TITLE: The role of circulating and exosomal miRNAs as biomarkers of drug-resistant epilepsy

Principle Investigator: Leila Zummo, MD, PhD Student (leilazummo@yahoo.it)

Department of Experimental Biomedicine and Clinical Neurosciences, University of Palermo, via del Vespro 129, 90127, Palermo, Italy

Other Investigators and other Italian Centers involved: Prof. Francesco Cappello (University of Palermo, Palermo, Italy; and Euro-Mediterranean Institute of Science and Technology, Palermo, Italy), Dott. Antonella Marino Gammazza (Euro-Mediterranean Institute of Science and Technology, Palermo, Italy), Dott. Celeste Caruso Bavisotto (Euro-Mediterranean Institute of Science and Technology, Palermo, Italy), Prof. Brigida Fierro (University of Palermo, Palermo, Italy), Prof. Ornella Daniele (University of Palermo, Palermo, Italy), Prof. A.T. Giallonardo (La Sapienza University of Rome, Rome, Italy), Prof. Giancarlo Di Gennaro (IRCCS Neuromed, Pozzilli, Italy)

Abstract

Epilepsy is estimated to affect about 65 million individuals worldwide, with up to 30 percent of drug-resistant patients who do not have remission despite appropriate therapy with antiepileptic drugs (AEDs). [1] Therefore, it is important to distinguish drug-resistant epilepsy early in the course of disease to start a specific therapeutical approach as soon as possible. [2]

Recently circulating miRNAs have been proposed as promising biomarkers for different neurodegenerative disorders, including epilepsy.

MiRNAs are a class of small non-coding RNA that regulate gene expression at a post-transcriptional level. The regulatory mechanisms controlling translation of mRNA transcripts represents to date a largely unexplored aspect of epilepsy. Several studies have demonstrated that miRNAs are differentially expressed in presence of drug-resistant epilepsy, with a specific expression pattern in brain regions connected to the epileptogenic activity[3-4-5].

Moreover miRNAs can be transmitted from one neuron to another across the synaptic cleft carried by exosomes.[6] Because of the ability of exosomes to mediate drug efflux, it could be useful to study how they participate in the pathogenesis of drug-resistant epilepsy.

Aims of this study are: 1) to evaluate the miRNA serum expression in patients with focal, non lesional, drug-resistant epilepsy, comparing differences of miRNA expression in patients with focal lesional epilepsy, in particular in presence of focal cortical dysplasia (FCDs). 2) to investigate miRNA expression in FCDs comparing sample to serum miRNA expression in the same patients.

In the study will be enrolled 60 clinically diagnosed patients with drug-resistant epilepsy [7] according the following groups: 30 idiopathic/cryptogenic and 30 lesional (FCDs) matched for age and gender with 60 healthy controls. A multiphase case-control study will be designed to evaluate circulating and exosomal miRNA expression profiling of patients with drug-resistant epilepsy and healthy controls.

There is a need to establish the relationship between miRNA levels in lesional and non lesional drug-resistant epilepsy in order to use miRNAs as biomarkers of specific pathological condition such as intractable epilepsy. We know that circulating miRNAs are stable in serum and their test in blood is broadly accessible, rapid and noninvasive. Previous studies already found a significant number of miRNAs differentially regulated in the epileptic state when compared to control animals, indicating a tight regulation of miRNAs associated with seizures in epilepsy models. Thus, circulating miRNAs could be used as non invasive biomarkers to indicate drug-resistance.

Then our intent is also to understand the role of miRNAs in signaling pathways during corticogenesis, identifying differences in miRNA expression between surgical and serum sample. This correlation would be a great goal for clinical practice, supporting the role of the miRNA as biomarkers in drug-resistant epilepsy associated to structural brain abnormalities.

To date few studies have investigated the possible role of miRNAs in the pathogenesis of FCD, the heterogeneity of the abnormal cell population, in fact, is a critical limitation in this field of research. Therefore taking into account the important role of balloon cells in epileptogenesis we expect that the evaluation of miRNA expression, in these particular cells, could be helpful to elucidate their fundamental role in pathogenethic mechanism of pharmacoresistant lesional epilepsy.

Surely the observation of new specific miRNAs associated with drug-resistant epilepsy and FCDs will broaden new horizons both for the clinical and the therapeutical approach of until now intractable epilepsy.

BACKGROUND/RATIONALE Epilepsy is estimated to affect about 65 million individuals worldwide, with an up to 30 percent of drug-resistant patients who do not have remission despite appropriate therapy with antiepileptic drugs (AEDs) [1]. Therefore, it is important to distinguish drug-resistant epilepsy with drug-responsive epilepsy early in the course of disease. To date, the early identification is mainly based on clinical manifestations, such as frequency of seizures and the response after the initial treatment with AEDs, or on neuroradiological evidence of such lesion; however, these features are indefinite and subjective [2].

Recently circulating miRNAs have been proposed as promising biomarkers for different neurodegenerative disorders, including epilepsy, supporting also their role in epileptogenesis.

MiRNAs are an endogenous class of small non-coding RNA functioning to regulate gene expression at a post-transcriptional level by targeting mRNAs and reducing protein production. The regulatory mechanisms controlling translation of mRNA transcripts represent to date a largely unexplored aspect of the molecular pathophysiology of epilepsy [3]. Several target studies and genome-wide miRNA expression profiling studies demonstrated that miRNAs were differentially expressed in presence of drug-resistant epilepsy, with a specific expression pattern in brain regions connected with the epileptogenic activity [4-5]. This could be connected with malformations of cortical development (MCD), and particularly with focal cortical dysplasia (FCD), a subgroup of lesions characterized by anatomic disorganization of the cellular layers and the presence of morphologically abnormal cells, which are common structural lesions in patients with drug-resistant epilepsy[6-7-8-9].

MicroRNAs can also be transmitted from one neuron to another across the synaptic cleft carried by exosomes. These are small lipoprotein vesicles, derived from multivesicular bodies and the cellular endosome system, that cross the external plasma membrane to enter the perisynaptic space. Exosomes may cross the blood-brain barrier (BBB) and reach the circulation delivering their cargo (proteins, RNA) between specific cells. [6] Then, taken into account all these evidences and the ability of exosomes to mediate also drug efflux, it could be useful to study how exosomes participate in the pathogenesis of drug-resistance in epilepsy, a mechanism that is not completely clarified.

Thus not only circulating miRNAs but also exosomal miRNAs show potential use as non-invasive biomarkers indicating disease states. This current growing evidence would change also both the pathophysiology and the therapeutical approach of epilepsy.

SPECIFIC AIMS

The intent of the present study is first to evaluate whether miRNAs, both circulating and exosomal, can be used as biomarkers for drug-resistant epilepsy analyzing the differences in serum miRNA levels between patients with neuroradiological evidence of focal cortical dysplasia type I, IIa and IIb (or Taylor FCD), patients without structural brain lesions and healthy controls. In addition we evaluate the role of miRNAs in the pathogenesis of epilepsy. In particular the study aims to:

1) evaluate the role of miRNA as biomarkers of drug-resistance in patients with epilepsy
2) investigate miRNA expression in FCDs, with a particular evaluation in balloon cells, the most epileptogenic area of FCD, to compared to serum expression in the same patients
3) evaluate differences of miRNA expression in lesional and non lesional intractable epilepsy
4) evaluate a new epigenetic therapeutic strategy in epilepsy

KEY WORDS

Drug-resistant epilepsy, miRNAs, biomarkers, focal cortical dysplasia, therapeutic strategy in epilepsy

RESEARCH DESIGNAND METHODS

Study design and patients

In the present research project will be enrolled, in about 24 months, 60 patients clinically diagnosed with drug-resistant epilepsy according the following groups:

1. 30 Idiopathic/cryptogenic;
2. 30 lesional (FCDs)

matched for age and gender with 60 healthy controls.

All patients will undergo electro-clinical phenotyping, through validated medical records, to investigate ictal semiology, seizure frequency and targeted MRI study to investigate the presence of FCD.

Epilepsy will be diagnosed and classified as lesional or non lesional according to guidelines from the International League against Epilepsy in 2010 [7]. Drug-resistant epilepsy will be defined as failure of adequate trails of two or more tolerated and appropriately chosen and used AEDs [8].

In patients with drug-resistant epilepsy and FCD, that will undergo to epilepsy surgery, we also obtain surgical specimens. Then that type of FCD will be confirmed by a pathologist according to the recent FCD' s classification [9].

A multiphase case-control study will be designed to evaluate circulating and exosomal miRNA expression profiling of patients with epilepsy and controls. Then, we will compare significant miRNAs between patients with drug-resistant epilepsy (with and without FCD) and healthy controls.

Blood processing

Up to 6 ml whole blood will be collected from each patient and will be processed for plasma isolation within 3 hours of collection by centrifugation at 3,000 r.p.m. for 5 min at room temperature. The plasma samples will be stored at − 80°C until use.

Circulating miR isolation and quantification

The total RNA will be isolated from 400 μl of plasma of each sample using the miRNeasy serum kit (Qiagen, Hiden, Germany) according to the manufacturer's protocol. The purity and concentration of all RNA samples will be quantified spectrophotometrically using the NanoDrop ND-1000system (NanoDrop, Wilmington, Del). The miRNA array profiling experiment will be performed using a service (Exiqon Services, Denmark, for example) as previously described. [10] The miScript PCR system (Qiagen, Hiden, Germany) will be used for the quantification and profiling of miRNA expression using SYBR green-based real time PCR and specific primers for selected miRNA.

Exosomes collection and characterization [11]

The exosomes from human plasma samples will be isolated according the following protocol:

1. Plasma obtained from citrate-treated blood will be incubated at 37 °C for 15 min with Thromboplastin-D to remove clotting factors and then will be centrifuged at 900×g for 30 min. Thereafter, the plasma will be diluted with an equal volume of PBS and centrifuged 30 min at 2,000×g at 4°C.
2. The supernatant obtained will be centrifuged 45 min at 12,000×g at 4°C and then transferred in clean tubes and ultracentrifuged 2 hr at 110,000×g at 4°C.
3. The pellet obtained will be resuspended in 1 ml PBS and diluted in large volume of PBS to fill the tube and filter the suspension through a 0.22-μm filter.
4. The suspension will be centrifuged 70 min at 110,000×g, 4°C.
5. The pellet will be resuspended in 1 ml PBS and then centrifuged 70 min at 110,000×g, 4°C.
6. The exosomal pellet obtained will be resuspended in 50 to 200 μl PBS. Store up to 1 year at −80◦C.
Exosomes characterization and quantification will be assessed by transmission electron microscopy (TEM) and by using the Nanosight™ technology. Exosomal markers will be detected using Western Blotting analysis (Alix, CD9, CD63, CD81, Hsc70, Lamp-1, Tsg101) [12].

Exosomal miRNAs isolation and profiling [13-14].

Isolation of exosomal miRNAs will be performed using the miRNeasy kit (Qiagen, Hiden, Germany) following manufacturers' instructions. The purity and concentration of all RNA samples will be quantified spectrophotometrically using the NanoDrop ND-1000 system (NanoDrop, Wilmington, Del). The exosomal miRNAs profiling will be performed as described in the previous section "Circulating miR isolation and quantification".

***In situ* hybridization**

The lesion tissue from patients will be fixed in 10% buffered formalin and embedded in paraffin. Paraffin-embedded tissue will be sectioned at 6 μm and mounted on pre-coated glass slides. The slices will be used for *in situ* hybridization using a 5′ fluorescein-labelled 19-mer antisense oligonucleotide specific for each miRNA selected.

EXPECTED RESULTS

1) use miRNAs as biomarkers of specific pathological condition such as intractable epilepsy. Previous studies already found a significant number of miRNAs differentially regulated in the epileptic state when compared to control animals, indicating a tight regulation of miRNAs associated with seizures in epilepsy models [15]. We expect that this research project may underlie the role of circulating miRNA as biomarkers for an accurate clinical diagnosis, faciliting the appropriate treatment, in particular in presence of drug-resistant epilepsy.

2) identify the role of miRNAs in regulation of signaling pathways during corticogenesis. Since FCDs are one of the most frequent epileptogenic malformation, susceptible to surgical treatment, it is of great importance to understand the mechanisms underlying epileptogenesis in FCDs [16-17-18]. Recent data support a role for miRNAs differentially regulated during normal mouse brain development [19-20]. Nevertheless, few studies have investigated the role of miRNAs in the pathogenesis of FCDs. We expect that the evaluation of differences in miRNA expression between surgical and serum sample, would be helpful to elucidate their role in lesional epilepsy. Their correlation would be something positive for the clinical practice using miRNA as biomarkers in drug-resistant epilepsy with structural brain abnormalities.

TOTAL BUDGET

Materials, supplies and services: 20.000

- *Glassware, plasticware and media for cell cultures: 2.000*
- *Chemicals, Immunochemicals (commercial primary and secondary antibodies): 3.000*
- *Kit for electron microscopy: 1.000*
- *Kit miRNeasy Mini Kit: 2.000*
- *IT service for maintenance of the clinical database: 2.000*
- *Service: 10.000*

Travels costs: 8.000

- *Participation at International and National Congresses: 4.000 (2000 per year)*
- *Publications: 2.000*
- *Shipment of biological samples to and from LICE partners: 2.000*

Salaries: 12.000

- *Salary for one year contract for laboratory technician*

Total: 40.000

This research project is financial supported by University of Palermo, Palermo, Italy (PhD bursary for Dr. Leila Zummo) and Euro-Mediterranean Institute of Science and Technology, Palermo, Italy (financial support of € 5.000,00).

REFERENCES

1. Moshe S. et al 2014 et al. Epilepsia. 2014 Apr;55(4):475-82.
2. Kwan P. et al. Seizure. 2000 Oct;9(7):464-8.
3. Bartel DP. et al Cell, 2009;136(2):215-33
4. Song YJ. et al Brain Res. 2011 Apr 28;1387:134-40.
5. Sun Z et al PLoS One. 2013 Oct 25;8(10):e78375.
6. Gupta A, Pulliam L. J Neuroinflammation. 2014 Apr 3;11:68.
7. Berg AT et al Epilepsia. 2010 Apr;51(4):676-85.
8. Blumcke I et al Epilepsia. 2011 Jan;52(1):158-74.
9. Kwan P et al Epilepsia. 2010 Jun;51(6):1069-77.
10. Bot AM et al PLoS One. 2013 Oct 11;8(10):e76051
11. Caby MP et al IntImmunol. 2005 Jul;17(7):879-87
12. Campanella C et al Oncotarget. 2015 Dec 19
13. Pfeffer SR et al J Clin Med. 2015 Dec 17;4(12):2012-27
14. Tadokoro H et al J Biol Chem. 2013 Nov 29;288(48):34343-51
15. Sun Z et al PLoS One. 2013 Oct 25;8(10):e78375.
16. Aronica E et al Brain Pathol. 2012 May;22(3):380-401.
17. Hauptman JS, Mathern GW. Epilepsia. 2012 Sep;53 Suppl 4:98-104.
18. Sakakibara T et al J Neuropathol Exp Neurol. 2012 Aug;71(8):741-9.
19. Dogini DB et al J Mol Neurosci. 2008 Jul;35(3):331-7.
20. Volvert ML et al Cell Death Differ. 2012 Oct;19(10):1573-81.