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Review

MicroRNAs as biomarkers and treatment targets in status epilepticus

Elizabeth Brindley [a], Thomas D.M. Hill [a,b], David C. Henshall [a,b,*]

[a] Department of Physiology and Medical Physics, Royal College of Surgeons in Ireland, Dublin, Ireland
[b] FutureNeuro Research Centre, RCSI, Dublin, Ireland

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ABSTRACT

Microribonucleic acids (miRNAs) are short noncoding ribonucleic acids (RNAs) that have been proposed as potential biomarkers for epilepsy, acute seizures, and status epilepticus. Various properties support their potential in this regard, including relative stability and amenability to rapid quantitation in biofluids. Several miRNAs are enriched in the brain and within specific cell types. Dysregulation of miRNAs has been reported in brain regions damaged by status epilepticus and in resected brain tissue from patients with drug-resistant epilepsy. Silencing miRNAs using antisense-like oligonucleotides termed antagomirs has been reported to suppress evoked and spontaneous seizures in animal models, indicating therapeutic applications. The prospect of miRNAs as mechanistic biomarkers is supported by recent studies showing blood levels of brain-enriched miRNAs increase after status epilepticus in rodents, and clinical studies have identified miRNAs upregulated in human cerebrospinal fluid after status epilepticus. It remains unproven, however, whether there are miRNAs that uniquely identify acute seizures, chronic epilepsy, or the process of epileptogenesis. Finally, efforts have turned to the challenge of proving that some of the circulating miRNAs actually originate from the brain. New models that feature a biochemically-labeled protein involved in miRNA function and restricted to specific brain cell types offer opportunities to resolve this issue. This review summarizes recent progress on miRNAs as diagnostic biomarkers of status epilepticus and considers some of the unanswered questions and future directions.

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1. Introduction

There is a major unmet need for biomarkers of status epilepticus and acute seizures to support the identification of individuals at risk of epilepsy development, to confirm nonconvulsive seizures, and to support differential diagnosis (e.g., patients with psychogenic nonepileptic seizures) [1,2]. Molecular biomarkers are particularly attractive since they offer the possibility to support diagnosis through minimally invasive tests of biofluids such as blood. A number of molecules have been explored to date, including proteins and ribonucleic acids (RNAs), but most have lacked specificity, sensitivity, or are impractical to measure in a point-of-care setting. Then, about 10 years ago, a report was published that showed changes to microRNA (miRNA) levels in the blood after status epilepticus in rats [3]. This finding, and the emergence of evidence that the same molecules could be targeted for the prevention of seizures, has driven efforts in multiple laboratories, including our own, to explore the potential use of biofluid miRNAs for the diagnosis of epilepsy, acute seizures, or to pinpoint an epileptogenic event. This review summarizes recent findings and some currently unpublished work on the potential use of biofluid miRNAs as diagnostic biomarkers and looks to the future at some of the applications and unanswered questions.

2. What are miRNAs and what do they do?

MicroRNAs are a class of short (or small) noncoding RNAs that perform a key posttranscriptional function, reducing protein levels by targeting protein-coding messenger RNA (mRNA) [4]. They help to buffer and confer precision to cellular protein noise [5]. The mature (functional) miRNAs are ~20 nucleotides in length but they begin as longer primary transcripts (pri-miRNAs) that include a hairpin loop structure. In the nucleus, they are cleaved to form a pre-miRNA, which is then exported to the cytoplasm for a second cleavage event mediated by the enzyme Dicer [6]. This produces a double-stranded miRNA molecule; the mature miRNA is derived from either the -3p or -5p arm of the remnant of the hairpin loop. One of the strands is selected and loaded into a binding pocket within the Argonaute family of proteins [7,8]. The Argonaute-miRNA complex (termed RISC; RNA induced silencing complex) then traffics along mRNAs seeking regions of base-pairing complementarity [9]. Where there is a region of 7–8 nucleotides

* Corresponding author at: Department of Physiology & Medical Physics, Royal College of Surgeons in Ireland, 123 St. Stephen's Green, Dublin D02 YN77, Ireland.
E-mail address: dhenshall@rcsi.ie (D.C. Henshall).

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complementarity between the miRNA and mRNA, the RISC complex halts, and this promotes mRNA destabilization or translational inhibition. This will lower protein levels in cells. As targeting requires only a 7–8 nucleotide match, an individual miRNA can potentially target many mRNAs [4,5].

The brain is the most miRNA-enriched organ in the body, expressing more than half of the known miRNAs [10–12]. By interfering with miRNA biogenesis using genetic tools, researchers have shown that miRNAs are required for brain development, cell differentiation, neurogenesis, and synaptic plasticity, among other functions [13,14]. Selective deletion of the Dicer enzyme from neurons in the adult mouse brain leads to neurodegeneration and, within a few weeks, is fatal [15]. One of the best understood functions of miRNAs is the regulation of local protein synthesis at synapses. By controlling levels of proteins involved in the expansion and retraction of dendrites, miRNAs have a potent influence on synaptic strength [13,16].

3. Why miRNAs as biomarkers?

There are several reasons to think that miRNA could be molecular biomarkers of brain insults, such as status epilepticus, acute seizures, or epilepsy. First, they are quite stable in biofluids, resistant to pH, and freeze–thaw cycles and other processing steps that can render other molecules such as mRNA difficult to work with and less reliable [17,18]. The stability of miRNAs derives, at least in part, from their enclosure in microparticles such as exosomes [19,20] and retained physical attachment to the Argonaute protein [21]. This is not to say, however, that reproducible detection and quantitation of miRNA in biofluids are trivial. There are multiple factors that should be standardized in order to produce reliable results [22,23]. Second, there are multiple platforms for their detection. This includes polymerase chain reaction (PCR)-type assays for individual miRNA, as well as genome-wide arrays, RNA sequencing, and the Nanostring platform which perform direct copy number analysis [24,25]. Several teams have also adapted these technologies or developed new methods for rapid direct detection of miRNAs in biofluids [26]. Our group and collaborators have developed a centrifugal microfluidic device that can process and directly detect levels of three miRNAs using an electrochemical approach within about an hour [27]. Others are developing other similar devices.

Beyond these technical factors, multiple miRNAs are enriched in the brain, and some are unique to specific cell types and subtypes [12,28]. The detection of a specific brain-enriched miRNA or a number of brain-enriched miRNA, perhaps in a particular combination, within a biofluid would therefore be an indicator of a brain injury. For example, a 50:20:20:10 ratio of neuron:astrocyte:microglia:oligodendrocyte miRNA could be unique to an epileptogenic injury or the chronic epileptic state [29]. We know that multiple miRNAs are dysregulated in brain tissue after status epilepticus in rodents and in resected tissue from patients with refractory epilepsy [30,31]. Finally, multiple studies have shown that miRNAs can be targeted to produce effects on seizures [32]. The most common way of doing this is by designing an antisense oligonucleotide sequence complimentary to a stretch of the mature miRNA. These “antagomirs” bind and prevent the function of the target miRNA [31]. We showed that inhibiting one of the miRNAs involved in local control of synaptic structure, miR-134-5p, could reduce evoked and spontaneous seizures in rat and mouse models [33–35]. Other teams have reported results targeting this, and other miRNAs, and there is significant interest in the development of a miRNA-based therapy for epilepsy [32]. Taken together, these studies strongly support the potential of miRNAs as mechanistic biomarkers. But what is the evidence so far?

4. Recent studies on miRNAs as biomarkers of status epilepticus

The first miRNA investigation to sample a biofluid after status epilepticus found a set of up- and downregulated miRNAs in the blood of rats after kainate seizures [3]. They found 10 upregulated miRNAs in blood after seizures, and some of the same miRNAs were upregulated by other known epileptogenic injuries [3]. Other studies have followed which examined specific miRNAs in blood after seizures in rodents [36,37]. Probably, the most comprehensive study on the miRNA profile in blood after status epilepticus to date was reported by Roncon and colleagues [38]. This study identified time specific changes to a number of miRNAs in the blood of rats after pilocarpine-induced status epilepticus. Among several important findings, they identified an early spike in blood levels of miR-9-3p, one of the most abundant brain miRNAs, after status epilepticus. This fits with a hypothesis in which an early insult triggers the release of brain-enriched miRNAs into the blood that could be a useful biomarker of epileptogenesis. Indeed, the levels of miR-9-3p returned to baseline at later time points, including in the epileptic rats. Several other studies have also reported changes to brain-expressed miRNAs in rodent studies [29].

We and our collaborators recently completed a comprehensive analysis of miRNAs in plasma at multiple time points in three rodent models of status epilepticus [39]. This identified a set of miRNAs which were up- and downregulated in several models, including both electrically stimulated and pharmacologically induced epilepsy. During the validation phase, however, few of the miRNAs were found to be unique to the epileptogenesis phase. That is, while the miRNA level in plasma changes shortly after status epilepticus, it remained elevated into the phase of recurrent spontaneous seizure and was not, therefore, a specific biomarker of the epileptogenesis phase. Nevertheless, several of the miRNAs identified in this project show promise as biomarkers of epilepsy. For example, their levels were unaltered by anticonvulsant drugs administered to the rodents, whereas injection of a disease-modifying therapy (antagomir targeting miR-134) returned levels of the biomarkers to baseline. This argues that levels of the miRNAs reflect the enduring underlying pathophysiology rather than being acute responses to seizures. Several of the same miRNAs were found to be altered in plasma from patients with focal intractable epilepsy. Again, levels did not appear to be altered by recent seizures indicating they were biomarkers of the underlying epilepsy and not recent ictal activity.

We know very little about how biofluid miRNAs change after status epilepticus in humans. As part of a recent study on three miRNA biomarkers of epilepsy, we included samples from patients with status epilepticus [40]. Generally, levels of the miRNAs followed a similar pattern to that found in people with epilepsy but small sample sizes limited meaningful insight. There is a need, therefore, for a well-designed, adequately-powered study to analyze blood miRNAs after status epilepticus in patients. In contrast, there has been a major human study focused on miRNAs in cerebrospinal fluid (CSF) after status epilepticus. Cerebrospinal fluid has the advantage of being in closer proximity to the area of tissue pathology but the disadvantage is that it is not routinely sampled and requires an invasive lumbar puncture. We performed genome-wide analysis of miRNAs in a set of 15 CSF samples from people who experienced status epilepticus, comparing results to people with TLE and controls (in this case, CSF from people who were admitted to hospital with a headache) [41]. This study used the OpenArray profiling platform, a genome-wide PCR-based technology that allows rapid screening of miRNAs. Fifteen miRNAs were found to be differentially expressed in CSF samples from people who had experienced status epilepticus. This included some high abundance brain-enriched miRNAs such as miR-9-3p. From the set, two in particular were highly upregulated: miR-451a and miR-21-5p (Fig. 1a). Levels of both were higher in people following status epilepticus than in both people with temporal lobe epilepsy (TLE) and controls, and miR-451a levels were also different compared to CSF samples from people with other neurological diseases (mainly multiple sclerosis and Alzheimer's disease) [41]. These differences were robust, and reproduced using other methods of detection including digital PCR (Fig. 1b). Using statistical analysis, the combination of the two miRNAs could distinguish people following status epilepticus from the other groups with high

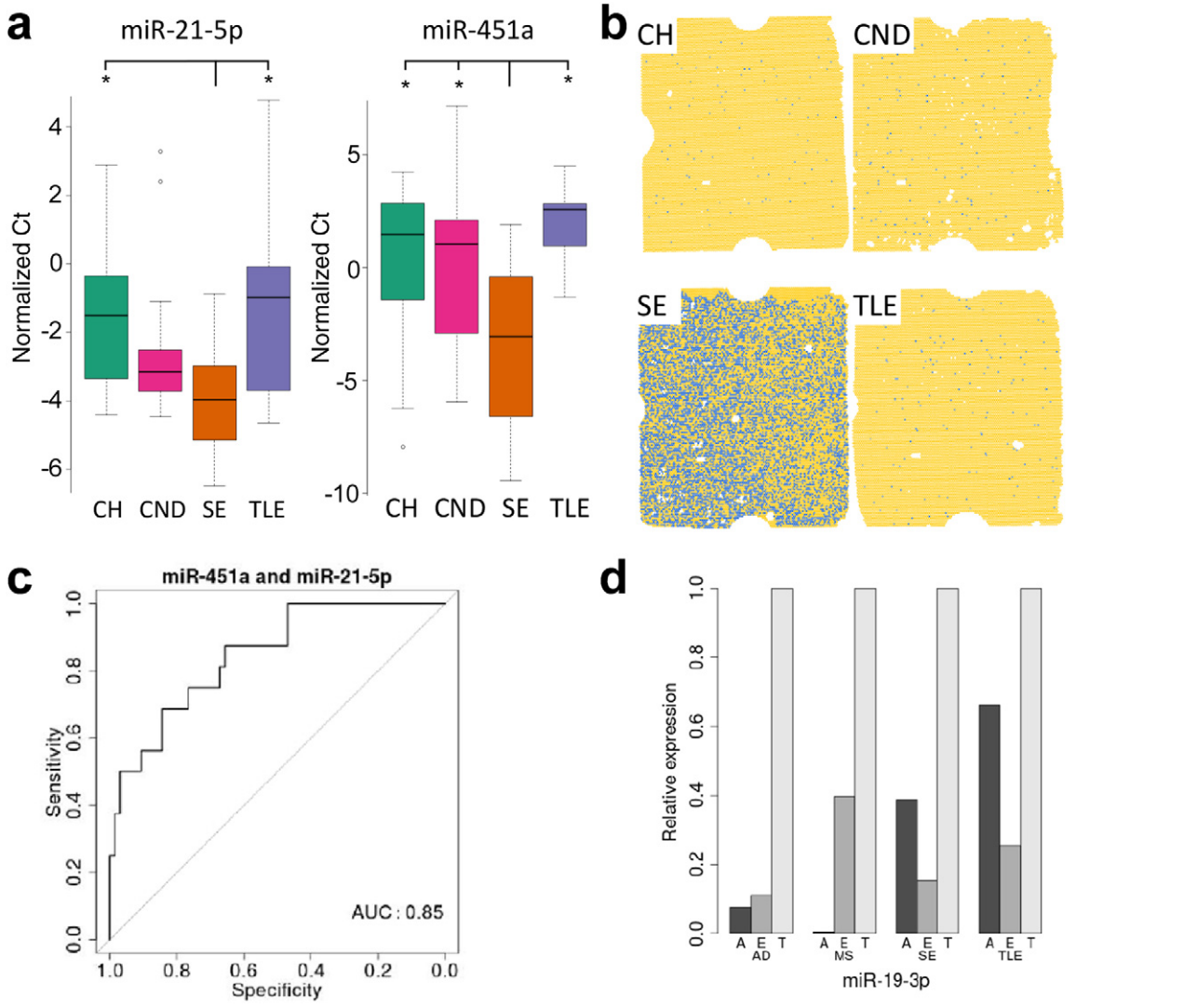

Fig. 1. Cerebrospinal fluid from patients with status epilepticus contains miRNA biomarkers. (a). Boxplots showing the distribution of normalized cycle threshold (Ct; the number of cycles required for the signal to exceed background level) following Taqman individual microRNA assays of miR-21-5p and miR-451a. CH = control (headache), CND (control, other neurological disease), SE (status epilepticus), and TLE (temporal lobe epilepsy). Note, the data shown are normalized and the lower the ct value, the greater the relative expression of the miRNA. (b) Digital PCR validation of increased miR-451a in status epilepticus. Representative chips show the number of miR-451a positive wells from CSF samples for each group. Blue dots represent positive signal from amplified miR-451a. (c) receiver operating characteristic (ROC) analysis showing sensitivity and specificity of miR-21-5p and miR-451a combination levels for distinguishing SE samples versus CH, CND, and TLE samples. The closer the area under the curve (AUC) to 1.0 the better the biomarker. (d) Relative expression of miR-19b-3p in Argonaute (A), exosome (E), and total (T) fractions from the CSF of patients with Alzheimer's disease (AD), multiple sclerosis (MS), SE, and TLE. Note, the amounts of the miRNA differ notably between the different brain diseases suggesting comparisons of fractions rather than total miRNA is a better approach to increase diagnostic yield. Data are adapted with permission from Raoof et al. [41].

sensitivity and specificity (Fig. 1c). A final part of this study looked at the ratios of these miRNAs bound to Argonaute and enclosed in exosomes. Overall, the results suggested that higher diagnostic accuracy can be achieved by analyzing these fractions compared to the "total" miRNA pool. Perhaps just as interesting, the distribution between these forms varied widely between diseases. For miR-19b-3p, levels of the miRNA bound to Argonaute were high in people following status epilepticus and people with TLE but virtually undetectable in samples from people with multiple sclerosis and Alzheimer's disease [41] (Fig. 1d). Thus, measuring the miRNAs within specific physical fractions within a biofluid may enhance diagnostic readout.

5. Can we prove that some of circulating miRNA really comes from the brain?

While the work by our group and others has found brain-enriched miRNAs increase in the blood after status epilepticus, this does not prove that they came from the brain. Nor does it tell us whether the miRNAs were released from neurons, glia, or some other cell types. It might also be useful to know whether the miRNA reached the circulation following lytic release (e.g., following neuronal necrosis) or more controlled (e.g., the exosome route or following apoptosis). Can we exclude that these assumed "brain miRNAs" in the circulation were actually synthesized by other organs? Convulsive status epilepticus is a multiorgan stress, and it is possible that some of the pool of circulating miRNAs reflects release from sources besides the brain, such as skeletal and cardiac muscles, lungs, liver, etc. Proving at least some of the circulating miRNA came from the brain is fundamental to providing a mechanistic basis for the use of miRNAs as biomarkers of status epilepticus or acute seizures. It might also lead to ideas about ways to increase the sensitivity and specificity of measuring miRNA levels to diagnose epileptogenesis or acute seizures.

We recently attempted to answer this key question by developing an animal model in which miRNAs in the blood could be definitely linked to the brain. The approach we took was to use a previously developed transgenic mouse that expresses an artificial amino acid sequence

(FLAG tag) on the Argonaute-2 protein [42,43]. By crossing that line with Cre recombinase lines under specific cell promoters (e.g., for neurons), we could generate mice that expressed the tagged form only within the brain either in excitable cells (neurons) or glia. The transgenic mice could then be subjected to status epilepticus and blood collected and checked for the presence of the tagged form of the Argonaute. Extraction and identification of the miRNA attached to the circulating FLAG-Argonaute would provide a high degree of confidence that at least some of the circulating miRNAs are really from the brain. By comparing between lines, it might also be possible to know which main cell type in the brain contributes the most to the miRNA "signal" in the blood. While these studies are ongoing, early results suggest that there is miRNA circulating bound to the FLAG-Argonaute in mice subject to status epilepticus. This is the first direct proof that a portion of the circulating miRNA in the brain comes from neurons.

6. Next steps and future questions

There are good reasons to think that measurement of biofluid miRNAs (or other short noncoding RNAs) could support the diagnosis of epileptogenesis, epilepsy, or acute seizures. The molecules have favorable biochemical characteristics; there is cheap and reliable technology for their detection which could be scaled in a way suitable for point-of-care use; and there are mechanistic links between their dysregulation in epileptic brain tissue and targeting for seizure control or disease modification. We now have direct evidence that some of the circulating miRNAs do indeed come from neurons. We are still a long way, however, from a clinical test or diagnostic marker. Some of the barriers to translation and the next steps were recently reviewed [29]. In particular, we need to identify and validate miRNAs that are unique to specific phases and link these to etiologies. Fractionating biofluids may improve signal-to-noise, and the identification of isomirs and editing of miRNA may further enhance diagnostic specificity or sensitivity [29]. We need multicenter clinical validations, and we need to continue to develop and refine the technology for their detection. Several potential applications of miRNAs as biomarkers have not yet been addressed. Can we use miRNA profiles to predict who will develop drug-resistant epilepsy or have favorable outcomes following resective surgery? The prospects for a miRNA diagnostic continue to improve although we will have to await key experiments in the coming months and years before we know the full potential of these important molecules.

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Disclosure

DCH holds US patent No. US 9,803,200 B2 "Inhibition of microRNA-134 for the treatment of seizure-related disorders and neurologic injuries".

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