions, with diagnostic and prognostic value. Perturbation of cortical connectivity and excitability by TMS and detection of HFOs are promising innovative electrophysiological biomarkers of epileptogenicity. In addition, they are useful to accurately identify SOZ. Future pre-clinical and

clinical studies are warranted to strengthen the role of these biomarkers in supporting routine clinical practice.

CONFLICT OF INTEREST:

Dr. Sara Gasparini is currently working with a research grant co-funded by Biogen s.r.l. Dr Simona Balestrini was supported from the Epilepsy Society and The Muir Maxwell Trust. Other authors declare that they have no conflict of interest.

ACKNOWLEDGMENTS

Chiara Sueri, Sara Gasparini, Umberto Aguglia, Edoardo Ferlazzo: conceived the study, performed the study, collected data, drafted and revised the manuscript.

Simona Balestrini, Angelo Labate, Antonio Gambardella, Emilio Russo, Antonio Leo, Silvia Casarotto, Francesca Pittau, Michele Trimboli, Vittoria Cianci, Michele Ascoli, Salvatore M. Cavalli and Giulia Ferrigno: performed the study, drafted and revised the manuscript.

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Table 1. Recent studies assessing miRNAs as diagnostic biomarkers in epilepsy.

miRNAs	Expression	Clinical studies	Human samples	Preclinical studies
miR-106b-5p [80]	Up-regulated	Multiphase case-control study on 147 PWE and 142 controls	Serum	None
miR-301a-3p [139-141]	Down-regulated	Multiphase case-control study on 107 patients with refractory epilepsy, 111 with responsive epilepsy and 85 controls	Serum	TLE rat model (lithium-pilocarpine model) and status epilepticus rat model evoked by amygdala stimulation
miR-129-2-3p [81,140]	Up-regulated	Multiphase case-control study on 25 patients with refractory TLE and 25 controls	Cortical brain tissue and plasma	Sstatus epilepticus rat model evoked by amygdala stimulation
miR-4521 [83]	Up-regulated	Randomized controlled study on 9 patients with refractory TLE (with HS) 8 controls	Cortical brain tissue and serum	None
miR-146a and miR-106b [82,142]	Up-regulated	Case-control study on 90 subjects with symptomatic, idiopathic or cryptogenic epilepsy and 90 controls	Serum	TLE rat model evoked by hippocampal electrical stimulation
miR-146° [142,143]	Up-regulated	Case control study on 10 patient with refractory TLE (6 with HS) and 5 controls	Cortical brain tissue	TLE rat model evoked by hippocampal electrical stimulation