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MicroRNAs as Potential Biomarkers for Childhood Epilepsy

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Abstract

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BACKGROUND: Epilepsy is the most frequent chronic neurologic condition in childhood. Its clinical diagnosis is based on electroencephalograms (EEG) and neuroimaging techniques. MicroRNAs (miRNAs) modulate gene expression of several genes and are aberrantly expressed in several diseases.

AIM: Evaluation of using circulating miR-106b and miR-146a as diagnostic and prognostic biomarkers in children patients with epilepsy.

METHODS: Thirty epileptic children and twenty controls were enrolled in our study. They were assessed for the expression pattern of miR-106b and miR-146a in plasma using quantitative real-time PCR and determination of plasma Immunoglobulin levels.

RESULTS: MiR-146a and miR-106b expression patterns were significantly up-regulated in children patients than that in normal controls. Plasma Immunoglobulins were differentially expressed in epileptic patients in comparison with healthy controls. No correlations were found between expression levels of miRNAs (miR-146a and miR-106b) and clinical data or immunoglobulin levels in children patients with epilepsy.

CONCLUSION: Our findings suggest that up-regulated plasma miR-106b and miR-146a could be used as biomarkers for epilepsy evaluation.

Introduction

Epilepsy is a common childhood neurological condition and a worldwide major public health concern [1]. It's estimated that about 4-10% of the world population is affected by it [2]. At the pediatric side, 0.5% to 1% of children are suffered from epilepsy. Nowadays, its occurrence is decreased in high-income countries. In Arab countries, the estimated prevalence of epilepsy in children ranges from 3.6 to 10.5/1000 [3]. It is characterised by recurrent seizures, which are either brief generalised or partial involuntary movements that may be accompanied by loss of consciousness [4]. Those seizures are due to excessive electrical discharges in a group of brain cells. They may be a very brief lapse of attention to severe long convulsions [5].

Diagnosis of epilepsy is challenging; the principles of the neurological diagnosis should be followed, including what? (clinical diagnosis), where?

(topographical diagnosis), and why? (etiological diagnosis) with detailed history, general and neurologic examination accompanied with the right choice of complementary evaluations as EEG which despite that normal EEG does not exclude epilepsy diagnosis and even 5-8% of normal children may have epileptiform discharges on the EEG, Cerebral imaging (MRI) may also be used [6], and hence biomarkers may be considered novel tools for diagnosis which could facilitate the treatment afterwards [7]. MiRNAs are a class of short non-coding RNAs which regulate the expression of a variety of genes and enter in various biological functions as cell differentiation, development, metabolism, immune responses, and carcinogenesis [8]. More than 50% of the identified miRNAs are expressed in the brain. They are incorporated in many brain functions which are of importance to epileptogenesis, including cell death, neurogenesis, and synaptic plasticity [9].

The changing miRNA profiles in biofluids may be considered as useful biomarkers of

epileptogenesis. Targeting Key miRNAs has been shown to suppress or exacerbate seizures and alter brain excitability, indicating a potential for miRNA-based therapeutics in epilepsy [10].

Recent reports have shown that certain miRNAs (e.g., miR-132) control several epileptogenesis-related processes, such as cell death and neuroinflammation [11], [12]. Mir-21 has also been reported to be increased in models of prolonged seizures in immature rats [13]. MiR-34a expression is controlled by p53, which is upregulated and contributes to neuronal death provoked by seizures [14], [15]. MiR-184 was known as the most upregulated miRNA in the hippocampus after an episode of brief, non-harmful seizures, a model of epileptic preconditioning and a rich source of neuroprotective pathways [16]. Silencing miR-184 significantly increased seizure-induced neuronal death in two animal models of status epilepticus (SE) [16]. Mice with a conditional deletion of miR-128 were found to develop fatal epilepsy [17]. MiR-134 is a brain-enriched miRNA overexpressed after SE and in experimental and human epilepsy [18], [19].

People with epilepsy may show various types of immunological abnormalities, such as low serum IgA levels, lack of IgG subclass and presence of certain types of antibodies [20].

In this study, we aim to evaluate using of circulating miRNAs (miR-106b and miR-146a) as diagnostic and prognostic biomarkers in epilepsy through investigating their expression patterns in epileptic patients compared to healthy controls, examining their correlations with the clinical characteristics and clinical data of patients, and comparing their expression levels with Immunoglobulin levels in epileptic patients.

Patients and Methods

This study was approved by the Research Ethics Committee, Faculty of Medicine for Girls, Al-Azhar University (Cairo, Egypt). Written informed consent from both parents/guardians and oral informed consent from children were taken after full explanation of the study according to ICMJE Recommendations for the Protection of Research Participants.

This study was a case-control study. Thirty patients with epilepsy and 20 healthy controls with matched age and sex and having age ranging from 5 to 15 years were involved in the study. Patients were recruited from Pediatrics Neurology clinic, Al-Zahraa University Hospital, Cairo, Egypt, from January 2019 to June 2019. Inclusion criteria: Idiopathic epilepsy by clinical examination and EEG. Exclusion criteria:

Patients with other chronic diseases or psychiatric disorders, history of autoimmune diseases, history of infection 2 weeks before sample collection. Patients in this study were subjected to full medical history with special emphasis on the character of seizures (type, age of onset and duration of the seizures) and full neurological examination.

RNA extraction and quantitative real-time PCR

MicroRNA was extracted and isolated from plasma of all subjects of the study populations using miRNeasy Mini kit of Qiagen (Germany) according to the manufacturer's instructions. For miRNA-specific reverse transcription, microRNA was reverse-transcribed to cDNA using TaqMan[®] MicroRNA Reverse Transcription Kit (Applied Biosystems) and using specific primers according to the manufacturer's instructions. Reverse transcription was performed under the following conditions: 30 min at 16°C, 30 min at 42°C and followed by 5 min at 85°C and the resulting cDNA was kept at -80°C until use.

A real-time quantitative PCR (qRT-PCR) was carried out to quantify the expression levels in triplicate of mature miR-146a and miR-106b using TaqMan[®] MicroRNA Assay kit and TaqMan[®] Universal Master Mix (Applied Biosystems) using 7500 fast real-time PCR system according to the manufacturer's instructions. RNU6B was used as an endogenous control to normalise the expression levels of target miRs. Relative quantification (Rq) of miRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ threshold cycle method. ΔCt was determined by subtracting the Ct values for RUN6B from the Ct values for the gene of interest. Q RT-PCR was performed under the following conditions: 2 min at 50°C, 10 min at 95°C, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min [21].

Determination of plasma Immunoglobulin levels (IgA, IgG and IgM)

Measurement of human Immunoglobulin in plasma was performed using the method of immunonephelometry [22] (MininephTM, the Binding Site Ltd, PO Box 11712, Birmingham, B14 4ZB, U.K).

Statistical analysis

Data were statistically analyzed using SPSS version 16.0 software (SPSS Inc., Chicago, Illinois, USA). Independent samples T-Test was used to compare gene expression levels of microRNA between patients with epilepsy and normal controls. Nonparametric Mann-Whitney U test was used for comparing Immunoglobulin levels between groups. Correlations between microRNA expressions, immunoglobulin levels and clinical parameters of

patients with epilepsy were analyzed using Spearman's rank correlation. Data were presented as mean \pm SEM. A P value of less than 0.05 was considered statistically significant. Receiver operating characteristic (ROC) curve was constructed for miR-146a and miR-106b to evaluate the efficiency of these miRNAs as biomarkers for epileptic patients against controls. Area under curve (AUC) values, specificity, sensitivity and 95 % confidence intervals for each miRNA were calculated.

Results

The total number of epileptic patients was 30, with 22 males and 8 females. Table 1 shows the clinical characteristics of patients. Most of our patients (45%) were diagnosed as generalised epilepsy, and the lowest percentage (10%) was diagnosed as focal epilepsy.

Table 1: Clinical Characteristics of patients

Characteristic	Epileptic patients	Normal healthy controls
No. of cases	30	20
Gender, no. male/female	22/8	10/10
Age, (range)	5-15	5-15
Disease duration (yrs) mean \pm S.E.	6.25 \pm 0.59	-
Idiopathic	25%	
Types (% of Epilepsy)	Focal 10%	
Generalised	45%	
Focal then Generalized	20%	
Medications (Tegretol, Depakine, Tiratam)	25/30	0/20

Expression pattern of microRNAs

Our results indicated that miR-146a expression pattern was significantly higher in children patients with epilepsy than that in normal controls. 14.65-fold up-regulation in expression levels of miR-146a was found in epileptic patients compared to healthy controls. Moreover, the expression level of miR-106b was significantly up-regulated in patients with epilepsy in comparison with healthy controls. The level of miR-106b expression was 11.6 fold higher in epileptic patients compared to normal controls (Table 2).

Table 2: Relative Quantification of miR-146a and miR-106b in plasma of epileptic patients in comparison with healthy controls

	N	Rq	S.E.	Sig.
Mir-146a	30	14.65	4.92	0.004*
Mir-106b	30	11.6	3	0.002*

* $P < 0.01$ versus controls (by Independent Samples T-Test).

ROC Curve of miR-146a and miR-106b for epileptic patients against normal controls

ROC curve of miR-146a showed a significant AUC value of 0.763, a sensitivity of 73.7% and a

specificity of 60% ($P < 0.05$). Moreover, miR-106b showed a ROC curve with a good significant AUC value of 0.885, a sensitivity of 80% and also a specificity of 80% ($P < 0.001$) as shown in Figure 1).

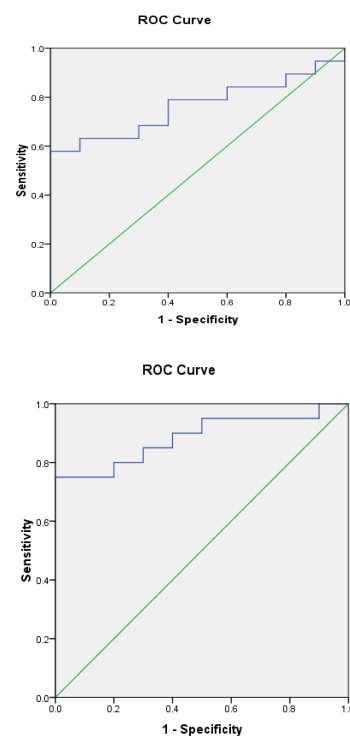


Figure 1: ROC curve of miR-146a (top); ROC curve of miR-106b (bottom)

Levels of plasma Immunoglobulins in epileptic patients

Plasma immunoglobulin IgA levels were significantly decreased in children patients with epilepsy in comparison with healthy children. Also, Immunoglobulin IgG levels were significantly lower in plasma of epileptic patients than in normal controls. However, levels of IgM in children patients with epilepsy were comparable to its levels in normal controls (Table 3).

Table 3: Immunoglobulin levels in the plasma of patients with epilepsy versus healthy controls

	groups	Mean	S.D.	S.E.	Sig.
IgA	Epileptic patients	0.798	0.478	0.119	0.038*
	Healthy controls	1.22	0.421	0.149	
IgG	Epileptic patients	8.79	5.234	1.309	0.024*
	Healthy controls	12.3	4.55	1.608	
IgM	Epileptic patients	0.796	0.454	0.11	0.815
	Healthy controls	0.805	0.286	0.101	

* Statistically significant at $P < 0.05$ versus controls (with Non-parametric Mann-Whitney U Test).

Correlations of miR-146a and miR-106b with Immunoglobulin levels of epileptic patients

Correlation analysis indicated that there are no correlations between miR-146a and Immunoglobulin levels in patients with epilepsy. Also, no correlations were found between miR-106b and

levels of immunoglobulins in epileptic patients (Table 4).

Table 4: Correlations of miR-146a and miR-106b with Immunoglobulin levels of epileptic patients

Parameters	R (Spearman Correlation)	Sig.
miR-146a expression ~ IgA	-0.024	0.467
miR-146a expression ~ IgG	-0.204	0.242
miR-146a expression ~ IgM	-0.096	0.362
Parameters	R (Spearman Correlation)	Sig.
miR-106b expression ~ IgA	0.096	0.362
miR-106b expression ~ IgG	-0.089	0.376
miR-106b expression ~ IgM	0.221	0.197

*Correlation is significant at the 0.01 level (2-tailed).

Correlations of miR-146a and miR-106b with clinical parameters of epileptic patients

Our analysis revealed that miR-146a expression is significantly correlated with the age of epileptic patients in a positive correlation. But there are no correlations between miR-146a expression and the other clinical parameters of epileptic patients (Table 5). Furthermore, the miR-106b expression has no correlations with any clinical parameter of patients with epilepsy (Table 5).

Table 5: Correlations of miR-146a and miR-106b with clinical parameters of epileptic patients

Parameters	R (Spearman Correlation)	Sig.
miR-146a expression ~ Age	0.554*	0.007
miR-146a expression ~ Gender	-0.330	0.084
miR-146a expression ~ disease duration	0.378	0.055
miR-146a expression ~ seizure duration	0.208	0.196
Parameters	R (Spearman Correlation)	Sig.
miR-106b expression ~ Age	-0.007	0.489
miR-106b expression ~ Gender	-0.190	0.211
miR-106b expression ~ disease duration	-0.046	0.423
miR-106b expression ~ seizure duration	0.202	0.197

* Correlation is significant at the 0.01 level (1-tailed).

Discussion

Epilepsy is considered a chronic severe neurological disorder that leads to recurrent seizures [23]. Nowadays, there are many emerging studies which undergone on animal models, and human patients with epilepsy had concluded that epilepsy pathogenesis is accompanied by non-neuronal and neuronal components [24]. Because of evidence-based results in experimental models and the clinical studies, there's a strong theory that inflammatory processes in the brain is included in the etiopathogenesis of seizures and then the formation of a chronic epileptic focus [25]. MiRNAs have an effective role in inflammatory pathways that occurs with epilepsy. MiR-146a and MiR-106b are key regulators of the innate immune response in the modulation of astrocyte-mediated inflammation [26]. Those miRNAs can be used as new therapeutic target in epilepsy treatment. MiRNAs are reported to be

regulated in blood, suggesting that blood miRNAs could be used as biomarkers for brain injury and many neuronal diseases [10].

In this study, we found that miR-146a and miR-106b levels were significantly higher in our epileptic patients than that in normal controls with 14.65-fold and 11.6-fold up-regulation in their expression levels respectively. These findings are in accordance with Wang *et al.*, [27] study which confirmed this result either in their training phase with 30 epileptic patients and 30 controls or in their validation phase with 117 epilepsy patients and 112 controls.

We also had found the ROC curve result of serum miR-106b with a good significant AUC value of 0.885 for prediction of epilepsy higher than of miR-146a which showed a significant AUC value of 0.763. Our findings go in the same way of An *et al.*, [28] whom had their study with measuring miRNAs using quantitative RT-PCR in 90 epileptic patients and controls.

We had found that miR-146a expression is significantly positively correlated with age of epileptic patients. But there are no correlations between miR-146a expression and miR-106b expression with the other clinical parameters of epileptic patients. Those results are the same as Wang *et al.*, [27] except what we had reached for positive correlation of miR-146a expression with age of patients which may indicate that miR-146a is affected by it.

Our study shows that plasma immunoglobulins IgA and IgG levels were significantly lower in plasma of epileptic patients than in normal controls whereas levels of IgM in children patients with epilepsy were comparable to its levels in normal controls. In fact, this may be attributed to drugs which taken for treatment of epilepsy that lowers levels of immunoglobulins despite they were high levels before treatment [29], [30].

Nowadays, there is an increased evidence supports miRNA changes in the pathogenesis of epilepsy. There is a subset of epilepsy miRNAs which should be studied more not only miRNA-146a and miRNA-106b to be able to have a clear image on biomarkers as our era now is seeking improvement of the ways used for current diagnosis and management of epilepsy. In other words, combination of the levels of miRNAs with EEG, Neuroimaging and clinical history will give a good diagnosis and prognosis which will pave the path to novel treatment options.

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